ABSTRACT

A method for the production of functional proteins including hormones by renal cells in a three dimensional culturing process responsive to shear stress uses a rotating wall vessel. Natural mixture of renal cells expresses the enzyme 1-α-hydroxylase which can be used to generate the active form of vitamin D: 1,25-diOH vitamin D₃. The fibroblast cultures and natural mixture of renal cells expresses the gene for erythropoietin which is expressed due to shear stress, such as toxin receptors megalin and cubulin (gp280). Also provided is a method of treating an in-need individual with the functional proteins produced in a three dimensional co-culture process responsive to shear stress using a rotating wall vessel.
OTHER PUBLICATIONS


Schwarz et al., Cell culture for three-dimensional modeling in rotating-wall vessels; An application of simulated microgravity, 1992, journal of Tissue Culture Methods, vol. 14, pp. 51-57.


* cited by examiner
Fig. 1A

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

Fig. 1B
Fig. 2A
Fig. 2B

No. of events/channel

Fluorescein fluorescence
(arbitrary fluorescence units)

Antibody binding
(arbitrary fluorescence units)
Fig. 2C
Antibody dilution

Antibody binding (arbitrary fluorescence units)

Rotating wall vessel

Stirred fermentor

Fig. 2D
Fig. 2E

Antibody binding (arbitrary fluorescence units)

Minimum | Maximum | Minimum | Maximum

gp280 | gp330

Fig. 3A
Band B
control  STLV

Band D
control  STLV

Fig. 3B
Fig. 4A
<table>
<thead>
<tr>
<th></th>
<th>ICAM</th>
<th>MnSOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT</td>
<td>1485</td>
<td>11876</td>
</tr>
<tr>
<td>C40</td>
<td>1977</td>
<td>7748</td>
</tr>
<tr>
<td>C80</td>
<td>6178</td>
<td>10881</td>
</tr>
<tr>
<td>AS40</td>
<td>2856</td>
<td>5902</td>
</tr>
<tr>
<td>AS80</td>
<td>2604</td>
<td>5116.7</td>
</tr>
</tbody>
</table>

Fig. 4B
MnSOD

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NT</td>
<td>11876</td>
</tr>
<tr>
<td>C40</td>
<td>7748</td>
</tr>
<tr>
<td>C80</td>
<td>10881</td>
</tr>
<tr>
<td>AS40</td>
<td>5902</td>
</tr>
<tr>
<td>AS80</td>
<td>5116.7</td>
</tr>
</tbody>
</table>

Fig. 4C
8 Day Bag Control

8 Day STLTV

Fig. 5
PRODUCTION OF FUNCTIONAL PROTEINS: BALANCE OF SHEAR STRESS AND GRAVITY

CLAIM OF BENEFIT OF PRIORITY OF PRIOR-FILED CO-PENDING NON-PROVISIONAL APPLICATION

This application is a continuation of commonly-owned U.S. patent application Ser. No. 10/947,786, filed Sep. 20, 2004, which is a continuation of U.S. patent application Ser. No. 09/532,001, filed Mar. 21, 2000, now U.S. Pat. No. 6,946,246, which is a divisional of U.S. application Ser. No. 09/085,365, filed Apr. 7, 1998, now U.S. Pat. No. 6,730,498, which claims the benefit of provisional U.S. Application Ser. No. 60/043,205, filed Apr. 8, 1997.

CROSS-REFERENCE TO RELATED APPLICATIONS


ORIGIN OF THE INVENTION

The present invention was funded by NIH Grant DK46117, NIH R21, and NASA NRA Grant 9-811. Consequently, the United States government has certain rights in this invention.

The jointly made invention described herein was made by an employee of the United States Government and may be manufactured and used by or for the Government of the United States of America for governmental purposes without the payment of any royalties thereon or therefor.

The invention described herein was also made by inventors in the performance of work under an agreement with Tulane Educational Fund and is subject to the provisions of Section 305 of the National Aeronautics and Space Act of 1958, Public Law 85-568 (72 Stat. 435; 42 U.S.C. §2457).

