# IN VITRO EXPANSION OF POSTPARTUM-DERIVED CELLS IN ROLLER BOTTLES

## CROSS-REFERENCE TO RELATED APPLICATIONS

**[0001]** This application claims benefit to United States Provisional Patent Application No. 60/751,550, filed December 19, 2005, the contents of which are incorporated by reference herein, in their entirety.

## FIELD OF THE INVENTION

**[0002]** This relates generally to the growth and expansion of mammalian cells. In particular, this relates to methods for the *in vitro* growth and expansion of postpartum-derived cells in containers such as roller bottles.

## BACKGROUND OF THE INVENTION

**[0003]** Commercial cell therapy products are preferably produced in aseptic systems that are closed. However, the growth of many cell lines used for commercial cell therapy products is anchorage-dependent. While stirred tank reactors, shaker flasks, spinner flasks, uplift reactors, and the like, are all useful for cells that grow in suspension (e.g. hybridomas for monoclonal antibody production, many cells used for recombinant DNA technology, and most insect cell cultures), the options for growing and expanding anchorage-dependent or anchorage-preferred cells are more limited.

**[0004]** Included among the anchorage-dependent cells are many normal diploid cell strains, as well as most primary cell lines. Options for large-scale production of such cells include roller bottles, fiber beds and hollow fiber systems, multi-plate or stacked-plate culture systems, cell cubes, and microcarriers, each of which has advantages and disadvantages.

**[0005]** Roller bottle-based methods of cell culture are probably the most commonly used method for growing anchorage-dependent and anchorage-preferred cells. Roller bottles are essentially cylindrical vessels, of glass or plastic, which are at least partially filled with a growth medium. Most modern roller bottles are made of a disposable plastic material. The bottles are placed on an apparatus that turns, causing the bottles to continuously "roll" or revolve at a

typically constant speed of between about 5 and 250 revolutions per hour. The rolling motion allows the cells, which attach to the inside surfaces of the bottle, to bathe in the medium while having ample exchange of gases with the atmosphere in the bottles.

**[0006]** Roller bottles are available in various sizes, each size providing a fixed amount of surface area and volume. Many bottles are available in the 1- 2 liter volume range. Two common sized commercial roller bottles provide 850 $cm^2$ and 1050$cm^2$, respectively. For some applications, large size can be a limitation because roller bottles that are too large are difficult to handle where microbiological safety is critical. More recently roller bottles with expanded inner surfaces have become commercially available to help address the issue. Handling of roller bottle cultures, such as manipulations for subculture should be minimized where possible.

**[0007]** Roller bottle-based culture systems provide many advantages including relatively low cost for equipment and set-up, relative ease of set-up, and ability to scale up or down according to needs. The bottles, which are typically clear, allow for visual and microscopic inspection of the cells and the growth. Contaminated samples are easy to spot and can be discarded.

**[0008]** The potential drawbacks include the relatively high level of skill required for seeding, transfers, harvest of cells or biologics produced, and other ongoing manipulation of the cells. The costs associated with ongoing operations may be high because of the skill level required. The risk of contamination is relatively high because of the amount of manipulation required. Notwithstanding the potential drawbacks, roller bottles are used still, even for the commercial production of some biologics.

**[0009]** Among the factors which should be considered in using roller bottles for cell culture are the attachment efficiency, as well as time to reach confluence, the growth parameters of attached cells including maximum attainable density per unit surface area, detachment techniques, which are required, and the efficiency of the detachment, scalability of the culture conditions, as well as homogeneity of the culture under scaled-up conditions, and the ability to successfully scale-up detachment procedures. Some of these considerations can be influenced by the inoculation parameters (such as rotational speed, media volume), culture conditions such as the rotational speed of the bottles, as well as the seeding density of the initial culture, the volume of medium used relative to the surface area and/or shape of the bottle, and the length of time the culture is incubated.

**[0010]** It is also important, particularly in cell therapeutic applications, that the characteristics of the cells grown under scaled-up roller bottle conditions be those of the desired cell type in terms of surface markers, gene expression, viability (over 70 to 80%), and the like.

[0011] There is a need to attempt to optimize the controllable culture parameters to improve roller bottle culture systems in terms of simultaneously maximizing the growth rate, the number of population doublings achieved, and the total cells available for harvest.

## SUMMARY OF THE INVENTION

[0012] In one of its several aspects, the invention provides methods of maximizing the growth parameters of postpartum cells in roller bottle culture systems. In accordance with the invention, provided are methods of maximizing the number doublings of a culture of postpartum cells in a roller bottle culture system. The methods comprise using a rotational speed of at least about 0.85 rpm, using a media volume of at least about 100 ml in an 850 square centimeter culture bottle; using a seeding density of less than about 2500 cells per square centimeter; and incubating for at least about 5.5 days.

[0013] Also in accordance with the present invention are methods of maximizing the rate of doubling of a culture of postpartum cells in a roller bottle culture system. The methods for maximizing the rate of doubling preferably comprise using a rotational speed of at least about 0.85 rpm, using about 300 ml of growth medium in an 850 square centimeter culture bottle; using a seeding density of less than about 2500 cells per square centimeter; and incubating for less than about 6 days.

[0014] In another of its aspects, the invention provides methods of maximizing the density of cells at harvest for postpartum cells in a roller bottle culture system. These methods comprise using a rotational speed of about 0.5-1 rpm, using about 300 ml of growth medium in an 850 square centimeter culture bottle; using a seeding density of about 10,000 cells per square centimeter; and incubating for between about 5.5 to 6.7 days.

[0015] Also provided in accordance with one aspect of the invention are methods of simultaneously maximizing doubling rate, harvest density, and the total number of population doublings for postpartum cells in a roller bottle culture system. These methods are also sometimes referred to herein as optimizing the roller bottle culture system for the foregoing parameters. The methods preferably comprise using a rotational speed of about 0.65-0.9 rpm, using at least about 300 ml of growth medium in an 850 square centimeter culture bottle; using a seeding density of less than about 2,500 cells per square centimeter; and incubating for between about 5.5 to 6.5 days.

[0016] The foregoing methods are particularly useful where the postpartum cells are umbilicus-derived cells, and presently preferred postpartum cells are those which are substantially similar, or even identical to ATCC NOS PTA-6067 and PTA-6068.

[0017] These and other aspects of the invention will be described with reference to the examples, figures, and detailed description of the various aspects of the invention which follow.

## BRIEF DESCRIPTION OF THE DRAWINGS

[0018] Figure 1: Calculated optimal rotational speed (1.0 rpm), media volume (112 ml), seeding density (2,500 cells/cm sq.), and days in culture (6.27 days) to achieve the maximal population doublings (3.04). The black line on each graph represents the plotted values of the factor levels (low to high along the x-axis) vs. population doublings (minimal to maximal along the y-axis). The blue line represents the single maximum y-axis value for all four graphs. The red line represents the point on the x-axis that the plotted value of the factors levels vs. population doublings value (black line) intersects the single maximum y-axis value (blue line) thus defining the optimal factor level.

[0019] Figure 2: Calculated optimal rotational speed (0.92 rpm), media volume (300 ml), seeding density (2,500 cells/cm sq.), and days in culture (5 days) to achieve the minimal hours per population doubling (29.71). The black line on each graph represents the plotted values of the factor levels (low to high along the x-axis) vs. hours per population doublings (minimal to maximal along the y-axis). The blue line represents the single maximum y-axis value for all four graphs. The red line represents the point on the x-axis that the plotted value of the factors levels vs. hours per population doublings value (black line) intersects the single minimum y-axis value (blue line) thus defining the optimal factor level.

[0020] Figure 3: Calculated optimal rotational speed (0.98 rpm), media volume (300 ml), seeding density (10,000 cells/cm sq.), and days in culture (6.67 days) to achieve the maximal harvest density (cell/cm sq.) (3.59E+04). The black line on each graph represents the plotted values of the factor levels (low to high along the x-axis) vs. maximal harvest density (minimal to maximal along the y-axis). The blue line represents the single maximum y-axis value for all four graphs. The red line represents the point on the x-axis that the plotted value of the factors levels vs. maximal harvest density (black line) intersects the single maximum y-axis value (blue line) thus defining the optimal factor level.

