The present invention provides a method for production of functional proteins including hormones by renal cells in a three dimensional co-culture process responsive to shear stress using a rotating wall vessel. Natural mixture of renal cells expresses the enzyme 1-alpha-hydroxylase which can be used to generate the active form of vitamin D: 1,25-diOH vitamin D3. The fibroblast cultures and co-culture of renal cortical cells express the gene for erythropoietin and secrete erythropoietin into the culture supernatant. Other shear stress response genes are also modulated by shear stress, such as toxin receptors megalin and cubulin (gp280). Also provided is a method of treating in-need individual with the functional proteins produced in a three dimensional co-culture process responsive to shear stress using a rotating wall vessel.

17 Claims, 16 Drawing Sheets
**Fig. 1A**

γ-glutamyl transpeptidase activity
as 585 ± 42 nm fluorescence
log scale

**Fig. 1B**

γ-glutamyl transpeptidase activity
as 585 ± 42 nm fluorescence
log scale
Fig. 2B

Antibody binding (arbitrary fluorescence units)

Antibody dilution

Rotating wall vessel

Stirred fermentor

10-2 10-3 10-4 10-5
Fig. 2C
Fig. 3A
Fig. 3B
Fig. 4A
Antisense ICAM RT-PCR

Fig. 4B
Antisence MnSOD RT-PCR

MnSOD

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Fig. 4B (cont.)
The present invention relates generally to the fields of protein chemistry, endocrinology and gene therapy. More specifically, the present invention relates to a method for production of functional proteins in culture in response to shear stress using a rotating wall vessel.

2. Description of the Related Art

A successful and documented modality to induce polarization and differentiation of cells in culture is the rotating wall vessel (1-4). In rotating wall vessels gravity is balanced by equal and opposite physical forces including shear stresses. In engineering terms this has been claimed to be simulated microgravity at boundary conditions [Wolf D. A. and R. P. Schwarz. (1991) NASA Technical Paper 3143]. Rotating wall vessels, including models with perfusion, are a quantum advance. The rotating wall vessel is a horizontally oriented cylindrical cell culture device with a coaxial tubular oxygenator (1, 5-7). The rotating wall vessel induces expression of select tissue-specific proteins in diverse cell cultures (1-2, 8-9). Examples of expression of tissue-specific proteins include carcinoembryonic antigen expression in MIP-101 colon carcinoma cells (2), prostate specific antigen induction in human prostatic fibroblasts (7), through matrix material induction during chondrocyte culture (8). The quiescent cell culture environment of the rotating wall vessel balances gravity with shear and other forces without obvious mass transfer tradeoff (1-2, 4). The rotating wall vessel provides a culture environment suitable for co-cultures of diverse cell types, and three dimensional tissue construct formation.

Rotating wall vessel technology is being used in clinical medical practice recently by facilitating pancreatic islet implantation (4, 10). Pancreatic islets are prepared in rotating wall vessels to maintain production and regulation of insulin secretion. The islets are alginate encapsulated to create a non-inflammatory immune haven, and are implanted into the peritoneal cavity of Type I diabetic patients. This implantation of pancreatic islets has maintained normoglycemia for 18 months in diabetic patients, and progressed to Phase III clinical trials (4, 10). These vessels have also been applied to, for example, mammalian skeletal muscle tissue, cartilage, salivary glands, ovarian tumor cells, and colon crypt cells (11-13). Previous studies on shear stress response in endothelial cells, and rotating wall vessel culture have been limited to structural genes (14-16). These studies did not address the issue of a process for the production of functional molecules such as hormones. Shear stress response elements have not previously been demonstrated in epithelial cells, either for structural genes of production of functional molecules.

Vitamin D dependent rickets has been a disease familiar to family farms and larger animal husbandry industries for centuries (17-18). The development of renal replacement therapy by dialysis in humans expanded vitamin D deficient bone disease from an occasional human clinical caveat to a common clinical problem. This led to identification of the active form of vitamin D as 1, 25-diOH D3 and the development of a multi-billion dollar per year worldwide market, predominantly in end-stage renal disease patients, to provide replacement hormone clinically (18). The active 1,25-diOH form of vitamin D3 is mainly used to treat bone disease in dialysis patients but has also been implicated as a therapy for osteoporosis, and some forms of cancer. Recently, the effects of vitamin D have been recognized to play a central role not only in other common bone lesions such as osteoporosis due to aging and steroid induced osteoporosis, but in immune function and surveillance, growth and development, and cardiac and skeletal muscle function (19-22).

Several active forms of vitamin D have been identified, vitamin D receptors cloned, and nuclear binding proteins for the hormone identified and cloned (17-22). Studies on the regulation of 1 alpha-hydroxylase activity are limited by the lack of a renal cell line with regulated expression of the enzyme. The only reports of 1-ct-hydroxylase activity in culture utilize freshly isolated chicken renal cortical cells in which the activity declines precipitously within 48 hours of plating in culture (28).

The importance of the renal 1-ct-hydroxylase is best understood by comparing the kinetics of the renal enzyme to other forms in the body (29-30). Demonstration that nephrectomy in pregnant rats did not completely abolish 1, 25-diOH-D3, formation sparked an intensive search for extrarenal sites of 1-ct-hydroxylase activity (29). Although 1 alpha-hydroxylase activity has been reported in monocytes, liver, aortic endothelium and a variety of placental and fetal tissues, the enzyme kinetics contrast sharply with the renal 1 alpha-hydroxylase. Extrarenal 1-ct-hydroxylase has a much higher Km indicating that much higher substrate levels are needed for activity (29). In the uremic patient, extrarenal 1,25-diOH-D3 production is very limited due to a relative lack of substrate. Administering large quantities of 25-OH-D3, substrate to anephric patients modestly boosts plasma 1,25-diOH-D3 levels (29).