BACKGROUND OF THE INVENTION

1. Field of the Invention

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 (References Cited Nos. 1-4 (hereinafter referred to simply by number)). In rotating wall vessels, gravity is balanced by equal and opposite physical forces including shear stresses. In engineering terms, this balance has been claimed to simulate 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 rotated 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, and 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 prostate 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, and 4). The rotating wall vessel provides a culture environment suitable for co-cultures of diverse cell types and for formation of three-dimensional tissue constructs.

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-inflamatory immune haven and are implanted into the periureal cavity of Type 1 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). The rotating wall vessels have also been applied to, for example, muscle, 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 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 problem led to the 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 it 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-α-hydroxylase activity are limited by the lack of a renal cell line with regulated expression of the enzyme. The only reports of 1-α-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-α-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 ablish 1,25-diOH-D3 formation sparked an intensive search for extrarenal sites of 1-α-hydroxylase activity (29). Although 1-α-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-α-hydroxylase. Extrarenal 1-α-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-dihydroxy 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, and 35). But these renally derived immortal cell lines such as MDCK or LLP-CK1 retain few if any of the differentiated features characteristic of renal epithelial cells. Similarly, primary cultures rapidly dedifferentiate and modulate as diverse as basement membrane matrices, growth supplements or Millipore 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) (Moestrop, et al., J. Biol. Chem. 273 (9):5325-5342 (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 previously known to express megalin in culture are immortalized placental cells. There is no known renal cell culture which expresses megalin. Cubulin (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. Megalin previously known to express cubulin (gp280) 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 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 longstanding need and desire in the art.

SUMMARY OF THE INVENTION

In one embodiment of the present invention, a method of producing a functional protein comprises the steps of: isolating mammalian cells; placing said cells into a rotating wall vessel containing a cell culture comprising culture media and cell 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, a method of inducing expression of at least one gene in a cell comprises 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.

In yet another embodiment of the present invention, a transcription factor decoy comprises 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 exemplary embodiments of the invention given for the purpose of disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

More particular descriptions of the exemplary embodiments of the invention briefly summarized above may be had by reference to illustrations thereof in the appended drawings. These drawings form a part of the specification. The appended drawings illustrate exemplary embodiments of the invention and therefore are not to be considered limiting in their scope.

FIGS. 1A-1D show homogeneity and structure of human renal epithelial cells in culture. Flow cytometry frequency histograms demonstrate number of cells positive for the proximal tubular marker gamma-glutamyl transferase. FIG. 1A shows the number of cells with gamma-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 the 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.

FIGS. 2A-2E show 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 entraped fluorescein dextran as an endosomal marker and antibody binding allows construction of three-dimensional frequency histograms displaying entraped 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 none of the.
somal membranes can be subdivided into cubulin positive and negative populations (middle panel). This pattern is repeated for anti-megalin binding in renal cortical cells (right panel).

FIG. 2D shows quantization of cubulin and megalin antibody binding to renal cell membranes under various culture conditions. Analysis of protein expression in cultured cells by antibody binding using 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 (upper left panel below). This increased cubulin antibody binding in the cells grown in the slow-turning lateral 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 antisera showed a similar pattern (not shown).

FIG. 2E depicts non-specific (minimum) and peak binding of each antisem 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 (0.1420 kDa) on the abscissa is displayed against isoelectric point (pH 3-10) on the ordinate.

FIGS. 3A-3D show 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 subcloned into the pGEM-T vector and sequenced.

FIGS. 3A-3D show structure and effects of an antisense probe for shear stress response element on rat renal cortical epithelial cells. FIG. 4A shows the probe with sequence CTCGAGCCGATAICGGCTCAG (SEQ ID No:1) has two possible conformations. As a single strand it would fold back on itself to form a binding element for the transcription factor. As a double strand it would then have two binding sites for the transcription factor, one in the sense orientation and one in the antisense orientation.

FIGS. 4B and 4C show effects of an antisense shear stress response element probe on time dependent gene expression. The antisense probe added to conventional 2-dimensional cultures of rat renal cortical cells at 80 nm increases MnSOD in a time dependent manner. Comparison is made to controls with the active binding site scrambled. In contrast the probe has no effect on villin gene expression.