[0021] Figure 4: Calculated optimal rotational speed (0.705 rpm), media volume (300 ml), seeding density (2,500 cells/cm sq.), and days in culture (6.1 days) to achieve the minimum hours/ population doubling (38.07 hours), maximum population doublings (3.17), and maximum

harvest density (cell/ cm sq.)(1.8E+04). The black lines on each graph represent the plotted values of the factor levels (low to high along the x-axis) vs. response values (minimal to maximal along the y-axis). The blue line represents the single maximum, or minimal, y-axis value for all four graphs. The red line represents the point on the x-axis that the plotted value of the factors levels vs. response value (black line) intersects the single maximum y-axis value for population doublings and harvest density and the minimum y-axis value for hours per population doubling (blue line) thus defining the optimal factor levels.

**[0022]** Figure 5: Population Doublings vs. Days in Culture of umbilical cell line 050604B expanded from passage six to passage nine under optimized roller bottle culture conditions

## DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

**[0023]** In several of its numerous aspects the invention provides separate methods of maximizing the amount of anchorage-dependent cells available for harvest from a population of cells grown in a roller bottle culture system, the growth rate of such a culture, or the total number of doublings of such a culture. The invention also provides methods for simultaneously maximizing all three of the foregoing parameters. Cells and populations of cells produced by the foregoing methods are also provided herein

**[0024]** Roller bottle culture systems are known in the art of cell culture. As used herein, roller bottle culture systems comprise at least a cell line of interest, growth medium, roller bottles, an apparatus for rotating the bottles, and means for harvesting the cells. The growth medium preferably comprises a basal medium, for example, Dulbecco's Modified Eagle's Medium (DMEM), Advanced DMEM, Ham's F12, or combinations thereof, for example 1:1 DMEM:F12. The medium can be supplemented with serum, for example in some embodiments the medium is supplemented with fetal bovine serum (FBS) or new born calf serum (NCS). The serum content can range in concentration from 0 (a serum-free media) to 20% of the total volume of the medium. Growth factors, for example, platelet derived growth factor BB (PDGF-BB), basic fibroblast growth factor (bFGF), and others, or combinations of such may be used to supplement the growth medium. Either serum-containing or serum-free media can be with or without growth factor supplementation.

**[0025]** Roller bottle culture systems typically further comprise means for controlling the temperature during the incubation, as well as means for aseptically handling the cultures, for example during initial seeding of the bottles with cells, or during subsequent transfers. Harvesting of the cells may be achieved through enzymatic treatment such as with trypsin, trypsin-EDTA, dispase, and collagenase, or other enzymes or combinations of enzymes with or

without other components. Other commercial products such as but not limited to TrypLE Express (Gibco, Inc.) can utilized. The cells be also be harvested by manual operations including, for example, batch centrifugation, or harvesting can be automated.

**[0026]** Presently the methods of maximizing the amount of cells available for harvest preferably are applied to postpartum-derived cells, particularly cells derived from the placenta or umbilicus. Cells of the type preferred here are described in U.S. Patent Application Nos: 10/877,446 (placenta-derived cells) and 10/877,012 (umbilicus-derived cells), each filed June 25, 2004. The entireties of these applications are incorporated by reference herein. Also preferred are cells of the types available from the American type Culture Collection as ATCC Accession Nos. PTA-6067; PTA-6068; PTA-6074; PTA-6075; or PTA-6079, the characterization and description of each of which is also incorporated by reference herein. Particularly preferred for the present methods are directed to maximized or optimized methods for culturing umbilical-derived cells, for example, ATCC Accession Nos. PTA-6067 and PTA-6068.

**[0027]** In one of its several aspects the invention provides methods of maximizing the number of population doublings achievable for a population of cells grown in a roller bottle culture system. Preferably the cells are postpartum-derived cells, and even more preferably the cells are umbilicus-derived cells. In a presently preferred embodiment the cells are ATCC Accession No: PTA-6067 or PTA-6068.

**[0028]** The independent variables which have been used to maximize the number of population doublings achievable in a roller bottle culture are rotational speed, seeding density of the cells into the bottles, time of incubation, and volume of medium placed in the bottle. Herein and throughout in the embodiments exemplified these independent variables have been tested, for reasons of practicality, within certain ranges. The skilled artisan will appreciate that other values outside of the tested ranges could be routinely tested using the same methodology, and these values may prove to offer incremental gains in the number of population doublings. Maximal response of the dependent variable, here the number of population doublings achieved is measured as a function of these parameters and embodiments not specifically exemplified herein are contemplated as part of this disclosure.

**[0029]** To help maximize the dependent variable as a function of the four independent variables, regression analysis was used. In particular, response surface methodology (RSM) is presently a preferred approach to optimize culture growth parameters. Applications of RSM to optimization techniques are known in the art. RSM allows optimization of multiple independent parameters to achieve a desired or optimal response. Parameter sets that provide maximal and minimal responses can be accurately determined using RSM.

**[0030]** As can be seen from the data exemplified herein and below, the independent variables tested can reproducibly affect the maximal number of population doublings. Thus, the statistical analysis of the data allows the skilled artisan to determine the optimal value for each of the four independent variables to maximize the number of doublings.

**[0031]** In a preferred embodiment, therefore, the bottles are filled about 100-300 ml of growth medium, in other embodiments about 100-200 ml are used, as can be seen from Figure 1. In one embodiment about 100-120 ml, or even 105-115 ml are placed in the bottles. In other embodiments, the bottles are filled with about 112 ml of growth medium to achieve maximal population doublings. The bottles are seeded with about 2500 to about 10,000 cells per square centimeter. In a preferred embodiment, the lower end of that range is used, for example seeding is with less than about 3000 cells per square centimeter. As can be seen from Figure 1, still more preferred are embodiments where the seeding is at an even lower end of the tested range – the seeding is done with about 2500 cells per square centimeter. The seeding bottles are rotated during attachment and growth. The rotational speed is set at between about 0.5 to 1 rpm. Preferably, the rotation is between about 0.75 and 1 rpm. More preferably, the bottles are rotated at about 0.8 to 1 rpm. Rotation near or about 1 rpm is preferred as can be seen in Figure 1.

**[0032]** The filled and seeded roller bottles are rotated and incubated for about 5 to 7 days to achieve maximal doublings. Presently, an incubation time of about 5.5 to about 6.5 days is preferred. As Figure 1 reflects, incubation for about 6.2 to 6.3 days is also preferred.

**[0033]** It can also be seen from Figure 1 that the independent variable can be selected as a set of parameters to maximize the number of population doublings. A roller bottle culture system that comprises a fill volume of about 112 ml of growth medium, and a seeding density of about 2500 cells per square centimeter, which is rotated at a speed of about 1 rpm for an incubation of about 6.2 days will provide the maximal population doublings achievable in such a system.

**[0034]** It may be noted that the roller bottles preferred for use are typically coated with an agent that aids in the attachment of the cells to the inner surface of the roller bottles, such as gelatin, extracellular matrix molecules (such as gelatin, laminin, vitronectin, fibronectin, collagen types I, IV, and VI), or the like. While for many of the embodiments exemplified, a gelatin coating was used, other coating are deemed suitable and the skilled artisan will appreciate that commercially-available coated bottles are completely compatible with the methods taught herein. One example of such commercially available coated bottles are those coated with CellBind (available from Corning as catalog number 3907). The use of CellBind bottles and a comparison with gelatin-coated bottles is exemplified in Example 4 below. It is envisioned that

various coating agents will be found acceptable for attachment and growth of cells in accordance with the methods provided herein.

