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Goodwin et al.

PRODUCTION OF NORMAL MAMMALIAN ORGAN CULTURE USING A MEDIUM CONTAINING MEM-ALPHA, LEIBOVITZ L-15, GLUCOSE, GALACTOSE, FRUCTOSE

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Related U.S. Application Data


References Cited

U.S. PATENT DOCUMENTS

3,883,393 5/1975 Knazek et al. 195/1.8
4,190,725 9/1980 Knazek et al. 435/285
4,352,887 10/1982 Reid et al. 435/240
4,757,017 7/1988 Cheung 435/240.23
4,940,853 7/1990 Vandenburgh 435/240.23
4,934,489 10/1990 Naughton et al. 435/240.1
4,988,623 1/1991 Schwarz et al. 435/286
5,026,637 6/1991 Soule et al. 435/29
5,026,650 6/1991 Schwarz et al. 435/286
5,032,508 7/1991 Naughton et al. 435/32
5,155,035 10/1992 Schwarz et al. 435/240.24

OTHER PUBLICATIONS


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ABSTRACT

Normal mammalian tissue and the culturing process has been developed for the three groups of organ, structural and blood tissue. The cells are grown in vitro under microgravity culture conditions and form three dimensional cells aggregates with normal cell function. The microgravity culture conditions may be microgravity or simulated microgravity created in a horizontal rotating wall culture vessel. The medium used for culturing the cells, especially a mixture of epithelial and mesenchymal cells contains a mixture of Mem-alpha and Leibovits L15 supplemented with glucose, galactose and fructose.

6 Claims, 6 Drawing Sheets
Fig. 10
PRODUCTION OF NORMAL MAMMALIAN ORGAN CULTURE USING A MEDIUM CONTAINING MEM-ALPHA, LEIBOVITZ L-15, GLUCOSE GALACTOSE FRUCTOSE

This is a division of application Ser. No. 08/066,292, filed May 25, 1993 now U.S. Pat. No. 5,496,722.

ORIGIN OF THE INVENTION

The invention described herein was made in the performance of work under a NASA contract and is subject to the provisions of Section 305 of the National Aeronautics and Space Act of 1958, Public Law 85-508 (72 Stat. 435; 42 U.S.C. 2457).

RELATED PATENTS AND APPLICATIONS


Normal mammalian tissue has been grown for limited periods of time as two-dimensional monolayers on gelled substrate or other surface for anchoring the cells. Adult colonic epithelial cells have been found to be more difficult to culture than fetal colonic epithelial cells. Buset et al. “Defining Conditions to Promote the Attachment of Adult Human Colonic Epithelial Cells”, In Vitro Cell. & Dev. Biol., Vol. 23. No. 6 pp. 403-412 (June 1987). To mimic a more normal environment using monolayer culture, cocultures were prepared using two cell types, often with a “feeder layer” of fibroblasts or other cells to supply the primary cells with nutrients and other factors difficult to incorporate into a substrate and to provide the cellular interaction believed to be necessary for the production of differentiated tissue. Reid et al., “Culturing Hepatocytes and Other Differentiated Cells”, Hepatology, Vol. 4, No. 3, pp. 548-559 (1984); Haake et al. “Retention of Differentiated Characteristics in Human Fetal Keratinocytes In Vitro”, In Vitro Cell. & Dev. Biol., Vol. 25 No. 25 pp. 592–600 (July 1989). Also, monolayers “conditioned” with fibroblast cells have been used to impart into the substrate the soluble growth factors for epithelial cells. Kabalin et al. “Clonal Growth of Human Prostatic Epithelial Cells Is Stimulated by Fibroblasts”, The Prostate, Vol. 14, pp. 251–263 (1989). Monolayers do not produce a three dimensional tissue, but rather a two-dimensional spread of cells. Often the cells developed by monolayer culture and coculture become undifferentiated and lack normal function.

Three dimensional in vitro models of differentiated tissue have been produced, however, the cells often do not demonstrate normal cellular activity. Embryonic avian skeletal muscle cells have been grown in vitro on expandable membranes which are gradually and substantially, continuously stretched to simulate the mechanical stimulation of cells experienced in vivo. U.S. Pat. No. 4,940,853, Method for Growing Tissue Specimens In Vitro, Vadenburgh, Jul. 10, 1990. The expandable support membrane supports development of three dimensional structures which more closely resemble tissue grown in vivo, however, normal independent cellular activity was not identified. Additionally, three
dimensional human mammary epithelial cells have been grown in collagen. U.S. Pat. No. 5,026,637, Soule, et al., Jun. 25, 1991. However, the cells, under the culture conditions, did not undergo terminal differentiation and cell senescence, but rather were “immortal” in that they retained the capacity to divide forever. Thus, normal cellular activity and naturalization was not observed.