FIG. 4 shows gene expression in a rotating wall vessel via automated gene analysis. Abundance of the expression of over 18,300 genes was assayed by annealing poly A RNA from human renal cortical epithelial cells grown in a rotating wall vessel for 8 days to a filter robotically loaded with oligonucleotide primers. Poly A RNA from a non-adherent bag culture serves as a control. The filters are shown at the top of the diagram, then the analysis of shear stress responsive genes, renal epithelium specific genes, and other genes germane to the current analysis.

DETAILED DESCRIPTION OF THE EXEMPLARY EMBODIMENTS

An exemplary embodiment of 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 of such physical forces include sedimentational shear stress, centrifugal forces, viscosity and Coriolis forces.

In one embodiment, 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 exemplary method include 1.25-dihydroxy-vitamin D₃ and erythropoietin. Representative examples of toxin receptors which can be produced according to the exemplary method include megalin and cubulin. Representative examples of shear stress dependent functional biomolecules which can be produced according to the exemplary method include biomolecules selected from the group consisting of villin, 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 monocytic chemotactic protein-1, megalin, cubulin, erythropoietin and 1-oxy-hydroxylase.

Generally, any mammalian cell could be used in the exemplary 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 cells and endothelial cells. Preferably, the cell that is selected contains...
shear stress response elements. Representative examples of shear stress response elements include GAGACC and GGTCTC.

In the methods of the exemplary embodiments, 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.

Another exemplary embodiment described herein is directed to 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. Generally, an 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. In one embodiment, the cell is selected from the group consisting of an epithelial cell and an endothelial cell. Representative examples of a type of cell which can be used in this method include a 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 two-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 nM.

Another exemplary embodiment of the present invention is directed to a method of 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 embodiment, 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 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” or “STLV” 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 two different conditions.
**Example 3**

**Culture Techniques: Rotating Wall Vessels**

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 vessel known as a 55 ml slow turning lateral vessel (STLV) (1, 45). To initiate cell culture, the slow turning lateral vessel was filled with medium and seeded by addition of cells to the vessel in a few hours. Beads were Cytodex-3 in all protocol except when electron microscopy was planned, when the vessel was filled with medium and seeded by addition of cell suspension (2x10⁶ 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 Cultisphere 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 concentrations of cells and beads from the same pool added to the slow turning lateral vessel (1, 31, and 46). Gas permeable Fluoro- seal bags (Fluoro seal Inc, Urbana III.) 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 Quantization 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 photographed using a Phillips EM/200 electron microscope. For electron microscopy the easily sectioned Cultisphere GL beads replaced Cytodex-3 beads, which are almost impossible to section.

**Example 6**

**Analysis of the Proximal Tubule Epithelial Marker, γ-glutamyl Transpeptidase**

The renal cortical cells were 75±4% (n=4) proximal tubules as determined by flow cytometry analysis of aliquots for the proximal marker γ-glutamyl transferase using Schiff base trapping of cleavage products of L-γ-glu-4-methoxy-4-β-naphthylamine (44) (FIGS. 1A-1B).

**Example 7**

**Analysis of the Endosomal Distribution of Megalin and Cubulin by Flow Cytometry**

To quantitatively and endosomal expression of cubulin, megalin, and aquaporin-2 cells in conventional culture, stirred fermentors, and slow turning lateral vessels, 0.3 mg/ml 10S fluorescein-dextran was added to each cell culture for 10 minutes at 37°C in the CO₂ incubator. This step loads an entrapped fluorescent dye into the early endosomal pathway (9, 47). Cells were then immediately diluted into ice cold phosphate buffered saline and washed once. Next, the cells were homogenized with 6 passes of a tight fitting glass-Teflon motor driven homogenizer. A post-nuclear supernatant was formed as the 11,000 g supernatant, 18,000 g pellet of membrane vesicles (FIGS. 2A-2C).