The lack of a differentiated polarized line of renal tubular epithelial cells for investigative purposes persists despite extensive searches by several laboratories (31-38). Renally derived cell lines transformed with viruses or tumor cells to produce immortality continue as some of the most popular cell biological tools to study polarized delivery (31, 33, 35). But these renally derived immortal cell lines such as MDCK or LLP-C1 retain few if any of the differentiated features characteristic of renal epithelial cells. Similarly, primary cultures rapidly dedifferentiate and lose basement membrane matrices, growth supplements or Milipore inserts achieve only modest degrees of polarity (37-38).
The pathognomonic structural features of renal proximal tubular epithelial cells are the abundance of apically derived microvilli, the glycoprotein content of associated intermicrovillar clefts, and the highly distinctive arrangement of subapical endosomal elements (39–40). Renal epithelial cells of the proximal tubule are characterized by thousands of long apical microvilli. The apical endosomal machinery begins in intermicrovillar clefts. The endosomal pathway is characterized by clathrin coated vesicles, small spherical endosomal vesicles, with deeper larger endosomal vacuoles (33, 39). From the endosomal vacuoles proteins and lipids either recycle to apical surface in dense apical tubules or shuttle to lysosomes to be degraded.

A cluster of apical proteins with homologous sequence repeats are especially desirable to express in cultured cells as they are thought to be molecular mediators of renal injury (41–43). Two of these proteins megalin (gp330) and cubulin (gp280) (Moesstrup, et al., J. Biol. Chem.,273 (9):5325–5242 (1998) are molecular mediators of tubular vacuolation and ensuing secondary damage. Megalin (gp330) is a receptor found on the luminal surface of the proximal tubular cells of the kidney. Megalin binds several proteins and drugs including aminoglycoside antibiotics and other polybasic drugs. Megalin is expressed in the kidney, lung, testes, ear, and placenta. The only cells which express megalin in culture are immortalized placentals cells. There is no known renal cell culture which expresses megalin. Gp280 is a receptor found on the luminal surface of the proximal tubular cells of the kidney. Gp280 binds several proteins and drugs including intrinsic factor-cobalamin (vitamin B12 bound to its carrier protein) and myeloma light chains. Cubulin (gp280) is expressed in the kidney, ear, and placenta. The only cells which express cubulin in culture are immortalized placental cells. There is no known renal cell culture which expresses cubulin (gp280).

Erythropoietin (EPO) is a hormone produced in the kidney, and secreted into the blood. Erythropoietin controls the rate of production of red blood cells by the bone marrow. Erythropoietin may be produced by the interstitial cells between the tubules or the proximal tubular cells or both. Erythropoietin production is lost in all known renal cell culture systems. Erythropoietin is mainly used to treat anemia in dialysis patients but is also popular to treat the anemia of AIDS patients and many forms of cancer.

The prior art is deficient in the lack of effective means of producing functional proteins including hormones in response to shear stress. Further, the prior art is deficient in the identification of shear stress response elements in epithelial cell genes. The present invention fulfills this long-standing need and desire in the art.

SUMMARY OF THE INVENTION

In one embodiment of the present invention, there is provided a method of producing a functional protein, comprising the steps of: isolating mammalian cells; placing said cells into a rotating wall vessel containing a cell culture comprising culture media and culture matrix; producing three-dimensional cell aggregates under simulated microgravity conditions; and detecting expression of the functional protein in the cell culture.

In another embodiment of the present invention, there is provided a method of inducing expression of at least one gene in a cell, comprising the steps of: contacting said cell with an transcription factor decoy oligonucleotide sequence directed against a nucleotide sequence encoding a shear stress response element; and determining the expression of said gene in said cell.

In yet another embodiment of the present invention, there is provided a transcription factor decoy, comprising an oligonucleotide sequence directed against a nucleotide sequence encoding a shear stress response element.

Other and further aspects, features, and advantages of the present invention will be apparent from the following description of the presently preferred embodiments of the invention given for the purpose of disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

The file of this patent contains at least one drawing executed in color. Copies of this patent with color drawing(s) will be provided by the Patent and Trademark Office upon request and payment of the necessary fee.

So that the matter in which the above-recited features, advantages and objects of the invention, as well as others which will become clear, are attained and can be understood in detail, more particular descriptions of the invention briefly summarized above may be had by reference to certain embodiments thereof which are illustrated in the appended drawings. These drawings form a part of the specification. It is to be noted, however, that the appended drawings illustrate preferred embodiments of the invention and therefore are not to be considered limiting in their scope.

FIGS. 1(A–D) shows homogeneity and structure of human renal epithelial cells in culture. Flow cytometry frequency histograms demonstrate number of cells positive for the proximal tubular marker γ-glutamyl transferase.

FIG. 1A shows the number of cells with γ-glutamyl transferase activity as the frequency of activity in 2000 cells compared to an unstained control with trapping agent alone. This is the raw digest of human renal cells.

FIG. 1B shows that following differential trypsinization, the percentage of proximal tubular cells present can be increased to 99±1%.

FIGS. 1C and 1D show transmission electron micrographs of human epithelial cells in culture. The intact renal cortex in vivo (far left panel), is compared to culture of the natural mixture of human renal cortical cells in conventional 2-dimensional culture (middle left panel) which is completely devoid of microvilli. Rotating wall vessel culture of pure proximal tubular cells shows some microvilli (middle right panel) but there are far more microvilli during rotating wall vessel culture of the natural mix of renal cortical cells (far right panel). Compared to these representative images, some areas of the natural mixture of cells in the rotating wall vessel show much greater abundance of microvilli, and well defined desmosomes (lower panel) which are lacking in the other cultures.

FIG. 2 shows protein expression in the rotating wall vessel. FIGS. 2A–2C show analysis of the expression and endosomal compartmentation of megalin, and cubulin in renal cells following rotating wall vessel culture. The ability of flow cytometry to make simultaneous measurements of entrapped fluorescein dextran as an endosomal marker and antibody binding allows construction of three dimensional frequency histograms displaying entrapped fluorescein dextran fluorescence against antibody binding on horizontal axes. A control sample shows vesicles negative for fluorescein on the left and fluorescein containing endosomes on the right (2000 vesicles depicted left panel). A control without fluorescein entrapped shows only the left population (not shown). Co-localization of anti-cubulin binding demonstrates that all the fluorescein positive endosomes are positive for cubulin, while non-endosomal membranes can be subdivided into cubulin positive and negative populations.
weight (14-220 kDa) on the abscissa is displayed against isoelectric point (pH 3-10) on the ordinate. FIG. 3A and FIG. 3B show differential display of renal cortical cells after 16 days culture in gas permeable bags as a control (left panel) or rotating wall vessel (right panel). This increased cubulin antibody binding in the cells grown in the rotating wall vessel (STLV) is more than five times the expression seen in stirred fermentors. Similarly, there was no detectable expression in the conventional cultures resulting in a flat line (not shown). Binding of normal serum and minimal dilution of primary antisera were not detectably different. Binding curves for anti-megalin antiserum showed a similar pattern (not shown).