The study of the organ systems exemplifies the complex interrelationship of neighboring cells in mammalian tissue necessary for normal three dimensional tissue growth. Mammalian organ systems have been studied to determine the relationship of the cell types that make up the organ as well as their effects on each other. Studies on normal tissue differentiation have indicated an important interaction between epithelial cells and mesenchymal cells and the production and differentiation of the cells from fibroblasts.

Kaye et al., “The Colonic Pericyrtial Fibroblast Sheath: Replication, Migration, and Cytodifferentiation of a Mesenchymal Cell System in Adult Tissue” Gastroenterology, Vol. 60, No. 4, pp. 515–536 (1971). Furthermore, small intestine tissue is exemplary of organ tissue types with epithelial cells with functional brush borders and organized columnar cells associated with mesenchymal cells. Other organs have similar organization. Small intestine tissue has physical support in vivo so that the tissue forms crypts which increase the surface area of the organ. In addition to the crypt formation in vivo, there are also immunocytochemical markers for particular cell types with normal functionality as well as cell types that are in developmental phases to produce functional organ tissue.

Immunocytochemical properties further characterize normal cells and tissue. Keratin, vimentin and Factor VIII antibodies detect epithelial, fibroblastic and endothelial cells respectively. Villin is a cytoskeletal protein only found in epithelial cells from the small intestine and colon. The angiomaker is present in certain precursor endothelial cells, particularly dividing cells. Sucrase is an enzyme found in the epithelial cell brush border of the small intestine. Basement membrane and extracellular matrix development and differentiation, and the deposition of proteoglycan as large-scale three dimensional configurations has not been shown in vitro by culturing chondrocytes. Typical culture durations achieved in the more successful instances range from 10 days to 30 days, the 30-day point being a transition towards static culture or cellular degeneration and death. The success achieved to date has had limited 3-dimensionality. Typical cell culture methodologies include isolating chondrocytes from various articular surfaces and plating the isolated chondrocytes as a monolayer in a T-flask. Various manipulations were then utilized to encourage chondrocyte expansion and differentiation, and the deposition of proteoglycan into the interstitial matrices. These maneuvers include various growth factor augmentations, an effort to emuliate in situ conditions. The extent to which the cells can be maintained in situ is on the order of tens of years as seen in the normal adult. Conversely, in standard cell culture the extent which cells can be maintained in a growth phase or a normal static maintenance phase is on the order of 22 days. It has been demonstrated that multiple cell-surface receptors exist that are important in cellular growth and differentiation, and extracellular matrix formation. Cell surface receptors are labile to destructive cell surface shear.

Cartilage cells were reportedly cultured for 13 months using a solid substrate in conventional culture vessels. U.S. Pat. No. 4,757,017 Cheung et al., Jul. 12, 1988. The solid substrate was a porous calcium compound. Multilayer cell growth was observed and the cells maintained their phenotype.

Bone marrow has been grown using conventional monolayer cell culture technique. Human bone marrow cell culture technique. Human bone marrow cell production declines over time in monolayer culture. Naughton et. al. disclosed a bone marrow culture system in co-culture with stromal cells or mixtures of cell types on a three dimensional support. U.S. Pat. No. 4,963,489. The three dimensional support was coated by cells such as fetal fibroblasts over a collagen layer on a mesh support.

Mammalian tissue has been grown in bioreactors providing low shear from abnormal cell types such as tumor cells or cocultures with normal and tumor cells. Goodwin et al., “Morphological Differentiation of Colon Carcinoma Cell Lines HT-29 and HT-29K in Rotating-Wall Vessels,” In Vitro Cell. Dev. Biol. Vol. 28A, pp. 47–60, January 1992. The bioreactor designs are disclosed in U.S. Pat. Nos. 4,988,623; 5,026,650 and 5,153,131. Co-culture of tumor and normal cells in solid-state culture has been reported as shown in U.S. Pat. No. 4,352,887 to Reid et al. issued Oct. 5, 1982. However, since tumor or abnormal cells grow under conditions that normal cells cannot tolerate, the growth of nonnormal cells does not predict success for normal mammalian culture under the same or similar culture conditions.

There are many potential uses of artificially produced tissue for different mammalian systems. For example, small intestine tissue can be used as a model for the molecular and clinical treatment of diseases such as inflammatory bowel disease (Crohn’s, ulcerative colitis), malabsorptive syndromes (short-gut syndrome), numerous infectious diseases and tumors of the small bowel. The tissue can be a model for the therapeutic trials prior to in vivo experimentation. However cultures longer than 7 weeks have been difficult to achieve, since crypt cells are unable to survive standard culture regimens, and two-dimensional organ cultures do not support the de novo assembly of stroma and its contribution to epithelial cell growth. Shamsuddin, “Colon Organ Culture as a Model for Carcinogenesis”, Colon Cancer Cells, Moyer and Poste, Eds. Academic Press, Inc. 1990.