Aliquots of membrane vesicles were labeled with megalin or cubulin antisera. The megalin and cubulin antisera were rabbit polyclonal raised to affinity purified and chromatographically pure receptor (43, 48). Membrane vesicles were first pre-incubated in 50% normal goat serum for 2 hours to reduce non-specific binding of secondary antisera raised in goat. After washing aliquots of membrane vesicles were stained with serial log dilution of antisera and incubated at 4°C overnight. After further washing, 1:40 of goat anti-rabbit affinity purified rat pre-absorbed phycoerythrin conjugated secondary antisera was added, and incubated for 4 hours at room temperature. Prior to flow cytometry, the membrane vesicles were washed and resuspended in 200 mM mannitol, 100 mM KCl, 10 mM HEPES, pH 8.0 with Tris to which had been added 10 mM nigericin. In the presence of potassium, nigericin collapses pH gradients, ensuring optimal fluorescence of the highly pH dependent fluorescein-dextran emission. Fluorescein-dextran and antibody staining tagged by phycoerythrin were now analyzed and co-localized on a vesicle-by-vesicle basis by flow cytometry (FIGS. 2A-2C).

**Example 8**

**Differential Display**

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 culture, gas permeable 2-dimensional bag controls (FIGS. 3A and 3B). Differential display was performed using Delta RNA Fingerprinting system (Clontech labs, Palo Alto Calif.). 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 (Promega, Madison Wis.) and sequenced using fMOL cycle sequencing system (Promega, Madison, Wis.). 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 of the changes by Northern blot analysis.**
Detection of Gene Expression in Cell Cultures by RT-PCR

Cell aggregates from the rotating wall vessel culture were washed once in ice cold phosphate buffered saline and snap frozen at -70°C until RNA was isolated. Total RNA was first isolated, followed by isolation of poly A+ RNA. Following reverse transcription, 10%-20% of each cDNA was amplified (Robocycler 40, Stratagene, La Jolla, Calif.) using 95°C C. denaturation, 63°C annealing and 72°C 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 Gene-tuner software. Twenty percent (20%) of the PCR reaction was electrophoresed on agarose/ethidium bromide gels and visualized under UV light so that a comparison of amplified gene fragments could be made to DNA standards (HaeIII digested X174 DNA, Promega) electrophoresed on the same gel (FIGS. 3C and 3D). Representative fragments amplified for each gene in question were isolated from gels and direct sequenced to assure identity of the PCR product. In addition, 5% of the same cDNA were subjected to PCR for expression of the housekeeping mRNA, glyceraldehyde 3-phosphate dehydrogenase, and β-actin to assure that similar amounts of input RNA and that similar efficiencies of reverse transcription were being compared. Each cDNA was run in at least three dilutions to ensure that measurements were made on the initial linear portion of the response curve.

Example 10

Genetic Decoys

Double stranded genetic decoys matching the sequence of a known shear stress response element were synthesized (Chemicon International Inc., La Jolla, Calif.) (structure and sequence shown in FIG. 4A). These decoys had a terminal phosphothiorate moiety to prevent intracellular lysis and a phosphodiester backbone to facilitate passage across cell membranes (49). 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 a known shear stress response element were synthesized 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 (PolyATtract 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 [LLNL](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 1α-hydroxylase Activity

As the 1α-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 Menten 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-dihydroxy vitamin D₃ generated in vitro was quantified as described (23-27). In vitro 3 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-dihydroxy D₃ are then subjected to normal phase HPLC on a Beckman model 344 liquid chromatography system. Normal-phase HPLC was performed with a Zorbax-Stil 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 and suspended in ethanol and counted by radio receptor assay or radio immunoassay (25-26). Plasma 1,25-dihydroxy vitamin D₃ was assayed in a similar fashion, but as the product is already formed, assay begins with extraction into acetonitrile (23-26). Hence, all measurement of 1α-hydroxylase activity in cells included determination of the Michaelis Menton Km and Vmax of the enzyme. The Michaelis Menton parameters were determined by automated curve fitting.