FIG. 2C depicts non-specific (minimum) and peak binding of each antiserum following rotating wall vessel culture and two-dimensional SDS-PAGE analysis of protein content of cells following rotating wall vessel culture. Analysis of the protein content of cultures of the natural mixture of rat renal cortical cells after 16 days culture in gas permeable bags as a control (left panel) or rotating wall vessel (right panel) depicts changes in a select set of proteins. Molecular weight (14-220 kDa) on the abscissa is displayed against isoelectric point (pH 3-10) on the ordinate.

FIGS. 3(A-D) shows gene expression in the rotating wall vessel. FIG. 3A and FIG. 3B show differential display of genetic expression of rat renal cortical cells grown in conventional culture or rotating wall vessels. Differential display of expressed genes was compared in aliquots of the same cells grown in a 55 ml rotating wall vessel (STLV) or conventional gas permeable 2-dimensional bag controls. For differential display, copies of expressed genes were generated by polymerase chain reaction using random 25 mer primers and separated on a 6% DNA sequencing gel (FIG. 3A). Bands of different intensity between control and STLV representing differentially expressed genes, were identified by visual inspection, excised and reamplified using the same sequence using the BLAST search engine. One expressed sequence representing differentially expressed genes, were identified by Northern hybridization (FIG. 3B). PCR products were then cloned into the pGEM-T vector and sequenced. Sequences were compared to the Genebank database to confirm Northern hybridization (FIG. 3B). PCR products were then cloned into the pGEM-T vector and sequenced. Sequences were compared to the Genebank database to confirm Northern hybridization (FIG. 3B). PCR products were then cloned into the pGEM-T vector and sequenced. Sequences were compared to the Genebank database to confirm Northern hybridization (FIG. 3B).

Generally, any mammalian cell could be used in the methods of the present invention. Representative examples of mammalian cells include renal cortical cells, renal fibroblast cells, hepatocytes, pancreatic islets, renal interstitial cells, parathyroid cells, thyroid cells, pituitary cells, ovarian cells and testicular cells. Generally, the cell is selected from the group consisting of epithelial cell and endothelial cell. Preferably, cells contain shear stress response elements.

The present invention is directed to a method of producing a functional protein, comprising the steps of: isolating mammalian cells; placing said cells into a rotating wall vessel containing a cell culture comprising culture media and culture matrix; producing three-dimensional cell aggregates under simulated microgravity conditions; and detecting expression of the functional protein in the cell culture. Generally, simulated microgravity conditions comprise a balance between gravity and oppositely directed physical forces. Representative examples such physical forces include sedimentational shear stress, centrifugal forces, viscosity and coriolus forces.

Preferably, the functional protein is selected from the group consisting of a hormone, a toxin receptor and a shear stress dependent functional biomolecule. Representative examples of hormones which can be produced according to the method of the present invention include 1,25-dihydroxyvitamin D3 and erythropoietin. Representative examples of toxin receptors which can be produced according to the method of the present invention include salivary shear stress dependent functional biomolecule which can be produced according to the method of the present invention include an unidentified gene that has a 76% homology to the mouse GABA transporter gene. FIG. 3C and FIG. 3D show RT-PCR of time dependent change in genes during rotating wall vessel culture. Semi quantitative RT-PCR shows increases in the epithelial genes megalin, cubulin, magnesium dependent superoxide dismutase, nitric oxide synthase, c-fos, c-jun, platelet derived growth factor-b, transforming growth factor-b, tissue-type plasminogen activator and monococyte chemotactic protein-1, megalin, cubulin, erythropoietin and 1-a-hydroxylase.

Generally, any mammalian cell could be used in the methods of the present invention. Representative examples of mammalian cells include renal cortical cells, renal fibroblast cells, hepatocytes, pancreatic islets, renal interstitial cells, parathyroid cells, thyroid cells, pituitary cells, ovarian cells and testicular cells. Generally, the cell is selected from the group consisting of epithelial cell and endothelial cell. Preferably, cells contain shear stress response elements.
Representative examples of shear stress response element include GAGACC and GGTCTC.

In the methods of the present invention, the rotating wall vessel is initiated and maintained from about 6 rotations per minute to about 16 rotations per minute. Preferably, the sedimentational shear stress is from about 0.2 dynes/cm² to about 1.0 dynes/cm². The culture matrix may contain a core structure selected from the group consisting of cell aggregates and microcarrier beads, although other components to such a culture matrix are well known to those having ordinary skill in this art.

The present invention is also directed to a method of inducing expression of at least one gene in a cell, comprising the steps of: contacting said cell with a transcription factor decoy oligonucleotide sequence directed against a nucleotide sequence encoding a shear stress response element; and determining the expression of said gene in said cell. Generally, oligonucleotide comprises a terminal phosphothiorate moiety and a phosphodiester backbone and a structure which allows the oligonucleotide to pass cell membranes and accumulate in the nuclear compartment of the cell. Generally, the cell is a cultured cell. Preferably, the cell is selected from the group consisting of an epithelial cell and an endothelial cell. Representative examples of which can be used in this method include renal cortical cell, renal fibroblast cell, hepatocyte, pancreatic islet, renal interstitial cell, parathyroid cell, thyroid cell, pituitary cell, ovarian cell and testicular cell. In one embodiment, the cell is grown in a three-dimensional culture. Representative examples of shear stress response elements include GAGACC and GGTCTC.

Preferably, the gene encodes a protein selected from the group consisting of megalin, cubulin, erythropoietin and 1-a-hydroxylase. The concentration of the oligonucleotide useful in this method generally ranges from about 10 nm to about 10 mm.

The present invention is also directed to a transcription factor decoy, comprising an oligonucleotide sequence directed against a nucleotide sequence encoding a shear stress response element. Preferably, the nucleotide sequence encoding a shear stress response element has a sequence selected from the group consisting of GAGACC and GGTCTC.