Also, a mammalian structural tissue such as a cartilage model of high fidelity is important in clinical studies.
are numerous maladies associated with cartilage, including
but not limited to knee-joint injuries, back injuries, articular-
surface injuries, inflammatory diseases such as arthritis and
temoral-mandibular joint disease. Beyond the diseases are
the natural processes of maturation through puberty and the
geriatric inability to repair and maintain articular surfaces. A
tissue model would be beneficial for the analyses and
development of therapeutic protocols. Moreover, these mod-
els become essential in tailoring pharmaceuticals, i.e.; non-
steroidal anti-inflammatories where the pharmaceutical may
actually become detrimental to the chondrocyte by way of
degradation into secondary metabolites.

The artificial production of functional blood tissue such as
normal bone marrow could be used in patients in need of
bone marrow replacement which have lost the ability to
produce the tissue. The tissue could be grown from a sample
and transplanted back to the patient. Also, bone marrow
material can be transplanted into a non-autologous recipient.

SUMMARY OF THE INVENTION

Aggregates of normal mammalian cells have been pro-
duced in vitro for each of the three major tissue groups. The
cell aggregates exhibited three dimensional tissue growth and
functional interrelationship by cell to cell contact. Functional
cells with normal morphology have been pro-
duced for organ, structural and blood producing tissues. Cell
function was in some cases confirmed immunohistochemi-
cally by antibodies specific to particular cellular components
characterizing cell types.

The aggregates were produced under "microgravity cul-
ture conditions" which can be microgravity or simulated
microgravity. Simulated microgravity was created in unit
gravity by controlling the horizontal rotation of a culture
vessel containing normal mammalian cells, culture matrix
and completely filled with culture media. In the preferred
process a horizontally rotating wall culture vessel produced the
simultaneous conditions of (1) collocation of cells and
culture matrix with similar or differing sedimentation prop-
erties in a similar spatial region, (2) freedom for three
dimensional spatial orientation of tissues formed by the
culturing of the cells and (3) low shear and essentially no
relative motion of the culture environment with respect to
the walls of the culture vessel. The culture conditions
included mass transfer under microgravity conditions with
exchange of nutrients for metabolic waste and appropriate
gas exchange in the culture system.

The process for producing the normal mammalian tissue is
particularly unique in that the resultant product is normal
tissue of a size of 2 millimeters and larger. The size of the
tissue cultured is significant because assembly of three
dimensional masses of this size is not possible without the
complex functional interrelationship of the normal mamma-
lan cells.

Functional three dimensional organ tissue masses have
been produced artificially from an inoculum of predomi-
nantly normal differentiated epithelial cells and predomi-
nantly normal differentiated mesenchymal cells. The epithe-
lial and mesenchymal cells were preferably disassociated
prior to introduction into the culture vessel. The epithelial
and mesenchymal cells were introduced in a vessel with a
culture matrix preferably of generally spherical microcarri-
ers. Tissue engineering was achieved by selected introduc-
tion of the mesenchymal cells and culture matrix for a
preselected culture period prior to transfer of epithelial cells
to the culture vessel.

A three dimensional normal organ tissue mass grew in
association with the culture matrix having functional epi-
thelial cells and functional mesenchymal cells. Also, the
tissue developed cells immunohistochemically characterized as
functional endothelial precursor cells in significantly greater
tissue conditions which can be microgravity or simulated
culture matrix of Type I rat tail collagen and

FIG. 1 is a view at 200x magnification of a three dimen-
sional normal organ tissue mass grown in vitro on stromal-like matrices and introduced into the
vessel. Also, culture matrix of Type I rat tail collagen and
biopolymers can be used.

Relatively large normal mammalian three dimensional
tissue masses were cultured using microgravity culture
conditions. The ability of the cells to grow into large
aggregates supports the functional relationship achieved by
cell to cell contact of normal cells. The process and normal
tissue of this invention can be used advantageously as an in
vitro produced mammalian product.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a view at 200x magnification of a three dimen-
sional artificial normal organ tissue mass.

FIG. 2 is a photograph of artificial normal organ tissue at
200x magnification.

FIG. 3 is a photograph of artificial normal organ tissue at
500x magnification.
patients, predominantly organ transplant donors, with no base medium supplemented with many growth factors and passaged as required by enzymatic dissociation with a solution of 0.1% Trypsin, 0.1% EDTA, for 15 minutes at 37°C. After incubation with the appropriate enzymes, the cells were centrifuged at 800xG for 10 minutes in Corning flasks by enzymatic digestion, washed once with calcium- and magnesium-free PBS (CMF-PBS), and assayed for viability by Trypan Blue dye exclusion (GIBCO). Cells were held on ice in fresh growth medium until inoculation. In the preferred process the primary inoculum introduced into the culture vessel was 2x10^6 mesenchymal cells/ml in a 125-ml volume with 5 mg/ml Cytodex-3 microcarrier beads (Pharmacia, Piscataway, N.J.). Cytodex-3 microcarriers were Type I, collagen-coated dextran beads, 175 microns in diameter. After the primary inoculum was prepared for seeding, it was transferred to a culture vessel filled with culture media and cultured at microgravity conditions.