**[0035]** In another of its aspects, the invention provides methods of minimizing the number of hours/population doubling (see Figure 2), or alternatively expressed, maximizing the population doubling rate for a population of cells grown in a roller bottle culture system. As used herein the population doubling rate is the number of population doublings per unit time, and is a reciprocal of the hours per population doubling. Achieving maximal population doubling rate reduces the amount of time required to produce a needed number of cell for therapeutic applications and increases the total throughput of a culture system of limited capacity. As above and throughout this disclosure, preferably the cells are postpartum-derived cells, and even more preferably the cells are umbilicus-derived cells. In a presently preferred embodiment the cells are ATCC Accession No: PTA-6067 or PTA-6068.

**[0036]** The independent variables which have been used to maximize the number of population doublings achievable in a roller bottle culture are the same as for achieving the maximal number of population doublings: rotational speed, seeding density of the cells into the bottles, time of incubation, and volume of medium placed in the bottle. Maximal response of the dependent variable, here the population doubling rate, is measured and computed as a function of these parameters.

**[0037]** Regression analysis, and particularly RSM was again employed to help maximize the dependent variable as a function of the four independent variables.

**[0038]** Thus, in a presently preferred embodiment, roller bottles are filled about 100-300 ml of growth medium, preferably about 300 ml are used, as can be seen from Figure 2. The bottles are seeded with about 2500 to about 10,000 cells per square centimeter. In a presently preferred embodiment, the lower end of that range is used, for example seeding is with less than about 3000 cells per square centimeter. Still more preferred are embodiments where the seeding is at about 2500 cells per square centimeter. The seeded bottles are rotated during attachment and growth. The rotational speed is set at between about 0.5 to 1 rpm, as can be seen from Figure 2. Preferably, the rotation is between about 0.75 and 1 rpm. More preferably, the bottles are rotated at about 0.8 to 1 rpm. Rotation near or about 0.9 -1.0 rpm is presently preferred, as shown in the figure.

**[0039]** To maximize the population doubling rate, the filled and seeded roller bottles are rotated and incubated for about 5 to 7 days, with an incubation time of about 5 to about 6 days preferred. Figure 2 shows that incubation for about 5 days is also preferred.

**[0040]** The independent variables can be selected as a set of parameters to maximize the population doubling rate, based on the results shown in Figure 2. A roller bottle culture system that comprises a fill volume of about 300 ml of growth medium, and a seeding density of about 2500 cells per square centimeter, which is rotated at a speed of about 0.9 rpm for an incubation of about 5 days will provide the maximal population doubling rate achievable in such a system.

**[0041]** In another of its several aspects the invention provides methods of maximizing the density of anchorage-dependent cells available for harvest from a population of cells grown in a roller bottle culture system. As above, preferably the cells are postpartum-derived cells, and even more preferably the cells are umbilicus-derived cells. In a presently preferred embodiment the cells are ATCC Accession No: PTA-6067 or PTA-6068.

**[0042]** In another of its aspect, the invention provides methods of maximizing the density of the cell population for harvest for a population of cells grown in a roller bottle culture system. Harvest density is expressed as the number of cells per square centimeter (of internal surface area in a roller bottle).

**[0043]** The independent variables which have been used to maximize the harvest density in a roller bottle culture are the same as those for maximizing the other responses discussed herein, i.e rotational speed, seeding density of the cells into the bottles, time of incubation, and volume of medium placed in the bottle. Maximal response of the harvest density is measured and computed as a function of these parameters.

**[0044]** Regression analysis, and particularly RSM was employed here as above to help maximize the dependent variable as a function of the four independent variables.

**[0045]** Maximal response of the dependent variable, here the density of cells available for harvesting is calculated as a function of these parameters. In a presently preferred embodiment, roller bottles are filled about 100-300 ml of growth medium, preferably about 300 ml are used, as can be seen from Figure 3. The bottles are seeded with about 2500 to about 10,000 cells per square centimeter, preferably the seeding density is selected from the lower end of that range, such as a value less than about 3000 cells per square centimeter. More preferred are those embodiments wherein seeding density is at about 2500 cells per square centimeter. The seeded bottles are rotated throughout attachment and growth at a speed between about 0.5 to 1 rpm, as shown in Figure 3. In preferred embodiments, rotation is between about 0.75 and 1 rpm. More preferably, the bottles are rotated at about 0.8 to 1 rpm. Rotation near or about 0.9-1.0 rpm is presently preferred, for example 0.98 rpm, as shown in the figure.

**[0046]** To maximize the harvest density of the cultured cells, the filled and seeded roller bottles are rotated and incubated for about 5 to 7 days, with an incubation time of about 6 to about 7 days preferred. Figure 3 shows that incubation for about 5.8 to less than about 6 days or for about 6.5 to about 6.7 days is also preferred.

**[0047]** In another aspect, the invention provides methods of simultaneously minimizing the number of hours/population doubling, while maximizing the number of population doublings and the harvest density (in cells per square centimeter) for a population of cells grown in a roller bottle culture system. Such optimization of the culture conditions makes the roller bottle culture system more useful for generating the number of cells required for a therapeutic application and increases the total throughput of a culture system of limited capacity. As above, preferably the cells are postpartum-derived cells, and even more preferably the cells are umbilicus-derived cells. In a presently preferred embodiment the cells are ATCC Accession No: PTA-6067 or PTA-6068.

**[0048]** The independent variables which have been adjusted to help simultaneously maximize the number of population doublings achievable, the rate of population doublings and the final harvest density of the cells in a roller bottle culture are the same as detailed above for maximizing the individual aspects: i.e. rotational speed, seeding density of the cells into the bottles, time of incubation, and volume of medium placed in the bottle. Maximal response of the dependent variables is measured and calculated as a function of these independent parameters.

**[0049]** As above, regression analysis, and particularly RSM was utilized to help simultaneously maximize the three dependent variables as a function of the four independent variables.

**[0050]** Thus, in preferred embodiments, roller bottles are filled about 100-300 ml of growth medium, preferably about 300 ml are used, as can be seen with reference to Figure 4. The bottles are seeded with about 2500 to about 10,000 cells per square centimeter. In a presently preferred embodiment, the lower end of that range is used, for example seeding is with less than about 3000 cells per square centimeter. Still more preferred are embodiments where the seeding is at about 2500 cells per square centimeter. Even lower seeding density is used is other embodiments. The seeded bottles are rotated during attachment and growth at speeds between about 0.5 to 1 rpm. With further reference to Figure 4, in can be seen that preferably, the rotation is between about 0.6 and 0.9 rpm. More preferably, the bottles are rotated at about 0.65 to 0.93 rpm. Also preferred are culture systems wherein the rotation is near or about 0.85 - 0.9 rpm as can be seen in the figure.

**[0051]** To further optimize the roller bottle culture, the filled and seeded roller bottles are rotated and incubated for about 5 to 7 days, with an incubation time of about 5.5 to about 6.5 days preferred. Figure 4 shows that incubation for about 6 days is also preferred, such as 5.9, 6.0, 6.1 or 6.2 days.

**[0052]** In a presently preferred culture system, the independent variables can be selected as a set of parameters to maximize the population doubling rate, based on the results shown in Figure 4. A roller bottle culture system that comprises a fill volume of about 300 ml of growth medium, and a seeding density of about 2500 cells per square centimeter, which is rotated at a speed of about 0.7 rpm for an incubation of about 6.1 days will provide the optimal population doubling rate, total number of doublings and harvest density for the cells achievable in such a system.

**[0053]** In another aspect, the invention provides postpartum cells, preferably umibilicus-derived cells, that are produced are produced by any of the methods of the invention, for example the maximized methods or optimized. In various embodiments, the cells are produced in populations for use as cell therapeutics, or to provide useful cellular product or by-products, such as useful cellular factors, or proteins.

