

SYSTEM FOR PRODUCING CULTIVATED MEATS, TISSUES AND ASSOCIATED PRODUCTS FROM CELLS

Technical Field

5 **[0001]** Embodiments discussed herein generally relate to improved systems and methods for growing cell culture. Embodiments discussed herein also generally relate to the improved systems and methods for meat production and tissue constructions/engineering.

Background

10 **[0002]** Animal meat is high in protein, and supplies all the amino acids needed to build the protein used to support body functions. Meat for consumption is traditionally obtained from animals or fish that are reared on farms. However, agriculture and aquaculture for producing animal meat require a large amount of energy and resources, and have a high carbon footprint. Meat produced by
15 agriculture or aquaculture may pose a public health risk as the production processes may expose the meat to diseases, pollutants, and toxins. A number of concerns such as a growing population, increasing demand for meat, environmental concerns, limited land and water resources, biodiversity loss, and the negative perception associated with animal slaughter have led
20 scientists to develop techniques to produce meat by alternative processes.

[0003] In vitro meat production is the process by which muscle tissue or organ tissue from animals are grown in laboratories using cell culture techniques to manufacture meat and meat products. As used herein, in vitro meat and meat products includes animal protein products as well as non-meat products
25 including soluble forms and solid forms. While still in an early stage of development, in vitro meat and meat products may offer a number of advantages over traditional meat products such as health and environmental advantages, and benefits to animal welfare. It is a next-generation and emerging technology that operates as part of a wider field of cellular
30 agriculture, or the production of agricultural products from cell cultures.

[0004] Cells for the production of in vitro meat may be cells (e.g., muscle cells, somatic cells, stem cells, etc.) taken from animal biopsies, which may then be grown separately from the animal in culture media in a bioreactor or other

type of sterile environment. The cells may grow into a semi-solid or solid form mimicking an animal organ by attaching to an edible three-dimensional scaffold that is placed in the bioreactor. The starter cells may be primary cells directly obtained from the animal's tissues, or continuous cell lines. If grown under the right conditions in appropriate culture media, primary cells will grow and proliferate, but only a finite number of times that is related to the telomere length at the end of the cell's DNA. Continuous cell lines, on the other hand, can be cultured in vitro over an extended period. Cell biology research has established procedures on how to convert primary cells into immortal continuous cell lines. Primary cells may be transformed into continuous cell lines using viral oncogenes, chemical treatments, or overexpression of telomerase reverse transcriptase to prevent the telomeres from shortening.

[0005] The culture media may contain components necessary for cell proliferation such as amino acids, salts, vitamins, growth factors, and buffering systems to control pH. Current methods add fetal bovine serum (FBS) to the media prior to use as it provides vital macromolecules, growth factors, and immune molecules. However, FBS is derived from unborn calves and, therefore, is incompatible with the objective of being free from animal products. Growing the cells in an animal component-free medium is an important factor considered by scientists involved in in vitro meat production research. Some growth factors may be derived from human sources.

[0006] Current in vitro meat production covers most commodity meat types, such as cell-based beef, pork and poultry meats. However, these types of meats have a complex tissue organization involving multiple cell types that are difficult and costly to produce using current biomedical technology techniques. There is also a lack of non-GM methods to increase the protein level and biomass yield in meat produced by cell culture techniques. Furthermore, as explained above, current cell culture technologies may rely on animal components (e.g., FBS) as a nutrient source, as well as expensive non-food grade growth factors.

[0007] In the production of cultivated meats or the clinical applications of regenerative medicine/tissue engineering/tissue construction, it is critical to harvest a sufficient cell number. In the application of production of cultivated

meat, 1 kg of protein contains approximately 8×10^{12} muscle cells. In the application of regenerative medicine, approximately 10^{10} to 10^{12} cells per treatment are required for most applications. For example, 1×10^9 to 2×10^9 cardiomyocytes would be required to replace damaged cardiac tissue in adults. Treating hepatic failure would require a cell number of 10^{10} hepatocytes.

[0008] The current cell culture approach is to seed cells on the 2D culture surface of culture vessels in the presence of a culture medium. In general, the culture medium contains glucose, vitamins, inorganic salts, amino acids and other nutrients. As the cells grow, nutrients are gradually depleted and metabolic wastes accumulate. Therefore, the culture medium is replaced every 2 to 3 days to replenish nutrients and remove wastes. There are several problems with this cell culture approach. Firstly, cells grow in suboptimal conditions between medium replacements. Particularly if the cells are highly proliferative and have a high metabolic rate, cells consume nutrients such as glucose and accumulate waste such as lactate and ammonia in a short time. An elevated level of metabolic wastes or growth inhibitors can inhibit cell growth. This hinders cells from growing at an optimal rate before the next round of medium replacement. Secondly, changing culture medium wastes nutrients and growth factors; and increases production costs. There are still nutrients in the spent culture medium when it is replaced. Particularly, growth factors in the spent culture medium, either from serum supplements or secreted by cells, are removed during medium replacement. This increases the use of serum supplements, which contributes to a significant portion of medium cost and production cost. Thirdly, the medium needs to be changed manually and this increases production costs and the chance of contamination in large-scale manufacturing.

Summary

[0009] The embodiments of the present disclosure apply methods for in vitro meat production for human consumption that provides a solution to the above challenges.

[0010] It is an objective of the present invention to provide an alternative system and method to scale up the cells production, thereby lowering the costs for and

scaling up the production of cultivated meats and tissue engineering/regenerative medicine/tissue construction applications. It is also an objective of the present invention to provide a system that maintains an optimal culture condition, stable level of nutrients and/or a minimum level of growth inhibitors for cell production. Yet another objective of the present invention is to provide a large-scale cell culture system that can automatically replenish nutrients and remove growth inhibitors produced by the cells while retaining of unused growth factors to enhance production and reduce cost.