In a preferred method the microgravity conditions were simulated in unit gravity by a horizontal rotating wall vessel (RWV). A preferred RWV bioreactor is described in U.S. Pat. No. 5,026,650 to Schwarz et al. issued Jun. 25, 1991 and is incorporated by reference herein. The rotation of the culture vessel (bioreactor) was controlled to produce the simultaneous conditions of (1) collocation of cells and culture matrix with similar or differing sedimentation properties in a similar spatial region, (2) freedom for three dimensional spatial orientation of tissues formed by the culturing of the cells and (3) low shear and essentially no relative motion of the culture environment with respect to the walls of the culture vessel. The trajectory of the cell aggregates was determined. The speed of the rotation of the culture vessel was increased if the cell aggregates fell excessively inward and downward on the downward side of the rotational cycle and excessively outward and insufficiently upward on the upgoing side of the rotational cycle to prevent wall impact. The rotation of the culture vessel was decreased in response to excessive accumulation of tissue aggregation near the outer wall of the culture vessel so as not to restrict three dimensional growth. As the tissue aggregates grew the rotation was adjusted to obtain minimal collision frequency and intensity.

The culture media used in the RWV can be varied. A 1:1 mixture of M3:2 medium and standard minimal essential medium-alpha (MEM-alpha) (GIBCO) supplemented with 10% FBS was used. A preferred media designated GTSF-2 was found to meet the growth requirements of the system without the need for unique growth factors and other complex components found in other media including M3:2. The GTSF-2 media is a trisugar-based medium with glucose, galactose and fructose supplemented with 6% FBS. A preferred formulation for GTSF-2 media is listed in Table 1 below. The pH was adjusted to 7.4 with 1N NaOH.

<table>
<thead>
<tr>
<th>Component</th>
<th>Source/Order or Designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEM-alpha supplemented with 2.25 g/ml</td>
<td>400 ml (40%) GIBCO/430-1900EB</td>
</tr>
<tr>
<td>NaHCO3</td>
<td>600 ml (60%) GIBCO/430-1300EB</td>
</tr>
<tr>
<td>NaHCO3</td>
<td>1.35 g/liter Sigma/S-5761</td>
</tr>
<tr>
<td>HEPES</td>
<td>3.0 g/liter Research Organics/603H-2</td>
</tr>
<tr>
<td>Folic acid</td>
<td>6.67 g/liter Sigma/T-8765</td>
</tr>
<tr>
<td>0.5% Nicotinic acid</td>
<td>0.67 g/liter Sigma/N-1426</td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>0.6 g/liter Difco/0118-01</td>
</tr>
<tr>
<td>I-Insulin</td>
<td>0.024 g/liter Sigma/I-5125</td>
</tr>
<tr>
<td>Fructose</td>
<td>0.13 g/liter Sigma/F-3510</td>
</tr>
<tr>
<td>Galactose</td>
<td>0.25 g/liter Sigma/G-5388</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>1.0 g/liter Sigma/G-5250</td>
</tr>
<tr>
<td>300 mM L-Glutamine</td>
<td>10 ml/liter Sigma/G-5763</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>1 ml/liter GIBCO/600-5750AD</td>
</tr>
<tr>
<td>Fungizone</td>
<td>2 ml/liter GIBCO/600-5295AE</td>
</tr>
<tr>
<td>Insulin-transferrin-sodium-selenite</td>
<td>5 ml/liter Sigma/I-1884</td>
</tr>
<tr>
<td>Fetal bovine serum</td>
<td>80 ml (6%) Hyclone/A-1111-1</td>
</tr>
</tbody>
</table>

The primary inoculum of mesenchymal cells was allowed to grow for a minimum of 2-3 days before the medium was changed. Then 2x10^6 epithelial cells/ml were added pre-
pared as described above for the mesenchymal cells. The
organ tissue can be bioengineered to develop desired pat-
terns of cell layering by preselecting the introduction of the
epithelial cells into the culture vessel. Also, the cells can be
introduced essentially simultaneously into the vessel to
produce normal organ tissue.

Culture conditions included mass transfer with exchange
of nutrients for metabolic waste and appropriate gas
exchange in the culture system. Fresh medium was
replenished by 65% of the total vessel volume each 20 to 24
hours. As metabolic requirements increased, fresh medium
was supplemented with an additional 100 mg/dl of glucose.
Viable cocultures grown in the RWVs were harvested over
periods up to 45 days and prepared for various assays.