In one preferred technique, the rotating wall vessel is generally initiated and maintained at 10 rotations per minute. Preferably, the rotating wall vessel provides a balance of forces comprising gravity and equal and opposite sedimentational shear stress. Useful sedimentational shear stress rates within the context of the claimed methods are from about 0.2 dynes/cm² to about 1.0 dynes/cm².

As used herein, rotating wall vessel refers to a cylindrical horizontal rotating culture vessel with a coaxial oxygenator.

As used herein, shear stress response element refers to a sequence of a family of genes in the cell nucleus which binds one or more transcription factors in response to shear stress on the cell. A representative example of a shear stress response element is GAGACC or its complementary sequence GGTCTC.

As used herein, shear stress conditions refers to flow of liquid, or current of liquid over cells which causes genes to turn on or off.

As used herein, slow turning lateral vessel refers to one specific size and shape of a rotating wall vessel.

As used herein, differential display refers to displaying on a filter, gel or chip a discrete set of genes turned on or off in a cell under different conditions.
When grown under conventional conditions in DMEM/F12 supplemented with fetal calf serum and an antibiotic cocktail such as ciprofloxacin and fungizone, both the highly purified cells as well as the cell mix form a monolayer. Fetal calf serum was used at optimal concentration: 2% for human cells and 10% for rat cells. In order to increase epithelial cell differentiation (1, 45), renal cells were cultured in a rotating wall vessels known as a 55 ml slow turning lateral vessel (STL) (1, 45). To initiate cell culture, the slow turning lateral vessel was filled with medium, and seeded by addition of cell suspension (2×10^6 cells/ml). Residual air was removed through a syringe port and vessel rotation was initiated at 10 rotations per minute, and maintained for 10–16 days. Medium was changed every 2 to 3 days depending on glucose utilization. Concomitant with cells, microcarrier beads were added an 5 mg/ml to promote aggregate formation in the slow turning lateral vessel. Without beads the cells became shattered in the vessel in a few hours. Beads were cytodex-3 in all protocol except when electron microscopy was planned when the much more expensive, but easily sectioned Cultispere GL cells were added to the vessels.

EXAMPLE 4
Stirred Controls and Static Controls
To provide a stirred control stirred fermentors which mixed in the horizontal plane were loaded with identical concentration of cells and beads from the same pool added to the slow turning lateral vessel (1, 31, 46). Gas permeable Fluoroseal bags (Fluoroseal Inc., Urbana Ill.) in 7 or 55 ml size were selected as conventional static controls. Culture beads were added to the conventional controls at the same density as the slow turning lateral vessel cultures (1, 45).

EXAMPLE 5
Electron Microscopy Quantitation of Number of Microvilli
Transmission electron micrographs were performed on cell aggregates from the rotating wall vessels and conventional monolayers. Cells were washed with ice cold phosphate buffered saline, then fixed for electron microscopy with 2.5% glutaraldehyde in phosphate buffered saline (9, 47). The samples were then transferred to 1% osmium tetroxide in 0.05 M sodium phosphate (pH 7.2) for several hours, dehydrated in an acetone series followed by embedding in Epon. Lead-stained thin sections were examined and by Northern hybridization. PCR products were then subcloned into the pGEM-T vector (Promega, Madison Wisc.) and sequenced using T7M cycle sequencing system (Promega, Madison, Wisc.). Sequences were compared to the Genebank sequences using the BLAST search engine (National Center for Biotechnology Information). For genes of interest the bands were labeled with 32P for confirmation following reverse transcription, 10%–20% of each cDNA was amplified (Robocycler 40, Stratagene, La Jolla, Calif.) using 95° C. denaturation, 63° C. annealing and 72° C. extension temperatures. Amplification was for a total of 30 cycles with the first three cycles having extended denaturation and annealing times. Positive and negative strand PCR primers, respectively, were derived from published sequences using
Assay of 1-a-hydroxylase Activity

Phosphate buffered saline, the cells were then lysed and supernatant. The media were concentrated 15x and assayed

32P dCTP. The cDNA probes were hybridized to identical samples so

...ropoietin response element were scrambled, and a fluorescein conjugated probe was exposed to Or active sequence decoy at 8o nm.

...sequence control in which the six bases of the shear stress sequence shown at top of FIG. 4. These decoys had a terminal phosphorothioate moiety to prevent intracellular lysis, and a phosphodiester backbone to facilitate passage across cell membranes. Passage to and accumulation in the nuclear compartment of cultured cells was confirmed by confocal imaging of a fluorescein tagged decoy. Three decoys were synthesized: the active decoy, a random sequence control in which the six bases of the shear stress sequence were scrambled, and a fluorescein conjugated form of the decoy. Decoys were placed in the cell culture medium of rat renal cortical cells grown as above in conventional two-dimensional culture. Aliquots of cells exposed to control or active sequence decoy at 80 nm concentration were harvested at 2, 6 and 24 hours after exposure.

EXAMPLE 11 Genetic Discovery Array

A sample of human renal cortical cells grown in conventional flask culture was trypsinized and split into a gas permeable bag control and a rotating wall vessel (55 ml slow turning lateral vessel). After 8 days of culture on 5 mg/ml cytodex-3 beads, cells were washed once with ice cold phosphate buffered saline, the cells were then lysed and MRNA was selected with biotinylated oligo(dT) then separated with streptavidin paramagnetic particles (PolyAtract System 1000, Promega Madison, Wis.). 32P labeled cDNA probes were then generated by reverse transcription with 32P dCTP. The cDNA probes were hybridized to identical Gene Discovery Array Filters (Genome Systems Inc. St. Louis, Mo.). The Gene Discovery Array filters contain 18,394 unique human genes from the I.M.A.G.E. Consortium ([LNL])(15) CDNA Libraries which are robotically arrayed on each of a pair of filter membranes. Gene expression was then detected by phosphor imaging and analyzed using the Gene Discovery Software (Genome Systems)(50).