**[0054]** Also provided are cell therapeutic compositions comprising cells cultured by the methods provided herein.

**[0055]** In another aspect of the invention, the cells that are cultured according to the methods provided are characterized as having substantially the same cell surface marker profile or gene expression profile as the starting cells. For many applications of cell-based therapies it is important that the cellular characteristics do not change when scaling up the culture conditions to increase quantities. For example, the morphology, cell surface markers, and expression of hallmark genes that help distinguish or denote the therapeutic cell should remain substantially unchanged if not identical. The cells provided in accordance with the invention and the methods taught therein are substantially unchanged, or preferably identical in such characteristics as the same cells grown under laboratory conditions ad scale. The preferred umbilicus-derived cells retain substantially the same the cell surface marker profile of the cells from which they are grown. Preferably, the cells produced according to the methods provided herein express more than one of surface markers for CD10, CD13, CD44, CD73, CD90, PDGFr-alpha, and HLA-ABC. More preferably they express all of these markers. The cells also preferably do not express more than one of the cell surface markers for CD31, CD34, CD45, CD117, CD141, and HLA-DRDPDQ. More preferably the cells do not express any of the foregoing. In a highly preferred embodiment, the cells express an identical cell surface marker profile with respect to

each of CD10, CD13, CD44, CD73, CD90, PDGFr-alpha, HLA-ABC, CD31, CD34, CD45, CD117, CD141, and HLA-DRDPDQ. Moreover, preferred cells are positive for expression of CD10, CD13, CD44, CD73, CD90, PDGFr-alpha, and HLA-ABC, but negative for expression of CD31, CD34, CD45, CD117, CD141, and HLA-DRDPDQ. The skilled artisan will appreciate that are several ways to assess the cell surface marker profile of a cell line. As used herein, if a cell surface marker protein can be detected through use of a fluorescently-labeled antibody the cell is deemed positive and if the cell surface marker cannot be detected via fluorescent antibody, the cell is deemed negative for that marker. Table 6 provides a summary of the preferred cell surface markers for the umbilicus-derived cells of the invention.

**[0056]** In similar fashion, the cells produced in accordance with the methods of the invention preferably retain substantially the same, or even identical gene expression profile, particularly for genes whose expression help to characterize the cell, or serve as hallmarks indicative of that cell line. The umbilicus-derived cells of the invention are noted for the expression genes for reticulon, LDL-R, IL-8, and GAPDH. The cells provided are substantially similar, or even identical in various embodiments with respect to their expression of these genes.

**[0057]** These and other aspects of the invention are further described with reference to the figures and examples provided below. The skilled artisan will appreciate that the examples are provided to exemplify only certain aspects of the invention and not to limit the invention.

*Example 1: Optimization Experiments for Determining Culture Condition Parameters*

**[0058]** Cells: The cells used were from umbilicus-derived cell line ATCC Accession Nos. PTA-6067 and/or PTA-6068.

**[0059]** Media: The growth medium used during the optimization experiments was Dulbecco's Modified Eagle Medium (DMEM) with low glucose, 15% Fetal Bovine Serum, 1% Penicillin-Streptomycin, and 1 ppm 2-Mercaptoethanol.

**[0060]** Bottles: 850 cm sq bottles were used (e.g. Corning catalog number 430851)

**[0061]** Gelatin Coating: Twenty (20) ml of 2% gelatin solution were added to each 850 cm sq bottle. The bottles were placed on the roller system for 20 minutes at 1 rpm. The excess gelatin solution was removed by aspiration and the bottles washed with 20 ml Phosphate Buffered Saline (PBS).

**[0062]** Cell Seeding: P13 Umbilical 022803 cells from frozen stock cells were thawed from a cryogenic vial and washed to remove dimethyl sulfoxide (DMSO). Cells were seeded

into a single 850 cm sq roller bottle pre-filled with 300 ml media and pre-gassed for 1 minute with compressed air containing 5% $CO_2$ , 95% atmospheric gas.

**[0063]** Incubation: Cells were cultured in a temperature-controlled room set at 37° C.

**[0064]** Harvesting: Media was removed from each roller bottle by aspiration and adherent cells were washed with 50 ml PBS. PBS was removed by aspiration and 10 ml of trypsin-ethylenediaminetetraacetic acid (EDTA) was added to aid in the release the cells from the roller bottle surface. Bottles were returned to the roller system and incubated for 5 minutes at 0.75 RPM. After incubation, 40 ml of media was added to each bottle. The media with cells was then transferred to a 50 ml conical tube and centrifuged for 5 minutes at 300 x g. After centrifugation, the media was removed by aspiration and each cell pellet was re-suspended in 10ml of media. Cells were counted using a Beckman-Coulter Cedex instrument.

**[0065]** Statistical Model: A Box-Behnken Response Surface model was used.

**[0066]** Experimental design and optimization were calculated using a Minitab 14.0. the parameters tested are shown in Table 1

*Table 1:* Factors tested and the range of values tested.

| Factor | Low | High |
|---|---|---|
| Rotational Speed (rpm) | 0.5 | 1 |
| Media Volume (ml) | 100 | 300 |
| Seeding Density ( cells/cm sq) | 2,500 | 10,000 |
| Days | 5 | 7 |

**[0067]** Experimental Design: Experiments as provided in Table 1 were set up in accordance with the above procedures.

*Table 2*: Box-Behnken Response Surface Experimental Design to evaluate the factors and factor interactions that significantly effect cell yield as calculated by Minitab 14.0 statistical software. The highest and lowest factor levels for rotational speed (low 0.5 rpm, high 1.0 rpm), media volume (low 100ml, high 500ml), seeding density (low 2,500 cell/cm sq., high 10,000 cells/cm sq.) and days in culture (low 5 days, high 7days) are user-defined and the mid point is calculated.

| Run Order | Rotational Speed (rpm) | Media Volume (ml) | Seeding Density (cells/cm sq) | Days |
|---|---|---|---|---|
| 1 | 0.5 | 200 | 6,250 | 5 |
| 2 | 1 | 200 | 6,250 | 7 |
| 3 | 0.75 | 300 | 6,250 | 7 |
| 4 | 1 | 200 | 10,000 | 6 |
| 5 | 0.75 | 200 | 6,250 | 6 |
| 6 | 0.75 | 200 | 2,500 | 5 |
| 7 | 1 | 300 | 6,250 | 6 |
| 8 | 0.5 | 200 | 6,250 | 7 |
| 9 | 0.5 | 300 | 6,250 | 6 |
| 10 | 1 | 100 | 6,250 | 6 |
| 11 | 0.5 | 200 | 10,000 | 6 |

| | | | | |
|---|---|---|---|---|
| **12** | 0.75 | 300 | 6,250 | 5 |
| **13** | 0.75 | 200 | 10,000 | 5 |
| **14** | 0.75 | 200 | 2,500 | 7 |
| **15** | 0.5 | 100 | 6,250 | 6 |
| **16** | 1 | 200 | 2,500 | 6 |
| **17** | 0.75 | 100 | 2,500 | 6 |
| **18** | 0.75 | 300 | 2,500 | 6 |
| **19** | 0.5 | 200 | 2,500 | 6 |
| **20** | 1 | 200 | 6,250 | 5 |
| **21** | 0.75 | 200 | 6,250 | 6 |
| **22** | 0.75 | 100 | 6,250 | 7 |
| **23** | 0.75 | 200 | 10,000 | 7 |
| **24** | 0.75 | 100 | 10,000 | 6 |
| **25** | 0.75 | 200 | 6,250 | 6 |
| **26** | 0.75 | 300 | 10,000 | 6 |
| **27** | 0.75 | 100 | 6,250 | 5 |

*Table 3:* Cells yield obtained from factor levels defined by Box-Behnken response surface experimental design. Harvest Density is expressed in cells/cm$^2$.