The artificial organ tissue mass was produced in the RWV
in both a 1:1 mixture of M3:2 and MEM alpha supplemented
with 10% fetal bovine serum (Hyclone Laboratories, Logan,
Utah) and tri-sugar based media shown in Table 1 on a
preferred culture matrix of 5 mg/ml Cytodex-3 generally
spherical microcarriers (Pharmacia, LKB). The three dimen-
sional tissue cultures grown in the M3:2 and MEM media
attained mean cell densities of 2.3-3.2 x 10^6 cells/ml although
the cultures did not reach plateau phase, suggesting the
potential for continued growth, the epithelial cell contingent
exhibited signs of considerable stress while the mesenchy-
mal cells proliferated well.

The preferred tri-sugar based media was found to meet the
growth requirements without the need for growth factors and
supplements. The cultures in the tri-sugar based media produced slightly higher average cell densities, 4.6 to
6.2 x 10^6 cells/ml as compared to the M3:2 and MEM media
which exhibited cell densities of 2.5-3.2 x 10^6 cells/ml. The
three dimensional tissue mass grown in the tri-sugar based
media exhibited an epithelial cell component which
appeared to proliferate in a more normal and appropriate
fashion. The cultures were not grown to the plateau phase,
indicating further capability for continued growth. The arti-
tficial tissue did not reach maximum cell densities after
38-45 days of culture.

The tissue masses grew to relatively large sizes as com-
pared to other in vitro methods. After 300 hours of culture
the tissue/microcarrier mass was about 0.2-0.3 cm in size
with masses increasing to about 0.4-0.6 cm after approxi-
mately 500 hours of culture and over 0.6 cm after 800 hours
of culture.

The epithelial and mesenchymal cells used to seed the
cultures were immunophenotyped and compared to the
three dimensional tissue cultured in the RWV. Samples were
harvested at intervals during the culture process for immu-
nochemistry. The antibodies used to immunochemically characterize the cell types are listed in Table 2 below.

### TABLE 2

<table>
<thead>
<tr>
<th>Antibody Specificity</th>
<th>Source/Order No. or Designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancytokeratin</td>
<td>pre-diluted DAKO, Inc/L1824</td>
</tr>
<tr>
<td>Vimentin</td>
<td>pre-diluted DAKO, Inc/L1843</td>
</tr>
<tr>
<td>Factor VIII</td>
<td>pre-diluted DAKO, Inc.L1805</td>
</tr>
<tr>
<td>Villin</td>
<td>1:20 Chemicon, Temecula, CAMAS1671</td>
</tr>
<tr>
<td>Sucrose</td>
<td>1:20 A. Elbein, UTHSCSA/KY</td>
</tr>
</tbody>
</table>

The keratin, vimentin, and Factor VIII antibodies detect epithelial, fibroblastic, and endothelial cells, respectively.
Villin is a cytoskeletal protein only found in epithelial cells from small intestine and colon. The angioblast marker is
present in subsets of precursor endothelial cells, particularly avoiding cells. Sucrase is an enzyme found in the epithelial
cell brush border of the small intestine. Basement membrane
and extracellular matrix components laminin, fibronectin,
Collagen IV, and proteoglycan were also assayed to deter-
mine their expression in the artificially produced three
dimensional tissues.

Cultured organ tissue grown on Cytodex-3 microcarriers
in the RWV were fixed in Omnifix, an alcohol-based
fixative not containing aldehydes or mercury (Xenetics
Biochemic, Tustin, Calif.). At all times, extreme care was
taken not to damage the delicate artificial tissue comprised
of cellular material and microcarrier beads. When an abun-
dance of beads was present, the supernatant fluid was
carefully decanted and a sample of beads was enclosed in a
biopsy bag, then placed in a cassette to prepare a paraffin
block. When bead clusters were scarce, a Shandon Cyto-
lock kit (Shandon Inc., Pittsburgh, Pa.) was used. Cassettes
were processed in a standard tissue processor. Five-micron
sections were cut from the paraffin-embedded tissues, depar-
affinized by standard procedures, then assayed by incubation
with the test antibodies (Table 2) followed by use of the
universal labeling streptavidin biotin (LSAB) kit (DAKO
Inc., Carpentry, Calif., No. K680), which detects mouse
monoclonal and rabbit polyclonal antibodies. The immuno-
cytophenotyping was confirmed by one positive and two
negative controls. The positive control was normal tissue
sections or normal cells positive for the primary antibody
used. The negative controls were 1) PBS only, to test for
false binding by “link” antibody and streptavidin, and 2) normal serum of the same species from which the primary
antibody was prepared.

Analysis by immunochemistry was used to confirm the
ability of the seed cells to produce a three dimensional
artificially produced tissue mass with functional epithelial
cells and functional mesenchymal cells as well as functional
precursor endothelial cells that were not present in the seed
culture. As the culture progressed and the tissue mass grew
it included functional angioblasts.