EXAMPLE 12 Assay of 1-a-hydroxylase Activity

As the 1-a-hydroxylase enzyme has never been isolated or cloned it is assayed functionally by the production of 1,25-dihydroxy-vitamin D3 from ultrapure exogenous 25-hydroxy vitamin D3. For each measurement, the classic Michaelis Menton kinetics of the enzyme are determined by assaying equal aliquots of renal cell aggregates in a curve of 25-OH D3 substrate concentrations from 0.1 to 10 mg/ml in 6 steps. All incubations are performed in the presence of the anti-oxidant DPED at 10 mM to ensure no contribution of non-enzymatic oxygenation (23-26). 1,25-dioH D3 generated in vitro was quantitated as described (23-27). In vitro incubations were terminated by adding a volume of acetonitrile equal to the incubation volume. Each incubation tube received 1,000 cpm of 3H-1,25 dihydroxy D3 to estimate recovery losses during the extensive extraction and purification scheme. The 1,25-dihydroxy D3 is extracted from the incubation medium by C18 solid-phase extraction (24-25). Following extraction, the samples are evaporated to dryness under N2 and dissolved in 2 ml of methylene chloride. The samples are then applied to silica Bond-Elut cartridges and the 1,25-dihydroxy D3-containing fraction is isolated and collected (26). The individual fractions containing 1, 25-dioH D3 and then subjected to normal phase HPLC on a Beckman model 344 liquid chromatography system. Normal-phase HPLC was performed with a Zorbax-Sil column (26) (4x25 cm) developed in and eluted with methylene chloride/isopropanol (96:4 v/v) with a flow rate of 2 ml/min. The 1,25-dihydroxy D3 eluted from this system was dried under N2 resuspended in ethanol and quantitated by radio receptor assay or radio immunoassay (25-26).

EXAMPLE 13 Culturing Renal Fibroblasts and Assay for Production of Erythropoietin

As renal fibroblasts are the source of erythropoietin secreted into the circulation, renal fibroblasts were cultured. Freshly dissected rat renal cortex was minced and collagenase/trypsin digested prior to removal of debris on a single discontinuous 5% albumin gradient. The mixture of rat renal cortical cells was placed into culture in DMEM/F12 with 20% fetal bovine serum. After two weeks to encourage fibroblast overgrowth in the rich medium, fibroblast growth factor was added. The resultant culture had fibroblastic features in the culture flask and was inoculated into a high aspect ratio vessel (HARV) for culture under increased shear stress conditions. The cells aggregate on the beads and slowly increasing their numbers. After 3 weeks growing the fibroblasts in a HARV, erythropoietin was assayed in the cell supernatant. The media were concentrated 15x and assayed via RIA. The media alone was also concentrated 15x as the control.

EXAMPLE 14 Culturing Hepatocytes and Assay for Production of Erythropoietin

As hepatocytes are a source of erythropoietin secreted into the circulation, immortalized human hepatocytes were cultured under control and applied shear stress conditions. The Hep3B cells were placed into culture in DMEM/ F12 with 10% fetal bovine serum in static flask culture. The resultant culture was split, one half remaining in static flask culture and the other half was inoculated into a HARV for culture under increased shear stress conditions. The cells aggregated on the beads. After 24 hours growing the Hep3B cells in a HARV, erythropoietin was assayed in the cell supernatant. The media were assayed by RIA. The static flask media was also assayed as the control.

EXAMPLE 15 Shear Stress Response Elements Mediate Changes in Erythropoietin Gene Expression

The immortal hepatic cell line, Hep3B, constitutively produces erythropoietin. The 5' promoter and 3' enhancer
regions of the gene contain putative shear stress response elements. The role of these elements in the enhancement of erythropoietin production in response to shear was tested by using integrated perfused rotating wall vessel culture to reintroduce graded shear. This protocol utilizes a library of promoters driving luciferase reporters genes, with various constructs lacking the putative shear stress response elements. It also allows DNA footprinting analysis of the histones which bind the promoter and enhancer elements.

**EXAMPLE 16**

**Results**

The proportion of proximal tubular cells in human renal cell fractions isolated by differential trypsinization was assayed using an entrapped fluorogenic substrate for the proximal enzyme marker γ-glutamyl-transferase (44). Flow cytometry analysis on a cell-by-cell basis showed the natural cell mixture in the human renal cortex to be 85±4%, n=4 proximal tubular cells (FIG. 1A, left panel). Following differential trypsinization, and selection of the pure fractions, proximal tubular enrichments as high as 99±1% could be achieved (right panel). As reported in other systems, rotating wall vessels were conducive to vigorous cell growth, as evidenced by high rates of glucose consumption assayed as 30 mg/dl glucose/100,000 cells/day. A cell doubling time of 4±3 days was assayed using Alamar blue in the rotating wall vessel compared to 4±2 days in conventional culture.

The ultrastructure of cultures of pure proximal tubular cells or renal cortical cell mixtures of human kidneys were grown in rotating wall vessels for 16 days, and were examined by transmission electron microscopy (FIGS. 1B and IC). Quantitation of the number of microvilli present by counting random plates at the same magnification demonstrates not only that the rotating wall vessel induces microvillus formation, but co-culture with the normal mix of renal cortical cells increases the effect (Table 1). Normal cortical cell mix in conventional two-dimensional culture has 21 microvilli per field; “pure” proximal tubular culture in rotating wall vessel has 104 microvilli per field; and the normal cortical cell mix in rotating wall vessel has 35 11 microvilli per field.

**TABLE 1**

Human proximal tubular cells microvilli counted on transmission electron-micrographs of cells grown for 16 days under various culture conditions.

<table>
<thead>
<tr>
<th>Culture Conditions</th>
<th>% Proximal Tubular Markers</th>
<th>% Microvilli Per Field</th>
</tr>
</thead>
<tbody>
<tr>
<td>conventional 2-D culture</td>
<td>85</td>
<td>2 1</td>
</tr>
<tr>
<td>pure culture (in rotating wall vessel)</td>
<td>99</td>
<td>10 4</td>
</tr>
<tr>
<td>normal cortical cell mix in rotating wall vessel</td>
<td>85</td>
<td>35 11</td>
</tr>
</tbody>
</table>

To examine the expression of megalin and cubulin in renal cells in culture, there are advantages to using human cells instead of rat cells. Specifically, the rat sequences of megalin and cubulin have not, and the antisera recognizes the rat but not the human isoforms of these proteins. Hence, the natural mixture of cells in the rat renal cortex was placed into culture in rotating wall vessels, stirred fermentors, and traditional culture for analysis of protein expression.