| Bottle | Seeded | Yield | Expansion | Doublings | Time (days) | Hours/doubling | Harvest Density |
|---|---|---|---|---|---|---|---|
| 1 | 5.31E+06 | 9.80E+06 | 1.84 | 0.88 | 5 | 136 | 1.15E+04 |
| 2 | 5.31E+06 | 1.68E+07 | 3.16 | 1.66 | 7 | 101 | 1.98E+04 |
| 3 | 5.31E+06 | 1.50E+07 | 2.82 | 1.5 | 7 | 112 | 1.76E+04 |
| 4 | 8.50E+06 | 1.86E+07 | 2.19 | 1.13 | 6 | 127 | 2.19E+04 |
| 5 | 5.31E+06 | 2.10E+07 | 3.95 | 1.98 | 6 | 73 | 2.47E+04 |
| 6 | 2.13E+06 | 1.23E+07 | 5.79 | 2.53 | 5 | 47 | 1.45E+04 |
| 7 | 5.31E+06 | 2.17E+07 | 4.08 | 2.03 | 6 | 71 | 2.55E+04 |
| 8 | 5.31E+06 | 1.62E+07 | 3.04 | 1.6 | 7 | 105 | 1.90E+04 |
| 9 | 5.31E+06 | 2.78E+07 | 5.23 | 2.39 | 6 | 60 | 3.27E+04 |
| 10 | 5.31E+06 | 1.96E+07 | 3.69 | 1.88 | 6 | 76 | 2.31E+04 |
| 11 | 8.50E+06 | 2.94E+07 | 3.46 | 1.79 | 6 | 80 | 3.46E+04 |
| 12 | 5.31E+06 | 1.58E+07 | 2.97 | 1.57 | 5 | 76 | 1.86E+04 |
| 13 | 8.50E+06 | 3.03E+07 | 3.56 | 1.83 | 5 | 65 | 3.56E+04 |
| 14 | 2.13E+06 | 1.50E+07 | 7.06 | 2.82 | 7 | 60 | 1.76E+04 |
| 15 | 5.31E+06 | 1.31E+07 | 2.47 | 1.3 | 6 | 111 | 1.54E+04 |
| 16 | 2.13E+06 | 1.59E+07 | 7.48 | 2.9 | 6 | 50 | 1.87E+04 |
| 17 | 2.13E+06 | 1.47E+07 | 6.92 | 2.79 | 6 | 52 | 1.73E+04 |
| 18 | 2.13E+06 | 1.84E+07 | 8.66 | 3.11 | 6 | 46 | 2.16E+04 |
| 19 | 2.13E+06 | 1.22E+07 | 5.74 | 2.52 | 6 | 57 | 1.44E+04 |
| 20 | 5.31E+06 | 1.63E+07 | 3.07 | 1.62 | 5 | 74 | 1.92E+04 |
| 21 | 5.31E+06 | 2.21E+07 | 4.16 | 2.06 | 6 | 70 | 2.60E+04 |
| 22 | 5.31E+06 | 1.77E+07 | 3.33 | 1.74 | 7 | 97 | 2.08E+04 |
| 23 | 8.50E+06 | 3.48E+07 | 4.1 | 2.04 | 7 | 83 | 4.10E+04 |
| 24 | 8.50E+06 | 3.55E+07 | 4.18 | 2.06 | 6 | 70 | 4.18E+04 |
| 25 | 5.31E+06 | 2.23E+07 | 4.2 | 2.07 | 6 | 70 | 2.62E+04 |
| 26 | 8.50E+06 | 3.40E+07 | 4 | 2 | 6 | 72 | 4.00E+04 |
| 27 | 5.31E+06 | 1.14E+07 | 2.15 | 1.1 | 5 | 109 | 1.34E+04 |

*Table 4*: Response Surface Regression Analysis: Harvest Density (cells/cm$^2$) versus Rotational Speed, Media Volume, Seeding Density and Days in Culture.

**Estimated Regression Coefficients for Harvest Density (coded units)**

| Term | Coef | SE Coef | T | P |
|---|---|---|---|---|
| Constant | 25647.1 | 2841 | 9.028 | 0.000 |
| Rotational Speed (rpm) | 43.1 | 1420 | 0.030 | 0.976 |
| Media Volume (ml) | 2029.4 | 1420 | 1.429 | 0.179 |
| Seeding Density (cells/cm sq) | 9229.4 | 1420 | 6.498 | 0.000 |
| Days | 1921.6 | 1420 | 1.353 | 0.201 |
| Rotational Speed (rpm)* Rotational Speed (rpm) | -4010.8 | 2131 | -1.882 | 0.084 |
| Media Volume (ml)*Media Volume (ml) | 9.8 | 2131 | 0.005 | 0.996 |
| Seeding Density (cells/cm sq)* Seeding Density (cells/cm sq) | 3898.0 | 2131 | 1.830 | 0.092 |
| Days*Days | -4887.3 | 2131 | -2.294 | 0.041 |
| Rotational Speed (rpm)* Media Volume (ml) | -3705.9 | 2460 | -1.506 | 0.158 |
| Rotational Speed (rpm)* Seeding Density (cells/cm sq) | -4264.7 | 2460 | -1.733 | 0.109 |
| Rotational Speed (rpm)*Days | -1723.5 | 2460 | -0.701 | 0.497 |
| Media Volume (ml)* Seeding Density (cells/cm sq) | -1529.4 | 2460 | -0.622 | 0.546 |
| Media Volume (ml)*Days | -2088.2 | 2460 | -0.849 | 0.413 |
| Seeding Density (cells/cm sq)*Days | 541.2 | 2460 | 0.220 | 0.830 |

S = 4920 R-Sq = 85.3% R-Sq(adj) = 68.2%

**Analysis of Variance for Harvest Density**

| Source | DF | Seq SS | Adj SS | Adj MS | F | P |
|---|---|---|---|---|---|---|
| Regression | 14 | 1691605592 | 1691605592 | 120828971 | 4.99 | 0.004 |
| Linear | 4 | 1115938309 | 1115938309 | 278984577 | 11.52 | 0.000 |
| Square | 4 | 408129068 | 408129068 | 102032267 | 4.21 | 0.023 |
| Interaction | 6 | 167538215 | 167538215 | 27923036 | 1.15 | 0.391 |
| Residual Error | 12 | 290519868 | 290519868 | 24209989 | | |
| Lack-of-Fit | 10 | 289163467 | 289163467 | 28916347 | 42.64 | 0.023 |
| Pure Error | 2 | 1356401 | 1356401 | 678200 | | |
| Total | 26 | 1982125460 | | | | |

Unusual Observations for Harvest Density

| Obs | StdOrder | Harvest Density | Fit | SE Fit | Residual | St Resid |
|---|---|---|---|---|---|---|
| 4 | 12 | 21882.350 | 30542.156 | 3757.991 | -8659.806 | -2.73 R |

(R denotes an observation with a large standardized residual.)

**Estimated Regression Coefficients for Harvest Density (uncoded units)**

| Term | Coef |
|---|---|
| Constant | -3.05951E+05 |
| Rotational Speed (rpm) | 195874.5033 |
| Media Volume (ml) | 281.8629 |
| Seeding Density (cells/cm sq) | 2.3578 |
| Days | 69013.6992 |
| Rotational Speed (rpm)* Rotational Speed (rpm) | -64172.5667 |
| Media Volume (ml)*Media Volume (ml) | 0.0010 |
| Seeding Density (cells/cm sq)* Seeding Density (cells/cm sq) | 0.0003 |

| | |
|---|---|
| Days*Days | -4887.2529 |
| Rotational Speed (rpm)* Media Volume (ml) | -148.2353 |
| Rotational Speed (rpm)* Seeding Density (cells/cm sq) | -4.5490 |
| Rotational Speed (rpm)*Days | -6894.1100 |
| Media Volume (ml)* Seeding Density (cells/cm sq) | -0.0041 |
| Media Volume (ml)*Days | -20.8824 |
| Seeding Density (cells/cm sq)*Days | 0.1443 |

*Table 5:* Response Surface Regression Analysis: Hours/Doubling versus Rotational Speed, Media Volume, Seeding Density and Days in Culture.