Immunophenotyping of the cell types used to seed the
coculture in the RWV confirmed the majority of epithelial and
mesenchymal cells seeded were keratin and vimentin
positive, respectively. In addition, the epithelial cells were at
various stages of differentiation. Only a small percentage
(<2%) of the cells was positive for Factor VIII, an endot-
helial cell differentiation marker. Table 3 is a summary of the
immunochemical staining reaction of the three dimensional

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**TABLE 2-continued**

<table>
<thead>
<tr>
<th>Antibody Specificity</th>
<th>Source/Order No. or Designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angioblasts</td>
<td>1:20 Accurate Chem, Westbury, CA</td>
</tr>
<tr>
<td>Laminin</td>
<td>1:20 ION, Costa Mesa, CA/60-</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>1:20 US BIOCH, Cleveland, OH/33752</td>
</tr>
<tr>
<td>Proteoglycan</td>
<td>1:20 Biological Products for Science, Oxford, UK/MCA 326</td>
</tr>
<tr>
<td>Collagen Type IV</td>
<td>1:20 DAKO, Inc/M785</td>
</tr>
</tbody>
</table>
tissue of the present invention. The tissue was harvested at various intervals up to 41 days as noted in Table 3.

An increased percentage ≥10% of cells present in the masses consisted of endothelial cells which were Factor VIII positive as confirmed by immunocytochemistry and growing as patches on the beads. Furthermore, a small percentage, 1–2% of cells, showed de novo expression of the HE3 angioblast antigen which was not present in the seed cultures of mesenchymal cells confirming the presence of angioblasts in the tissue by immunochemistry. Functional cell brush borders were confirmed immunocytochemically by the presence of sucrase.

FIG. 1 is a photograph of the small intestine artificially produced organ tissue at 200x magnification showing the three dimensional cell aggregate generally indicated by the arrows. FIG. 2 is a photograph of the small intestine organ tissue. The triangular markers at 2 and 4 in the photograph are for reference on the microcarriers. A mass of epithelial cells is shown at 6. Elongated mesenchymal cells are growing on the microcarriers as shown at 8 and 10. FIG. 3 is a higher magnification (500x) of the epithelial mass shown at 6 in FIG. 2. FIG. 4 shows the organized columnar epithelial cells of the small intestine organ tissue at arrows 12 and 14. FIG. 5 shows the three dimensional organ tissue masses joined by cord-like structures of fibroblasts (mesenchymal cells) covered by epithelial cells.

<table>
<thead>
<tr>
<th>TABLE 3 SUMMARY OF IMMUNOCHEMICAL STAINING ON TISSUE GROWTH IN RWV*</th>
<th>Days Grown in RWV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibodies</td>
<td>Specificity or Cell Stained</td>
</tr>
<tr>
<td>Keratin</td>
<td>Epithelial, Cyto-skeletal</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Fibroblasts, some endothelial</td>
</tr>
<tr>
<td>Factor VIII</td>
<td>Endothelial</td>
</tr>
<tr>
<td>Villin</td>
<td>Epithelial, small intestine</td>
</tr>
<tr>
<td>HE3</td>
<td>Angioblasts</td>
</tr>
<tr>
<td>Sca-1</td>
<td>Small intestine, cell brush border</td>
</tr>
<tr>
<td>Laminin</td>
<td>Basement membrane</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>Basement membrane</td>
</tr>
<tr>
<td>Proteoglycan</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>Collagen</td>
<td>Type IV</td>
</tr>
</tbody>
</table>

*Slides were observed and scored on a relative scale as – (negative) to ++++ (maximum staining; very dark positive for >90% of the cells); + indicates weaker staining for ~25–50% of the cells: ++ indicates moderate staining for greater than 50–75% of the cells.

Samples from the RWV cultures were taken for scanning electron microscopy at the same times as those taken for immunocytochemistry. After removal from the reactor vessels, samples were washed once with CMF-PBS. The samples were suspended in a buffer containing 3% glutaraldehyde and 2% paraformaldehyde in 0.1M cacodylate buffer at pH 7.4, then rinsed for 5 minutes with cacodylate buffer three times and postfixed with 1% osmium tetroxide (Electron Microscopy Sciences, Fort Washington, Pa.) in cacodylate buffer for 1 hour. Samples were then rinsed for 5 minutes with distilled water three times and then treated for 10 minutes with Millipore (Millipore Corp., Bedford, Mass.) 0.2 µm filtered, saturated solution of 2% uranyl acetate (Electron Microscopy Sciences), then washed for 5 minutes with distilled water five times and fixed with 1% buffered osmium tetroxide for 10 minutes. This last step was necessary to prevent the microcarriers from collapsing. Samples were then rinsed with distilled water three times and dehydrated with increasing concentrations of EtOH followed by three changes in absolute methanol. After transfer to 1, 1, 1, 3, 3, 3-hexamethyldisilazane (HMDS) (Electron Microscopy Sciences) samples were allowed to soak for 10 minutes, drained, and air dried overnight. Dried samples were sprinkled with a thin layer of silver paint on a specimen stub, dried, coated by vacuum evaporation with platinum-palladium alloy, and then examined in the JEOL T330 Scanning electron microscope at an accelerating voltage of 5 to 10 kV.