As the endosomal pathway has been implicated to play a central role in the function and pathophysiology of cubulin and megalin, entrapped endosomal markers were co-localized with receptor antibody binding. The ability of flow cytometry to make simultaneous measurements of entrapped fluorescein dextran as an endosomal marker and antibody binding allows construction of three dimensional frequency histograms displaying entrapped fluorescein dextran fluorescence against antibody binding on horizontal axes and number of vesicles in each channel up out of the page (FIGS. 2A-2C). A control sample shows vesicles negative for fluorescein on the left and fluorescein containing endosomes on the right (200 vesicles depicted, left panel). A control without fluorescein entrapped shows only the left population (not shown). Co localization of anti-cubulin binding demonstrates that all the fluorescein positive endosomes were positive for cubulin, while non-endosomal membranes could be subdivided into cubulin positive and negative populations. (middle panel). This pattern was repeated for anti-megalin binding in renal cortical cells (right panel) in culture.

Next, analysis of protein expression in cultured cells by antibody binding used classic serial log dilution antibody curves. An increase in binding with a decrease in dilution is pathognomonic for specific antibody binding during flow cytometry analysis. Binding of anti-cubulin antisera to membrane vesicles prepared from renal cortical cells after 16 days in culture, detected by the fluorescence of a phycoerythrin tagged secondary antibody, shows an almost two log increase in binding with antibody dilution (FIG. 2B). This increase in the cells grown in the rotating wall vessel (slow turning lateral vessel) is more than five times the expression seen in stirred fermentors. Similarly there was no detectable expression in the conventional cultures resulting in a flat line (not shown). Comparison of maximal binding of the anti-cubulin antibody to minimum taken to be the antibody dilution at which there is no further decline in signal with primary antibody dilution is shown in FIG. 2C. Binding of normal serum and minimal dilution of primary antisera were not detectably different. Binding curves for anti-megalin antisera showed a similar pattern (not shown) but the peak binding was a little lower (FIG. 2C). Again stirred fermentor has much less expression than the rotating wall vessel (slow turning lateral vessel) and the conventional cell membranes have no detectable binding (not shown).

To examine the proportion of proteins changing in the rotating wall vessel, two-dimensional SDS-PAGE analysis of cultures grown in the rotating wall vessel and bag controls were performed (FIG. 2B). The results shown in FIG. 2B demonstrates changes were in a selected group of proteins.

To identify the genes changing during rotating wall vessel culture, differential display were performed. Differential display of expressed genes was compared in aliquots of the same cells grown in a 55 ml rotating wall vessel (slow turning lateral vessel) or conventional gas permeable 2-dimensional bag controls. Differential display of copies of expressed genes were generated by polymerase chain reaction using random 25 mer primers and separated on a 6% DNA sequencing gel. Bands of different intensity between control and slow turning lateral vessel, representing differentially expressed genes, were identified by visual inspection, excised and reamplified using the same primers. Differential expression and transcript size were confirmed by Northern hybridization. PCR products were then subcloned into the pGEM-T vector and sequenced. Sequences were compared to the Genebank sequences using the BLAST search engine. One expressed gene which decreased in the slow turning lateral vessel (band D on gel, FIG. 3A) was identified as rat manganese-containing superoxide dismutase (98% match 142 of 144 nucleotides). Two genes
which increased in the slow turning lateral vessel, band A was identified as the interleukin-1 beta gene (100% match for 32 of 32 nucleotides) and Band B which corresponded to a 20 kbp transcript on a Northern blot appears to be a unidentified gene that has a 76% homology to the mouse GABA transporter gene.

To examine the genetic changes in specific genes, the expression of tissue specific epithelial cell markers and classic shear stress response dependent genes were examined by RT-PCR (FIG. 3c). Several genes specific for renal proximal tubular epithelial cells, including megalin, cubulin, the extracellular calcium sensing receptor, and the microvillar structural protein villin, increase early in rotating wall vessel culture. Similarly there were dynamic time dependent changes in classic shear stress dependent genes including intercellular adhesion molecule 1 (ICAM) and vascular cell adhesion molecule (VCAM) (increased) and manganese dependent superoxide dismutase (suppressed). Many but not all of these changes were prolonged, as after 16 days in culture gene expression of megalin, ICAM, VCAM and the extracellular calcium sensing receptor were still elevated, while villin and manganese dependent superoxide dismutase were at control levels. Expression of control GADPH, b-actin and 18S genes did not change throughout the time course.

To test for a role of a putative endothelial shear stress response element in these renal cortical cell changes, an antisense probe for the sequence was synthesized (FIG. 4A). A control probe had the active motif scrambled. Confocal imaging of a fluorescein conjugated form of the probe confirmed nuclear delivery of the probe (images not shown). Culture of rat renal cortical cells in 80 nm of the probe, resulted in a time dependent increase in magnesium dependent superoxide dismutase, but no change in villin gene expression (FIG. 4B). The control probe had no effect.

In order to confirm the genetic responses to rotating wall vessel culture and the analysis with human cells, automated gene display analysis expressed of a various rat renal cell cultures detected as production of 1,25-dihydroxy D3 (FIG. 3c). The results shown in TABLE 2 indicate that rat renal cells show increased structural differentiation during culture in simulated microgravity conditions, and express much greater 1-a-hydroxylase activity than under conventional culture conditions.

TABLE 3 indicates that human embryonic kidney cells show increased structural differentiation during culture in simulated microgravity conditions, and express 10 fold greater 1-a-hydroxylase activity than under conventional culture conditions.

The results shown in TABLE 2 indicate that rat renal cells show increased structural differentiation during culture in simulated microgravity conditions, and express much greater 1-a-hydroxylase activity than under conventional culture conditions.

To determine whether renal cells grown in simulated microgravity have 1-a-hydroxylase activity, the 1-a-hydroxylase activity of cell cultures were compared grown in traditional 2-D culture in gas permeable bags, and NASA rotating wall vessels. Both rat renal cells (Table 2) and human embryonic renal cells were assayed (Table 3).
also indicate that the expression of the erythropoietin gene was upregulated by those shear stress response elements during graded gravitational sedimentation shear in the vessel.

**EXAMPLE 17**

**Discussion**

Rotating wall vessels have been used by other investigators as “simulated microgravity”. The present invention contends that gravity is still active, and that in a rotating wall vessel gravity is balanced by equal and opposite sedimentational shear stress. A centrifugal force due to spinning the cell, quantitatively much smaller than gravity, is also present and offset by equal and opposite sedimentational shear stress. Thus, the present invention presents a new concept that rotating wall vessels provide this new balance of forces, including application of sedimentational shear, rather than microgravity.