**Estimated Regression Coefficients for Hours/Doubling (coded units)**

| Term | Coef | SE Coef | T | P |
|---|---|---|---|---|
| Constant | 70.740 | 10.095 | 7.008 | 0.000 |
| Rotational Speed (rpm) | -4.098 | 5.047 | -0.812 | 0.433 |
| Media Volume (ml) | -6.351 | 5.047 | -1.258 | 0.232 |
| Seeding Density (cells/cm sq) | 15.516 | 5.047 | 3.074 | 0.010 |
| Days | 4.068 | 5.047 | 0.806 | 0.436 |
| Rotational Speed (rpm)* Rotational Speed (rpm) | 14.990 | 7.571 | 1.980 | 0.071 |
| Media Volume (ml)*Media Volume (ml) | 2.919 | 7.571 | 0.386 | 0.707 |
| Seeding Density (cells/cm sq)* Seeding Density (cells/cm sq) | -14.948 | 7.571 | -1.974 | 0.072 |
| Days*Days | 17.023 | 7.571 | 2.248 | 0.044 |
| Rotational Speed (rpm)* Media Volume (ml) | 11.188 | 8.742 | 1.280 | 0.225 |
| Rotational Speed (rpm)* Seeding Density (cells/cm sq) | 13.635 | 8.742 | 1.560 | 0.145 |
| Rotational Speed (rpm)*Days | 14.528 | 8.742 | 1.662 | 0.122 |
| Media Volume (ml)* Seeding Density (cells/cm sq) | 1.885 | 8.742 | 0.216 | 0.833 |
| Media Volume (ml)*Days | 12.015 | 8.742 | 1.374 | 0.194 |
| Seeding Density (cells/cm sq)*Days | 1.222 | 8.742 | 0.140 | 0.891 |

S = 17.48 R-Sq = 76.4% R-Sq(adj) = 48.9%

**Analysis of Variance for Hours/Doubling**

| Source | DF | Seq SS | Adj SS | Adj MS | F | P |
|---|---|---|---|---|---|---|
| Regression | 14 | 11898.4 | 11898.42 | 849.89 | 2.78 | 0.042 |
| Linear | 4 | 3773.1 | 3773.06 | 943.27 | 3.09 | 0.058 |
| Square | 4 | 5439.2 | 5439.24 | 1359.81 | 4.45 | 0.020 |
| Interaction | 6 | 2686.1 | 2686.12 | 447.69 | 1.46 | 0.270 |
| Residual Error | 12 | 3668.4 | 3668.38 | 305.70 | | |
| Lack-of-Fit | 10 | 3663.0 | 3662.98 | 366.30 | 135.71 | 0.007 |
| Pure Error | 2 | 5.4 | 5.40 | 2.70 | | |
| Total | 26 | 15566.8 | | | | |

Unusual Observations for Hours/Doubling

| Obs | StdOrder | Hours/Doubling | Fit | SE Fit | Residual | St Resid |
|---|---|---|---|---|---|---|
| 4 | 12 | 127.460 | 95.835 | 13.354 | 31.625 | 2.80 R |

(R denotes an observation with a large standardized residual.)

**Estimated Regression Coefficients for Hours/Doubling (uncoded units)**

| Term | Coef |
|---|---|
| Constant | 1322.8425 |
| Rotational Speed (rpm) | -905.2233 |
| Media Volume (ml) | -1.2682 |
| Seeding Density (cells/cm sq) | 0.0036 |
| Days | -269.8567 |
| Rotational Speed (rpm)* Rotational Speed (rpm) | 239.8467 |
| Media Volume (ml)*Media Volume (ml) | 0.0003 |
| Seeding Density (cells/cm sq)* Seeding Density (cells/cm sq) | -0.0000 |
| Days*Days | 17.0229 |
| Rotational Speed (rpm)* Media Volume (ml) | 0.4475 |
| Rotational Speed (rpm)* Seeding Density (cells/cm sq) | 0.0145 |
| Rotational Speed (rpm)*Days | 58.1100 |
| Media Volume (ml)* Seeding Density (cells/cm sq) | 0.0000 |
| Media Volume (ml)*Days | 0.1202 |
| Seeding Density (cells/cm sq)*Days | 0.0003 |

*Table 6:* Response Surface Regression Analysis: Population Doublings versus Rotational Speed, Media Volume, Seeding Density and Days in Culture.

**Estimated Regression Coefficients for Doublings (coded units)**

| Term | Coef | SE Coef | T | P |
|---|---|---|---|---|
| Constant | 2.03667 | 0.15586 | 13.067 | 0.000 |
| Rotational Speed (rpm) | 0.06167 | 0.07793 | 0.791 | 0.444 |
| Media Volume (ml) | 0.14417 | 0.07793 | 1.850 | 0.089 |
| Seeding Density (cells/cm sq) | -0.48500 | 0.07793 | -6.223 | 0.000 |
| Days | 0.15250 | 0.07793 | 1.957 | 0.074 |
| Rotational Speed (rpm)* Rotational Speed (rpm) | -0.25542 | 0.11690 | -2.185 | 0.049 |
| Media Volume (ml)*Media Volume (ml) | -0.03417 | 0.11690 | -0.292 | 0.775 |
| Seeding Density (cells/cm sq)* Seeding Density (cells/cm sq) | 0.47208 | 0.11690 | 4.038 | 0.002 |
| Days*Days | -0.35667 | 0.11690 | -3.051 | 0.010 |
| Rotational Speed (rpm)* Media Volume (ml) | -0.23500 | 0.13498 | -1.741 | 0.107 |
| Rotational Speed (rpm)* Seeding Density (cells/cm sq) | -0.26000 | 0.13498 | -1.926 | 0.078 |
| Rotational Speed (rpm)*Days | -0.17000 | 0.13498 | -1.259 | 0.232 |
| Media Volume (ml)* Seeding Density (cells/cm sq) | -0.09500 | 0.13498 | -0.704 | 0.495 |
| Media Volume (ml)*Days | -0.17750 | 0.13498 | -1.315 | 0.213 |
| Seeding Density (cells/cm sq)*Days | -0.02000 | 0.13498 | -0.148 | 0.885 |

S = 0.2700 R-Sq = 89.5% R-Sq(adj) = 77.3%

**Analysis of Variance for Doublings**

| Source | DF | Seq SS | Adj SS | Adj MS | F | P |
|---|---|---|---|---|---|---|
| Regression | 14 | 7.45883 | 7.45883 | 0.532773 | 7.31 | 0.001 |
| Linear | 4 | 3.39682 | 3.39682 | 0.849204 | 11.65 | 0.000 |
| Square | 4 | 3.29139 | 3.29139 | 0.822846 | 11.29 | 0.000 |
| Interaction | 6 | 0.77063 | 0.77063 | 0.128438 | 1.76 | 0.190 |
| Residual Error | 12 | 0.87456 | 0.87456 | 0.072880 | | |
| Lack-of-Fit | 10 | 0.86969 | 0.86969 | 0.086969 | 35.74 | 0.028 |
| Pure Error | 2 | 0.00487 | 0.00487 | 0.002433 | | |
| Total | 26 | 8.33339 | | | | |

Unusual Observations for Doublings

| Obs | StdOrder | Doublings | Fit | SE Fit | Residual | St Resid |
|---|---|---|---|---|---|---|
| 4 | 12 | 1.130 | 1.570 | 0.206 | -0.440 | -2.52 R |

(R denotes an observation with a large standardized residual.)

**Estimated Regression Coefficients for Doublings (uncoded units)**

| Term | Coef |
|---|---|
| Constant | -20.9241 |
| Rotational Speed (rpm) | 14.0700 |
| Media Volume (ml) | 0.0221 |
| Seeding Density (cells/cm sq) | -0.0003 |
| Days | 5.3308 |
| Rotational Speed (rpm)* Rotational Speed (rpm) | -4.0867 |
| Media Volume (ml)*Media Volume (ml) | -0.0000 |
| Seeding Density (cells/cm sq)* Seeding Density (cells/cm sq) | 0.0000 |
| Days*Days | -0.3567 |
| Rotational Speed (rpm)* Media Volume (ml) | -0.0094 |
| Rotational Speed (rpm)* Seeding Density (cells/cm sq) | -0.0003 |
| Rotational Speed (rpm)*Days | -0.6800 |
| Media Volume (ml)* Seeding Density (cells/cm sq) | -0.0000 |
| Media Volume (ml)*Days | -0.0018 |
| Seeding Density (cells/cm sq)*Days | -0.0000 |

*Example 2: Validation of Optimized Culture Conditions*

**[0068]** Cells: The cells used were umbilicus-derived cells identified as CBAT 050604B P6 (passage 6).

**[0069]** Media: Dulbecco's Modified Eagle Medium (DMEM) -low glucose, 15% Fetal Bovine Serum, Penicillin-Streptomycin, 2-Mercaptoethanol was used for the validation experiments.