Micrographs taken of 6- to 7-day cocultures showed partial coverage of the microcarriers by normal small intestine mesenchymal cells. Additionally, large developing masses of small intestine epithelium were evident, growing on the microcarrier bead packs. Samples harvested at approximately 12 days of culture contained small microcarrier packs which were totally engulfed in proliferating small intestine epithelium. Micrographs of samples at 13 days displayed large tissue-like masses that were entirely covered with small intestine epithelium grown on a base layer of mesenchymal cells. Areas of organized columnar epithelium were prominent. As the cultures matured into mid-late-stage cultures, extremely complex tissue-like masses comprised of mesenchymal and epithelial cells were seen from approximately 16 days of culture until termination at 41 days. These tissues were assembled from smaller masses which were joined by cord-like structures of fibroblasts and covered by epithelial cells several layers deep. In addition, available microcarriers were drawn to the surface of these large masses which were approximately 0.3 to 0.5 cm in diameter. Finally, columnar epithelium was observed to be growing even in the recessed areas of the microcarrier bead packs.

The artificially produced mammalian structural tissue was generated from normal mouse embryonic femurs. The fetal cells are a mixture of chondrocytes and stromal cells. The cells were plated in a standard media commonly designated as CMRL 1066 (Gibco Labs) containing 10% Fetal Bovine Serum. The cells were dissociated by standard enzymatic digestion. The single cells produced by enzymatic digestion were washed several times in PBS and transferred into a culture vessel with fluid culture media. The preferred culture vessel is described in U. S. Pat. No. 5,026,650 and referenced above. The vessel was steam sterilized by autoclaving at standard temperature and pressure prior to transfer of the cells.

A preferred culture matrix of generally spherical microcarrier Cytodex-3 beads were transferred into the culture vessel. The approximate total cell inoculum was 132,000, 000 at a ratio of 30 cells per Cytodex-3 microcarrier bead. The bead concentration of the vessel was 10 mg/ml (4000 beads/mg). The culture media in the vessel was CMRL 1066 with ascorbic acid 10 mg/ml added.

Microgravity conditions were simulated in culture vessel as described and referenced herein. The vessel provided the quiescent well-perfused environment for maintenance of cell surface receptors and undisturbed cell-to-cell co-location. Co-location is important for autocrine-paracrine factor formation and exchange between cellular types, and nurturing of differentiation with consequential matrix deposition. The cells were cultured for up to 65 days. The tissue masses grew to at least 0.4 cm in length after 1000 hours of culture. The development of normal cartilage tissue was evidenced by normal morphology of the chondrocyte and stromal cell formation and cartilaginous tissue substructures. Also, tissue with calcification was produced. Also,
proteoglycan deposition was identified. The normal pattern of Type II collagen deposition followed by down regulation of Type II collagen deposition and enhanced formation of Type IX and Type X collagen was observed.

Photographs taken during the culture period of the structural cells show the growth and normal development of the tissue. FIG. 6 is a micrograph taken at 500x magnification at 300 to 600 hours of culture of normal fetal mouse chondrocytes. The cells grew around the generally spherical microcarriers. FIG. 7 is a higher magnification showing preliminary calcification at the arrow. FIGS. 8 and 9 were taken after 900 to 1100 hours of culture with different are fibroblastic chondrocytes shown in FIG. 9 at 22. Also, stromal cells are show in FIG. 9 at 24.

The artificially produced blood tissue can be made from human bone marrow samples or other sources of mammalian origin. Human bone marrow samples are obtained by standard clinical techniques under aseptic conditions. In general, a bone marrow tool is used to puncture the bone and obtain a bone marrow sample that is fully inclusive of normal constituents. The normal constituents will include, but are not limited to, pleuripotential stem cells, fully maturated white blood cells and red blood cells, stromal components, and cells in intermediate stages of maturation. Laboratory animals such as mice can be used as a source of bone marrow for selected studies. After sacrificing mice, the femur and tibia are removed and placed in lx Calcium- and Magnesium-free PBS with 200 µg/ml pen-strep. Muscle is removed and endcaps of the bones are cut. With a syringe and 26G needle, the bone is flushed with cold complete growth medium known to those skilled in the art such as CMRL 1066. Cells are then poured through collector screens to remove large pieces of tissue and suspended cells are placed in 50-cc sterile tubes on cold complete growth medium. Various cell matrices can be used. The preferred culture vessel is described in U.S. Pat. No. 5,026,650 previously cited and incorporated by reference herein. A fluid culture media is introduced into the sterilized vessel. A preferred fluid culture media is a standard tissue culture media commonly designated as RPMI-1640 (available from Gibco and other sources).