The rotating wall vessel bioreactor provides quiescent co-localization of dissimilar cell types (1, 46), mass transfer rates that accommodate molecular scaffolding and a micro-environment that includes growth factors (1, 46). Engineering analysis of the forces active in the vessel is complex (1, 5–7). This degree of shear damages or kills most epithelial cells (1, 5, 46). Second, impeller trauma in the stirred fermentor, is absent in the rotating wall vessel. This explains why there was far more cubulin and megalin induced in renal cultures in rotating wall vessel culture than in a stirred fermentor, and both receptors were not detectable in conventional 2-dimensional culture.

Rotating wall vessel culture induced changes in a select set of genes, as evidenced by the genetic differential display gels and 2-dimensional protein gel analysis. For example, erythropoietin production is controlled by a shear stress element which mediates changes observed during graded gravitation sedimentation shear. 1-α-hydroxylase activity is maintained and increased in both renal cortical epithelial cells and human embryonic kidney cells, wherein the induction of the enzyme (1-α-hydroxylase) converts 25-hydroxyvitamin D3 to the active 1, 25-dihydroxy-vitamin D3 form. The present invention is the first demonstration of a process for production of molecules including hormones and other biomolecules induced by shear stress and other forces. The mechanistic information can be interpreted from knowledge of the pattern of response and distribution of certain gene products.

Megalin and cubulin represent the first pattern of change, as these proteins are restricted in distribution to renal cortical tubular epithelial cells. The increase in megalin mRNA and protein, and cubulin protein expression is therefore unequivocal evidence for changes in the epithelial cells. This provides an important new tool for studies of nephrotoxicity. Long suspected to play a role in renal toxicity, the tissue restricted glycoprotein receptors megalin and cubulin, have recently been shown to be receptors for common nephrotoxins. Megalin is a receptor for polybasic drugs such as the aminoglycoside antibiotic gentamicin (48) and vitamin D binding protein (51), and cubulin is the receptor for vitamin-B12 intrinsic factor (52). Although these receptors are expressed by transformed placental cells in culture (9, 43), there is currently no renal model expressing these markers for toxicology investigations (53). Rotating wall culture provides a fresh approach to expression of renal specific markers in culture for study on the pharmacology, biochemistry and toxicology which define the unique properties and sensitivities of renal epithelial cells.

The second pattern of change is represented by villin. Message for the microvilli protein villin increases in the rotating wall vessel in the first day of culture, and soon reformation of microvilli was observed. A decoy matching, the nuclear binding motif of a putative shear stress response element failed to induce similar changes. Although the promoter for villin has not been cloned, this suggests the changes in villin were induced by other transcription factors which may be due to shear stress or other stimuli in the bioreactor. Villin is also restricted to brush border membranes such as renal proximal tubular cells, or colonic villi (54–55). The observed increases in villin message resolved after 16 days of rotating wall vessel culture.

Manganese dependent superoxide dismutase represents a third pattern of response: a mitochondrial enzyme, ubiquitously is distribution, modulated by the classic shear stress response element in endothelial cells (56–57). Magnesium dependent superoxide dismutase message decreased early in the first day of rotating wall vessel culture, and this was persistent after 16 days in culture. These changes were confirmed when magnesium dependent superoxide dismutase was identified as suppressed in the differential display analysis of gene changes, and Northern blot confirmation was performed. A decoy matching, the nuclear binding motif of a putative shear stress response element induced an increase in magnesium dependent superoxide dismutase (MnSOD), which indicates that similar changes to the rotating wall vessel can be induced by the use of genetic decoys. Thus, the biological process of genetic induction by defined shear stress elements can be produced by multiple means including genetic decoys or use of the rotating wall vessel. Other shear stress response element dependent genes, specifically, intercellular adhesion molecule 1 (ICAM) and vascular cell adhesion molecule (VCAM) had changes in the rotating wall vessel opposite to magnesium dependent superoxide dismutase, mirroring observations made during low induced stress in endothelial cells (56–57). This provides three lines of evidence consistent with a role for shear stress as one mediator of genetic changes induced in the rotating wall vessel.

Differential display of the genes activated and deactivated under rotating wall vessel culture conditions showed rotating wall vessel culture was associated with decreased expression of manganese dependent superoxide dismutase mRNA and increased expression of interleukin-1 b gene mRNA. This greatly extends and brings together previous observations on the interactions of stress, manganese dependent superoxide dismutase expression and interleukin-1. Topper et al. reported an oppositely directed effect, i.e., differential display of vascular endothelial cells exposed to high stress demonstrates increased manganese dependent superoxide dismutase gene expression (57). Other direct evidence links superoxide dismutase and interleukin-1 as increases in manganese superoxide dismutase levels and decreases in interleukin-1 levels in H1-1080 fibrosarcoma cells (58). In more indirect evidence overexpression of mitochondrial manganese superoxide dismutase promotes the survival of tumor cells exposed to interleukin-1 (59). The present study provides direct evidence that modest shear...
stress decreases magnesium dependent superoxide dismutase in association with an inverse effect on interleukin-1.

The data here demonstrates internal consistency. The changes in magnesium dependent superoxide dismutase were observed on differential display, confirmed by Northern blot analysis, and matched responses were detected by RT-PCR. Megalin demonstrated matched changes in RT-PCR gene and protein expression. Changes in villin observed by RT-PCR were associated with dramatic reformation of microvilli, in which villin is a major structural protein. Although semi-quantitative RT-PCR is prone to inherent variation due to the massive amplification of signals, the use of multiple controls which remain unchanged (b-actin, GAPDH and 18S), and experimental confirmation that reactions were linearly related to cDNA concentration, minimizes these problems. The internally consistent findings by other methods strongly suggests this RT-PCR data is valid.