**[0070]** Bottles: The bottles used were 850 cm sq culture bottles (e.g. Corning catalog number 430851).

**[0071]** Gelatin Coating: Twenty milliliters of 2% gelatin solution were added to each 850 cm sq bottle. The bottles were placed on the roller system for 20 minutes. The gelatin solution was removed by aspiration and each bottle was washed with 20 ml PBS.

**[0072]** Cell Seeding: 5.0E+06 P9 umbilical-derived cells (# 050604B) were thawed from a single cryogenic vial and washed to remove DMSO. Cells (2.12E+06) were seeded into a single 850 cm sq roller bottle pre-filled with 300 ml media and pre-gassed for 1 minute with compressed air containing 5% $CO_2$ , 95% atmospheric gas.

**[0073]** Incubation: Cells were cultured in a temperature-controlled room set at 37° C.

**[0074]** Speed: 0.7 RPM

**[0075]** Passaging: At passage media was removed from each roller bottle by aspiration and adherent cells were washed with 50 ml PBS. PBS was aspirated and 10 ml of trypsin-EDTA were added. Bottles were returned to the roller system and incubated for 5 minutes at 0.75 RPM. After incubation, 40 ml of media were added to each bottle. The growth medium with cells was then transferred to a 50 ml conical tube and centrifuged for 5 minutes at 300 x g. After centrifugation, the media was removed by aspiration and the cell pellet was re-suspended in 10 ml of media. Cells were counted using a Beckman-Coulter Cedex instrument. Cells were seeded into gassed roller bottles at about 2,500 cells/cm sq.

**[0076]** *Cell Yield per Passage*

*Table 7:* Growth kinetics from Umbilical cell line 050604B expanded from passage six to passage nine under optimized roller bottle culture conditions

| Passage | Seeded | Yield | Expansion | Doubling | Total Doublings | Time (days) | Hours/Doubling |
|---|---|---|---|---|---|---|---|
| | | 2.13E+06 | 1 | | | | |
| 6 | 2.13E+06 | 3.10E+07 | 1.46E+01 | 3.87E+00 | 3.87E+00 | 6.00 | 37.24 |
| 7 | 2.13E+06 | 3.55E+07 | 1.67E+01 | 4.06E+00 | 7.93E+00 | 7.00 | 41.36 |
| 8 | 2.13E+06 | 2.54E+07 | 1.20E+01 | 3.58E+00 | 1.15E+01 | 6.00 | 40.23 |
| 9 | 2.13E+06 | 2.56E+07 | 1.20E+01 | 3.59E+00 | 1.51E+01 | 6.00 | 40.10 |

*Example 3: Characterization of Cells Expanded on Optimized Culture Conditions*

**[0077]** Cells: Cells used for characterization experiments were umbilicus-derived cells at passage 6 (050604B P6)

**[0078]** Growth Medium: Dulbecco's Modified Eagle Medium (DMEM) -low glucose, 15% Fetal Bovine Serum, Penicillin-Streptomycin, 2-Mercaptoethanol was used as the growth medium.

**[0079]** Bottles: 850 cm sq bottles (e.g. Corning catalog number 430851)

**[0080]** Gelatin Coating: Twenty (20) ml of 2% gelatin solution was added to each 850 cm sq bottle. The bottles were placed on the roller system for 20 minutes. The gelatin solution was removed by aspiration and each bottle was washed with 20 ml PBS.

**[0081]** Cell Seeding: Umbilicus-derived cells at passage 9 (5.0E+06 P9) were thawed from a single cryogenic vial and washed to remove DMSO. Cells (2.12E+06) were seeded into 850 cm sq roller bottles pre-filled with 300 ml media and pre-gassed for 1 minute with compressed air containing 5% $CO_2$ , 95% atmospheric gas.

**[0082]** Incubation: Cells were cultured in a temperature-controlled room set at 37° C.

**[0083]** Speed: 0.7 RPM

**[0084]** Cell Harvesting: Media was removed from each roller bottle by aspiration and adherent cells were washed with 50 ml PBS. PBS was removed by aspiration and 10 ml of trypsin-ethylenediaminetetraacetic acid (EDTA) was added. The bottles were incubated for 5 minutes at 0.75 RPM on the roller system. After incubation, 40 ml of media were added to the bottles. Media with cells was then transferred to a 50 ml conical tube and centrifuged for 5 minutes at 300 x g. After centrifugation, the media was removed by aspiration and the cell pellet was re-suspended in 10ml of media. Cells were counted using a Beckman-Coulter Cedex instrument.

**[0085]** Antibody Staining for Flow Cytometry Analysis: Cells were and re-suspended in 3% (v/v) FBS in PBS at a cell concentration of 1x107 per milliliter. Antibody is added as per manufacture's specifications and incubated with cells in the dark for 30 minutes at 4 °C. After incubation, cells were washed with PBS and centrifuged to remove unbound antibody. Cells were re-suspended in 500 microliter PBS and analyzed by flow cytometry.

**[0086]** Flow Cytometry Analysis: Flow cytometry analysis was performed with a FACScalibur (Becton Dickinson San Jose, CA) instrument.

**[0087]** Antibodies: The following antibodies were used:

| Antibody | Manufacture | Catalog Number |
|---|---|---|
| CD10 | BD Pharmingen (San Diego, CA) | 555375 |
| CD13 | BD Pharmingen (San Diego, CA) | 555394 |
| CD31 | BD Pharmingen (San Diego, CA) | 555446 |
| CD34 | BD Pharmingen (San Diego, CA) | 555821 |
| CD44 | BD Pharmingen (San Diego, CA) | 555478 |
| CD45RA | BD Pharmingen (San Diego, CA) | 555489 |
| CD73 | BD Pharmingen (San Diego, CA) | 550257 |
| CD90 | BD Pharmingen (San Diego, CA) | 555596 |
| CD117 | BD Biosciences (San Jose, CA) | 340529 |

| | | |
|---|---|---|
| CD141 | BD Pharmingen (San Diego, CA) | 559781 |
| PDGFr-alpha | BD Pharmingen (San Diego, CA) | 556002 |
| HLA-A, B, C | BD Pharmingen (San Diego, CA) | 555553 |
| HLA-DR, DP, DQ | BD Pharmingen (San Diego, CA) | 555558 |
| IgG-FITC | Sigma (St. Louis, MO) | F-6522 |
| IgG- PE | Sigma (St. Louis, MO) | P-4685 |

**[0088]** *Total RNA isolation:* RNA was isolated with a RNeasy Mini Kit according to manufacture's specifications (RNeasy Mini Kit; Qiagen, Valencia, CA). RNA was eluted with 50 μL DEPC-treated water and stored at -80°C.

**[0089]** *Reverse transcription:* RNA was reversed transcribed using random hexamers with the TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA) at 25°C for 10 minutes, 37°C for 60 minutes and 95°C for 10 minutes. Samples were stored at -20°C. Genes termed "signature genes" (oxidized LDL receptor, interleukin-8, renin and reticulon), were further investigated using real-time PCR.

**[0090]** *Real-time PCR:* PCR was performed on cDNA samples using Assays-on-Demand™ gene expression products: oxidized LDL receptor (Hs00234028), renin (Hs00166915), reticulon (Hs00382515) CXC ligand 3 (Hs00171061), GCP-2 (Hs00605742) IL-8 (Hs00174103) and GAPDH (Applied Biosystems, Foster City, CA) were mixed with cDNA and TaqMan Universal PCR master mix according to the manufacturer's instructions (Applied Biosystems, Foster City, CA) using a 7000 sequence detection system with ABI prism 7000 SDS software (Applied Biosystems, Foster City, CA). Thermal cycle conditions were initially 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min.