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/M12 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 rotating vessel (HARV) for culture under increased shear stress con-
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 subjected to shear stress conditions. The Hep3B cells were placed into culture in DMEM with 10% fetal bovine serum in a static flask culture. The resultant culture was split, one half remaining in static flask culture and the other half inoculated into an HARV for culture under increased shear stress conditions. The cells aggregated on the beads. After 24 hours of growing the Hep3B cells in an 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 14

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 an integrated perfused rotating wall vessel culture to reintroduce graded shear. This protocol utilizes a library of promoters driving luciferase reporter 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. Results

The proportion of proximal tubular cells in human renal cell fractions isolated by differential trypsinization was assayed using an entrapped fluogenic 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 85–8% (n=44 proximal tubular cells (FIC. 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 the high rates of glucose consumption assayed as 30 mg/dl glucose/100,000 cells/day. A cell doubling time of 4a3 days was assayed using Alamar blue in the rotating wall vessel compared to 4a2 days in conventional culture (n=4).

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. 1C and 1D). Quantification 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 also that 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 3511 microvilli per field.

<table>
<thead>
<tr>
<th>Culture Conditions</th>
<th>% Proximal Tubular Markers</th>
<th>Micr0villi Per Field</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional 2-D culture</td>
<td>85</td>
<td>21</td>
</tr>
<tr>
<td>Pure culture in rotating wall vessel</td>
<td>99</td>
<td>104</td>
</tr>
<tr>
<td>Normal cortical cell mix</td>
<td>85</td>
<td>3511</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 been cloned, while the human sequences 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 fluoresceine of a phycoerythrin tagged secondary antibody, shows an almost two log increase in binding with antibody dilution (FIG. 2D). 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. 2E. Binding of normal serum and minimal dilution of primary antisera were not detectably different. Binding curves for anti-megalin antisemur showed a similar pattern (not shown), but the peak binding was a little lower.
The 1-α-hydroxylase activity of the various rat renal cell cultures detected as production of 1,25-diOH D$_3$.

<table>
<thead>
<tr>
<th>Cell Sample</th>
<th>1,25-diOH D$_3$ concentration (pg/ml)</th>
<th>Volume of Supernatant (ml)</th>
<th>1,25-diOH D$_3$ production (pg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boiled static control</td>
<td>&lt;2, not detectable</td>
<td>7 ml</td>
<td>Not detectable</td>
</tr>
<tr>
<td>Boiled static control II</td>
<td>&lt;2, not detectable</td>
<td>7 ml</td>
<td>Not detectable</td>
</tr>
<tr>
<td>Static control</td>
<td>&lt;2, not detectable</td>
<td>7 ml</td>
<td>Not detectable</td>
</tr>
<tr>
<td>Static control II</td>
<td>&lt;2, not detectable</td>
<td>7 ml</td>
<td>Not detectable</td>
</tr>
<tr>
<td>Boiled rotating wall vessel</td>
<td>&lt;2, not detectable</td>
<td>55 ml</td>
<td>Not detectable</td>
</tr>
<tr>
<td>Rotating wall vessel</td>
<td>14.2</td>
<td>55 ml</td>
<td>781</td>
</tr>
</tbody>
</table>

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-α-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-α-hydroxylase activity than under conventional culture conditions.
Erythropoietin production is controlled by a shear stress element which mediates changes observed during graded gravitational 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-hydroxy-vitamin D3 to the active 1,25-dihydroxy-vitamin D3 form. The exemplary embodiment of 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.

Magelin and cubulin represent the first pattern of change, as these proteins are restricted in distribution to renal cortical tubular epithelial cells. The increase in magelin mRNA and protein and cubulin protein expression is therefore unequivocal evidence for changes in the epithelial cells. This result provides an important new tool for studies of nephrotoxicity.

Long suspected to play a role in renal toxicity, the tissue restricted giant glycoprotein receptors magelin and cubulin have recently been shown to be receptors for common nephrotoxins. Magelin 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). Culture within a rotating wall vessel 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 result 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.