Study of the mechanisms of action of the rotating wall vessel to induce gene and protein expression during cell culture has been hampered by nomenclature. First, the attachment of the moniker “simulated microgravity”, based on engineering analysis of boundary conditions, clouds intuitive analysis of the cell biology as there is no cellular equivalent for this term (1, 6-7). Similarly the reduced shear stress in the rotating wall vessel compared to stirred fermentors leads to the term “reduced shear stress culture” (1), whereas there is increased shear stress compared to conventional 2-dimensional culture (1, 5). As cell aggregates remain suspended in the rotating wall culture vessels, gravity is balanced by an equal and opposite force. Engineering arguments on the relative contributions of fluid shear, drag, centrifugal force, coriolus motion, and tangential gravity-induced sedimentation are themselves tangential to the cell biology. Several lines of evidence are documented that shear stress responses are one of the component of the biological response. This opens the door for analysis of other biological response mediators in the vessels, and investigation as to whether unloading of gravity plays as big a role as the oppositely directed balancing forces.

Using the rotating wall vessel as a tool, data here provide the first evidence that shear stress response elements, which modulate gene expression in endothelial cells, are also active in epithelial cells, although other investigators failed to see an effect of shear stress on epithelial cells. The present invention demonstrates that epithelial cells have shear stress response elements and change gene expression in response to physical forces including but not limited to sedimentational shear stress. As the rotating wall vessel gains popularity as a clinical tool to produce hormonal implants it is desirable to understand mechanisms by which it induces genetic changes (10, 60), if necessary to prolong the useful life of implants. Several lines of evidence are provided that shear stress response elements are the first mechanism identified by which the rotating wall vessel induces genetic changes. Using a putative endothelial cell shear stress response element binding site as a decoy, the role of this sequence in the regulation of selected genes in epithelial cells was validated. However, many of the changes observed in the rotating wall vessel are independent of this response element. It remains to define other genetic response elements modulated during rotating wall vessel culture, and whether the induced changes are secondary to the balancing forces, or primarily related to unloading gravity.
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and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 1

(2) INFORMATION FOR SEQ ID NO: 1

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 bp
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single-stranded
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE:

(A) DESCRIPTION: cDNA to mRNA

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: yes

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(vii) IMMEDIATE SOURCE:

(viii) POSITION IN GENOME:

(ix) FEATURE:

(x) PUBLICATION INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CTAGACCCG ATG TTC AG

What is claimed is:

1. A method of producing a biomolecule in a bioreactor under simulated microgravity culture conditions comprising the steps of:

   selecting mammalian cells for cell culture wherein said cells are capable of producing at least one functional biomolecule selected from the group consisting of functional proteins, non-peptide hormones, vitamins and mixtures thereof;

   placing the mammalian cells into a rotating wall vessel containing a culture media and a culture matrix;

   culturing the mammalian cells in the culture media and culture matrix to produce the functional biomolecule;

   separating at least one functional biomolecule expressed by the mammalian cells from the culture media and culture matrix;

   and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.

2. The method of producing a biomolecule in a bioreactor under simulated microgravity culture conditions of claim 1 further comprising separating the functional biomolecule from a source selected from the group consisting of lysed cells, the culture media and mixtures thereof;

3. The method of producing a biomolecule in a bioreactor under simulated microgravity culture conditions and detecting their production of claim 1 wherein the biomolecule is selected from the group consisting of a hormone, a toxin receptor and a shear stress dependent functional biomolecule
not associated with a degenerative cell process and isolating the selected functional biomolecule.

4. The method of producing a biomolecule in a bioreactor under simulated microgravity culture conditions of claim 1 wherein the functional biomolecule is selected from the group consisting of magnesium dependent superoxide dismutase, nitric synthase, c-fos, c-jun, platelet derived growth factor-b, transforming growth factor-b, tissue-type plasminogen activator, monocyte chemotactic protein-1, megalin, cubulin, erythropoietin 1-α-hydroxylase and 1,25-dihydroxy-vitamin D3.

5. The method of producing a biomolecule in a bioreactor under simulated microgravity culture conditions of claim 1 wherein the mammalian cells are selected from the group consisting of epithelial cells, endothelial cells and mixtures thereof capable of producing functional biomolecules.

6. The method of producing a biomolecule in a bioreactor under simulated microgravity culture conditions of claim 1 wherein said mammalian cells are selected from the group consisting of epithelial cells, endothelial cells and mixtures thereof capable of producing functional biomolecules.

7. The method of producing a biomolecule in a bioreactor under simulated microgravity culture conditions of claim 1 wherein said culture matrix is micro carrier beads.

8. The method of producing a biomolecule in a bioreactor under simulated microgravity culture conditions of claim 1 wherein the functional biomolecules are suitable for therapeutic use.

9. The method of producing biomolecules in a bioreactor under simulated microgravity culture conditions of claim 7 wherein the functional biomolecules are suitable for therapeutic use.

10. The method of producing a biomolecule in a bioreactor under simulated microgravity culture conditions of claim 1 wherein the mammalian cells are human cells.

11. The method of producing a biomolecule in a bioreactor under simulated microgravity culture conditions of claim 1 wherein the organ cells are human organ cells.

12. The method of producing biomolecules in a bioreactor under simulated microgravity culture conditions of claim 1 further comprising the steps of:

selecting mammalian cells capable of producing erythropoietin;
culturing the mammalian cells under conditions to produce erythropoietin;
collecting the culture media and cells; and
separating the erythropoietin from the source consisting of the group of culture media, lysed cells and mixtures thereof.

13. The method of claim 12 further purifying erythropoietin wherein the erythropoietin is suitable for human therapeutic use.

14. The method of producing a biomolecule in a bioreactor under simulated microgravity culture conditions of claim 1 further comprising the steps of:

selecting mammalian cells capable of producing 1,25-dihydroxy-vitamin D3;
culturing the cells under conditions to produce 1,25-dihydroxy-vitamin D3;
collecting the cells; lysising the cells; and
separating the 1,25-dihydroxy-vitamin D3.

15. The method of claim 14 of producing and purifying 1,25-dihydroxy-vitamin D3 in a bioreactor under simulated microgravity culture conditions wherein the 1,25-dihydroxy-vitamin D3 is suitable for human therapeutic use.

16. The method of producing a biomolecule in a bioreactor under simulated microgravity culture conditions of claim 1 wherein the isolated functional biomolecules are suitable for therapeutic use.

17. The method of producing a biomolecule in a bioreactor under simulated microgravity culture conditions of claim 1 wherein the functional biomolecules are suitable for diagnostic use.