**[0091]** ***Results:***

*Table 6:* Expression of cell surface proteins expressed by Umb 050604B cells expanded with static TC flask methods or with the optimized roller bottle method as analyzed by flow cytometry.

| **Cell Surface Marker** | **Static TC Flasks** | **Roller Bottles** |
|---|---|---|
| CD 10 | (+) | (+) |
| CD 13 | (+) | (+) |
| CD 31 | (-) | (-) |
| CD 34 | (-) | (-) |
| CD 44 | (+) | (+) |
| CD 45 | (-) | (-) |
| CD 73 | (+) | (+) |

| | | |
|---|---|---|
| CD 90 | (+) | (+) |
| CD 117 | (-) | (-) |
| CD 141 | (-) | (-) |
| PDGFr-alpha | (+) | (+) |
| HLA-ABC | (+) | (+) |
| HLA-DRDPDQ | (-) | (-) |

**[0092]** *Real Time PCR Analysis of Cells Expanded in Optimized Roller Bottle Culture Conditions*

*Table 7:* Comparison of CT values for genes expressed by cells (Umb 050604B) expanded in static plastic with those grown TC flasks in the optimized roller bottle conditions.

| | CT Values | | | | CT Values-Normalized | | |
|---|---|---|---|---|---|---|---|
| **Cell Line** | **reticulon** | **LDL-R** | **IL-8** | **GAPDH** | **reticulon** | **LDL-R** | **IL-8** |
| Umb-static culturc | 27.79 | 32 | 28.7 | 27.02 | 0.972 | 0.844 | 0.941 |
| Umb -roller bottles | 24.22 | 27.37 | 19.91 | 20.49 | 0.846 | 0.749 | 1.029 |

Example 4: VALIDATION OF OPTIMIZED CULTURE CONDITIONS IN CELLBIND ROLLER BOTTLES

**[0093]** *Cells:* 050604B P7

**[0094]** *Media:* Dulbecco's Modified Eagle Medium (DMEM) -low glucose, 15% Fetal Bovine Serum, Penicillin-Streptomycin, 2-Mercaptoethanol.

**[0095]** *Bottles:* Corning 850 cm sq bottles (catalog number 430851), CellBind Corning 850 cm sq bottles (catalog number 3907)

**[0096]** *Gelatin Coating:* 20 ml of 2% gelatin solution is added to Corning 850 cm sq bottles (catalog number 430851) and placed on the roller system for 20 minutes. The gelatin solution is rcmovcd by aspiration and washcd with 20 ml PBS. CellBind Corning 850 cm sq bottles are not coated.

**[0097]** *Cell Seeding:* 5.0E+06 P9 Umbilical 050604B cells were thawed from a single cryogenic vial and washed to remove DMSO. 2.12E+06 cells were seeded into a single 850 cm sq roller bottle pre-filled with 300 ml media and pre-gassed for 1 minute with compressed air containing 5% CO2 , 95% atmospheric gas.

**[0098]** *Incubation:* Cells were cultured in a temperature controlled room set at 37°C.

**[0099]** *Speed:* 0.7 RPM

**[0100]** *Passage:* At passage media was removed from the roller bottle by aspiration and adherent cells were washed with 50 ml PBS. PBS was aspirated and 10 ml of trypsin-EDTA is added. Bottles are returned to roller system and incubated for 5 minutes at 0.75 RPM. After incubation 40 ml of media is added to the bottles. Media with cells are then transferred to a 50 ml conical tube and centrifuged for 5 minutes at 300 xg. After centrifuge, the media was removed by aspiration and the cell pellet was re-suspended in 10 ml of media. Cells are counted using a Beckman-Coulter Cedex instrument. Cells were seeded into gassed roller bottles at 2,500-cells/cm sq.

**[0101]** ***Results:***

*Table 8:* Actual Cell Yield per Passage for Cells Grown in Gelatin-coated Roller Bottles versus Bottles with CellBind.

Umb050604B Optimal Roller Bottle- Gel Coat

| Passage | Seeded | Yield | Date | Expansion | Doubling | Time (days) | Time (hrs) | Hours/ doubling | Harvest Density |
|---|---|---|---|---|---|---|---|---|---|
| | | 2.13E+06 | 4-Jan | 1 | | | | | |
| 7 | 2.13E+06 | 2.45E+07 | 10-Jan | 1.15E+01 | 3.53E+00 | 6.00 | 144.00 | 40.83 | 2.88E+04 |

Umb050604B Optimal Roller Bottle- CellBind

| Passage | Seeded | Yield | Date | Expansion | Doubling | Time (days) | Time (hrs) | Hours/ doubling | Harvest Density |
|---|---|---|---|---|---|---|---|---|---|
| | | 2.13E+06 | 4-Jan | 1 | | | | | |
| 7 | 2.13E+06 | 3.17E+07 | 10-Jan | 1.49E+01 | 3.90E+00 | 6.00 | 144.00 | 36.93 | 3.73E+04 |

**[0102]** The present invention is not limited to the embodiments described and exemplified above, but is capable of variation and modification within the scope of the appended claims.

What is Claimed:

1. A method of maximizing the number doublings of a culture of postpartum cells in a roller bottle culture system comprising using a rotational speed of at least about 0.85 rpm, using a media volume of at least about 100 ml in an 850 square centimeter culture bottle; using a seeding density of less than about 2500 cells per square centimeter; and incubating for at least about 5.5 days.

2. The method of claim 1 wherein the rotational speed is about 1 rpm; the media volume is about 112 ml in an 850 square centimeter culture bottle; and the incubation time is about 6.2.

3. The method of claim 1 which results in about 3 population doublings.

4. The method of claim 1 wherein the postpartum cells are umbilicus-derived cells.

5. The method of claim 4 wherein the postpartum cells are ATCC Nos PTA-6067 and PTA-6068.

6. A method of maximizing the rate of doubling of a culture of postpartum cells in a roller bottle culture system comprising using a rotational speed of at least about 0.85 rpm, using about 300 ml of growth medium in an 850 square centimeter culture bottle; using a seeding density of less than about 2500 cells per square centimeter; and incubating for less than about 6 days.

7. The method of claim 6 wherein the rotational speed is about 0.93 rpm and the incubation time is less than about 5.5 days.

8. The method of claim 6 wherein the time for a population to double is less than about 30 hours.

9. The method of claim 6 wherein the postpartum cells are umbilicus-derived cells.

10. The method of claim 9 wherein the postpartum cells are ATCC Nos PTA-6067 and PTA-6068.

11. A method of maximizing the density of cells at harvest for postpartum cells in a roller bottle culture system comprising using a rotational speed of about 0.5-1 rpm, using about 300 ml of

growth medium in an 850 square centimeter culture bottle; using a seeding density of about 10,000 cells per square centimeter; and incubating for between about 5.5 to 6.7 days.

12. The method of claim 11 wherein the rotational speed is about 0.98 rpm and the incubation time is about 6.7 days.

13. The method of claim 11 wherein the density of the cells is about at least about 3 x E+4 cells/cm sq.

14. The method of claim 11 wherein the postpartum cells are umbilicus-derived cells.

15. The method of claim 14 wherein the postpartum cells are ATCC Nos PTA-6067 and PTA-6068.

16. A method of simultaneously maximizing doubling rate, harvest density, and the total number of population doublings for postpartum cells in a roller bottle culture system comprising using a rotational speed of about 0.65-0.9 rpm, using at least about 300 ml of growth medium in an 850 square centimeter culture bottle; using a seeding density of less than about 2,500 cells per square centimeter; and incubating for between about 5.5 to 6.5 days.

17. The method of claim 16 wherein the rotational speed is about 0.7, and the incubation time is about 6 to 6.3 days.

18. The method of claim 16 wherein the rotational speed is about 0.85, and the incubation time is about 6 to 6.3 days.

19. The method of claim 16 wherein a population doubling time is les than about 39 hours, the population achieves at least about 3 doublings, and the density of cells at harvest is at least about 1.8 E+4 cells/cm sq.

20. The method of claim 16 wherein the postpartum cells are umbilicus-derived cells.

21. The method of claim 20 wherein the postpartum cells are ATCC Nos PTA-6067 and PTA-6068.