Magnesium dependent superoxide dismutase represents a third pattern of response: a mitochondrial enzyme, ubiquitous in 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 condition 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 for the classic 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
biological response mediators in the vessels and for investigating protein expression. Changes in villin observed by RT-PCR have shear stress response elements and change gene 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 HT-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 exemplary embodiments herein present direct evidence that modest shear stress decreases magnesium dependent superoxide dismutase in association with an inverse effect on interleukin-1.

The results herein demonstrate 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 semiquantitative 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, 67). 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, Coriolis motion, and tangential gravity-induced sedimentation are themselves tangential to the cell biology. Several lines of evidence are documented to indicate that shear stress responses are one of the components of the biological response. This research opens the door for analysis of other biological response mediators in the vessels and for 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 described herein 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.

The following references were cited herein:
9) Hammond, T. G., et al., J. Mem. Biol. (in press);
10) Soon-Shiong, P., et al., PNAS USA 90:5843-5847, 1993;
14) Goodwin et al., U.S. Pat. No. 5.496,722, 1996;
15) Wolf et al., U.S. Pat. No. 5,155,034, 1992;
16) Goodwin et al., U.S. Pat. No. 5,153,132, 1992;
23) Hollis, B. W., et al., PNAS USA 87:6009-6013, 1990;
30) Galli et al., et al., Am J Physiol 268(4):F746-F753, 1995;
31) Langer et al., Science 260:920 926, 1993;
46) Topper, J. N., et al., J. Vasc Res 33:S100A
49) Schlingensiepen, R., Antisense—From Technology to
Therapy, eds. Schlingensiepen, et al., (Blackwell Science) pp. 1-87, 1997;
51) Christensen, E. I., et al., JASN 8:59A, 1997;

Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. These patents and publications are incorporated herein by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

This application hereby incorporates by reference the sequence listing and other material found in the text file named “Sequence_ID_NO_1_ST25.txt”, which was created on Nov. 10, 2008, has a size of 1.07 KB, and has been submitted as a text file via EFS-Web.

Although only a few exemplary embodiments of this invention have been described in detail above, a skilled artisan will readily appreciate that many modifications are possible in the exemplary embodiments without materially departing from the novel teachings and advantages of this invention. Accordingly, all such modifications are intended to be included within the scope of this invention as defined in the following claims. In the claims, means-plus-function and step-plus-function clauses are intended to cover the structures or acts described herein as performing the recited function and not only structural equivalents, but also equivalent structures. Thus, although a nail and a screw may not be structural equivalents in that a nail employs a cylindrical surface to secure wooden parts together, whereas a screw employs a helical surface, in the environment of fastening wooden parts, a nail and a screw may be equivalent structures.

SEQUENCE LISTING

<table>
<thead>
<tr>
<th>SEQ ID NO.</th>
<th>LENGTH</th>
<th>TYPE</th>
<th>ORGANISM</th>
<th>OTHER INFORMATION</th>
<th>PUBLICATION INFORMATION</th>
<th>TITLKE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22</td>
<td>DNA</td>
<td>Unknown</td>
<td>Single-stranded, Linear Topology, Hypothetical: no; Antisense: yes</td>
<td></td>
<td>Production of Functional Proteins: Balance Of Shear Stress and Gravity</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PATENT DOCUMENT NUMBER</th>
<th>PATENT FILING DATE</th>
<th>PATENT PUBLICATION DATE</th>
</tr>
</thead>
<tbody>
<tr>
<td>6,730,498</td>
<td>1998-04-08</td>
<td>2004-05-04</td>
</tr>
</tbody>
</table>

Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. These patents and publications are incorporated herein by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

This application hereby incorporates by reference the sequence listing and other material found in the text file named “Sequence_ID_NO_1_ST25.txt”, which was created on Nov. 10, 2008, has a size of 1.07 KB, and has been submitted as a text file via EFS-Web.

Although only a few exemplary embodiments of this invention have been described in detail above, a skilled artisan will readily appreciate that many modifications are possible in the exemplary embodiments without materially departing from the novel teachings and advantages of this invention. Accordingly, all such modifications are intended to be included within the scope of this invention as defined in the following claims. In the claims, means-plus-function and step-plus-function clauses are intended to cover the structures or acts described herein as performing the recited function and not only structural equivalents, but also equivalent structures. Thus, although a nail and a screw may not be structural equivalents in that a nail employs a cylindrical surface to secure wooden parts together, whereas a screw employs a helical surface, in the environment of fastening wooden parts, a nail and a screw may be equivalent structures.