The sterile bone marrow cells are then transferred to fluid culture medium and suspended. Suspended cells are transferred, at approximate densities of 1x10⁶ cells/ml, to the culture vessel. Various cell matrices can be used. The collected bone marrow cells can be encased in stromal-like matrices. The matrices can be disc-shaped. Other matrices are generally known can be used including, but are not limited to, collagen matrices of Type I rat tail collagen or other biopolymeric matrices. The biopolymers used can be biodegradable such as suture materials. Cross-linked polymers such as collagen, fibronectin and mixtures thereof may be used. These matrices may have spherical diameters ranging from 1 millimeter to 10 millimeters and include the necessary cellular constituents to facilitate primary bone marrow culture. Cytodex-3 microcarriers can be introduced into the vessel at a density of 5 mg/ml. Bone marrow cells contained in the matrix or supported in suspension are cultured at microgravity conditions and mass transfer as described herein and the incorporated references.

Early in the initiation it is preferable that the cells are allowed to grow and metabolize without sampling interruption. The first sampling may begin one to seven days after inoculation. Consequently, cellular aggregates are not disturbed and bone marrow growth factors are not diluted by media repletion. After the lag phase, media repletion is based upon requirements as previously described. Cellular phenotyping is carried out by flow cytometry and light microscopy.

FIG. 10 is a schematic drawing of the bone marrow cell production. The disc-shaped culture matrices are shown at 30, 32 and 34. The bone marrow cells are shown as circular figures at 36, 38 and 40. The arrows with the GF generally indicate the excretion of growth factors from the normal bone marrow cells that enhance tissue growth in the culture system.

In the case of preparing bone marrow for recipients, volumes are expanded as cellular densities or metabolic requirements dictate. The limited parameters may depend upon the rotating vessel’s ability to suspend large aggregates, in which case if unable to suspend large aggregates, smaller aggregates would be distributed through several vessels. If the metabolic requirements were such that mass transfer of oxygen and removal of carbon dioxide were not sufficient to maintain cellular growth and expansion, then cells would be aliquoted into additional vessels.

The examples included are not intended to limit the scope of the present invention. Normal mammalian cells have been cultured to produce aggregates of cells of three dimensional tissue masses. Other substitutions, modifications and variations are apparent to those skilled in the art without departing from the disclosure and scope of the invention.

What we claim is:

1. A process for producing functional non-neoplastic mammalian organ tissue comprising culturing the mammalian organ tissue in a culture medium, consisting essentially of:
   (a) Minimal Essential Medium-alpha (MEM-alpha) medium
   (b) Leibovitz L-15 medium;
   (c) tri-sugar supplement comprising glucose, galactose, and fructose;
   (d) serum;
   (e) NaHCO₃;
   (f) HEPES;
   (g) folic acid;
   (h) nicotinic acid;
   (i) bactopeptone;
   (j) L-inositol;
   (k) L-glutamine;
   (l) gentamycin;
   (m) fungizone; and
   (n) insulin-transferin-sodium selenite.

2. The process of claim 1, wherein culturing the mammalian organ tissue in the culture medium comprises the following steps:
   (a) inoculating isolated non-neoplastic mammalian cells into a culture vessel containing said culture medium;
   (b) culturing the non-neoplastic cells under simulated microgravity culture conditions; and
   (c) maintaining the simulated microgravity culture conditions whereby three dimensional tissue growth is achieved, thereby producing functional non-neoplastic mammalian organ tissue.

3. The process of claim 2 wherein the simulated microgravity culture conditions are created by the culture vessel.

4. The process of claim 2 wherein the simulated microgravity culture conditions are created by a horizontal rotating wall culture vessel in unit gravity, and wherein the
horizontal rotation of the culture vessel is controlled to produce the simultaneous conditions of:

(a) collocation of cells with similar or differing sedimentation properties in a similar spatial region;

(b) freedom for three dimensional spatial orientation of tissues formed by the culturing of the cells; and

(c) low shear and essentially no relative motion of the culture environment with respect to the walls of the culture vessel.

5. The process of claim 1, wherein culturing the mammalian organ tissue in the culture medium comprises the following steps:

(a) inoculating isolated non-neoplastic, differentiated epithelial cells and isolated non-neoplastic, differentiated mesenchymal cells into a horizontal rotating wall culture vessel containing said culture medium;

(b) culturing the epithelial cells and the mesenchymal cells under simulated microgravity culture conditions and

(c) maintaining the simulated microgravity culture conditions whereby three dimensional tissue growth is achieved thereby producing non-neoplastic mammalian organ tissue.

6. The process of claim 5 wherein microgravity culture conditions are created by a horizontal rotating wall culture vessel in unit gravity, and wherein the horizontal rotation of the culture vessel is controlled to produce the simultaneous conditions of:

(a) collocation of cells with similar or differing sedimentation properties in a similar spatial region;

(b) freedom for three dimensional spatial orientation of tissues formed by the culturing of the cells; and

(c) low shear and essentially no relative motion of the culture environment with respect to the walls of the culture vessel.