SEQUENCE LISTING

<table>
<thead>
<tr>
<th>SEQ ID NO.</th>
<th>LENGTH</th>
<th>TYPE</th>
<th>ORGANISM</th>
<th>OTHER INFORMATION</th>
<th>PUBLICATION INFORMATION</th>
<th>TITLKE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22</td>
<td>DNA</td>
<td>Unknown</td>
<td>Single-stranded, Linear Topology, Hypothetical: no; Antisense: yes</td>
<td></td>
<td>Production of Functional Proteins: Balance Of Shear Stress and Gravity</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PATENT DOCUMENT NUMBER</th>
<th>PATENT FILING DATE</th>
<th>PATENT PUBLICATION DATE</th>
</tr>
</thead>
<tbody>
<tr>
<td>6,730,498</td>
<td>1998-04-08</td>
<td>2004-05-04</td>
</tr>
</tbody>
</table>

Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. These patents and publications are incorporated herein by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

This application hereby incorporates by reference the sequence listing and other material found in the text file named “Sequence_ID_NO_1_ST25.txt”, which was created on Nov. 10, 2008, has a size of 1.07 KB, and has been submitted as a text file via EFS-Web.

Although only a few exemplary embodiments of this invention have been described in detail above, a skilled artisan will readily appreciate that many modifications are possible in the exemplary embodiments without materially departing from the novel teachings and advantages of this invention. Accordingly, all such modifications are intended to be included within the scope of this invention as defined in the following claims. In the claims, means-plus-function and step-plus-function clauses are intended to cover the structures or acts described herein as performing the recited function and not only structural equivalents, but also equivalent structures. Thus, although a nail and a screw may not be structural equivalents in that a nail employs a cylindrical surface to secure wooden parts together, whereas a screw employs a helical surface, in the environment of fastening wooden parts, a nail and a screw may be equivalent structures.

SEQUENCE LISTING

<table>
<thead>
<tr>
<th>SEQ ID NO.</th>
<th>LENGTH</th>
<th>TYPE</th>
<th>ORGANISM</th>
<th>OTHER INFORMATION</th>
<th>PUBLICATION INFORMATION</th>
<th>TITLKE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22</td>
<td>DNA</td>
<td>Unknown</td>
<td>Single-stranded, Linear Topology, Hypothetical: no; Antisense: yes</td>
<td></td>
<td>Production of Functional Proteins: Balance Of Shear Stress and Gravity</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PATENT DOCUMENT NUMBER</th>
<th>PATENT FILING DATE</th>
<th>PATENT PUBLICATION DATE</th>
</tr>
</thead>
<tbody>
<tr>
<td>6,730,498</td>
<td>1998-04-08</td>
<td>2004-05-04</td>
</tr>
</tbody>
</table>
What is claimed is:

1. A method of producing active 1,25-dihydroxy vitamin D₃ comprising the steps of:
   isolating at least one renal cell;
   placing said at least one renal cell in a rotating wall vessel containing culture media and a cell culture matrix;
   culturing said at least one renal cell in the rotating wall vessel to produce three-dimensional cell aggregates and a supernatant; and
   collecting 1,25-dihydroxy vitamin D₃ from the supernatant.

2. A method of producing active 1,25-dihydroxy vitamin D₃ comprising the steps of:
   isolating a natural mixture of cells from a renal cortex and isolating purified renal proximal tubular cells;
   placing said isolated natural mixture of cells from a renal cortex and said isolated purified renal proximal tubular cells in a rotating wall vessel containing culture media and a cell culture matrix;
   co-culturing said isolated natural mixture of cells and said isolated purified renal proximal tubular cells in the rotating wall vessel to produce three-dimensional cell aggregates and supernatant; and
   collecting 1,25-dihydroxy vitamin D₃ from the supernatant.