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10 [0011] Another objective of the present invention is to provide a method for making meat products using the cell culture system. Furthermore, an objective of the present invention is to provide a method for tissue engineering/construction using this culture system. Finally, an objective of the present invention is to provide a method for expanding cells to a clinically relevant number for regenerative medicine applications.

15 [0012] The present invention provides the following advantages: (1) keeping nutrients such as glucose at optimal level and waste such as ammonia and lactate at low level in the culture medium for optimal cell viability and cell growth; and (2) retaining growth factors secreted by cells and reducing the use of animal-derived serum in the culture medium. Growth factors are expensive,
20 therefore the present invention helps lower the cost and optimizes medium use by retaining the growth factors in the culture medium while removing the wastes.

[0013] As it will be discussed in more detail below, the present invention dramatically (1) reduces wastes discharged from the cell culturing system and
25 (2) increases the cell mass production compared to the conventional technique.

[0014] According to one embodiment of the present disclosure, a cell culture system comprising a cell culture unit configured to hold at least one type of cell; a fresh medium unit configured to supply and receive a first fluid to the cell culture unit; a waste removal unit configured to supply and receive a
30 second fluid from the fresh medium unit; a first pump connected between the cell culture unit and the fresh medium unit configured to circulate the first fluid; a second pump connected between the fresh medium unit and the waste removal unit configured to circulate the second fluid, wherein the cell culture

unit, the fresh medium unit and the waste removal unit are separated from each other to avoid cross contaminations.

5 [0015] According to another embodiment of the present disclosure, a method of growing cell culture using the cell culture system of the present invention comprising the steps of placing at least one type of cell into the cell culture unit; circulating the first fluid between the cell culture unit and the fresh medium unit; circulating the second fluid between the fresh medium unit and the waste removal unit; extracting waste from the first fluid to the second fluid at the fresh medium unit; and replenishing nutrient from the second fluid to the first fluid at the fresh medium unit.

[0016] Embodiments disclosed herein apply systems and methods for in vitro meat production for human consumption and/or tissue engineering/tissue constructions/regenerative medicine applications that provide solutions to the above challenges.

15 [0017] **Brief Description of the Drawings**

[0018] The disclosure may be better understood by reference to the detailed description when considered in connection with the accompanying drawings. The components in the figures are not necessarily to scale, emphasis instead being placed upon illustrating the principles of the disclosure.

20 [0019] FIG. 1 is a flowchart of a method for meat production by in vitro cell culture, according to one embodiment of the present disclosure.

[0020] FIG. 2 is a schematic representation of a method for post-transcriptional enhancement of protein expression, according to one embodiment of the present disclosure.

25 [0021] FIG. 3 is a schematic representation of a method for post-transcriptional enhancement of collagen, type 1, alpha 1 (COL1A1) expression, according to one embodiment of the present disclosure.

[0022] FIG. 4 is a schematic representation of a method for post-transcriptional enhancement of collagen, type 1, alpha 2 (COL1A2) expression, according to one embodiment of the present disclosure.

30 [0023] FIG. 5 is a schematic or conceptual cross-sectional view of a bioreactor used for in vitro meat production having a solid phase support, according to one embodiment of the present disclosure.

[0024] FIG. 6 is a schematic or conceptual cross-sectional view of a bioreactor similar to FIG. 5 but having a second solid phase, according to one embodiment of the present disclosure.

5 [0025] FIG. 7 is a schematic view of a cell culture system according to one embodiment of the present disclosure.

[0026] FIG. 8 is a schematic view of a fresh medium unit according to one embodiment of the present disclosure.

[0027] FIG. 9 is a schematic view of a waste removal unit according to one embodiment of the present invention.

10 [0028] FIG. 10A shows Brightfield images and LIVE/DEAD staining images of HEK293 cells cultured in a collagen-based scaffolds on day 0. FIG. 10B shows Brightfield images and LIVE/DEAD staining images of HEK293 cells cultured in a collagen-based scaffolds on day 4. FIG. 10C shows Brightfield images and LIVE/DEAD staining images of HEK293 cells cultured in a
15 collagen-based scaffolds on day 11.

[0029] FIG. 11 shows images of extracted cell mass produced after 11 days of culture in the cell culture system of FIG. 7, the cell mass was extracted from the collagen-based scaffold by trypsinization and centrifugation. Cells grew in the cell culture system were grown to form microtissues of a much higher
20 volume than those in control. Clumps of microtissues were observed in the cell culture system but not in control. Bioreactor, cells cultured in the working cell culture system prototype. Control, cells cultured in a 6-well plate.

[0030] FIG. 12 is a graph of the change in glucose concentration in the culture medium over 11 days of cell culture in the cell culture system and in a 6-well
25 plate.

Detailed Description

[0031] Referring now to the drawings, and with specific reference to FIG. 1, a method 10 for in vitro meat production is shown. As used herein, "in vitro meat production" refers to a cell-based meat production process or cell-based
30 agriculture process in which tissues from animals and/or plants are grown in laboratories using cell culture techniques to manufacture meat and meat products. At a block 12, tissue from an animal or a plant is isolated. In one embodiment, the tissue is derived from bony fish of the class Osteichthyes

including saltwater fish such as a grouper, sea bass, or a yellow cocker. In other embodiments, other types of animal tissue, such as cow tissue, may be isolated. In some embodiments, the block 12 may involve collecting organ tissue, such as a swim bladder, from a fish and making a cell suspension.

5 Although the following description primarily describes tissues derived from fish sources, it will be understood that the concepts may be applied to tissues derived from other types of animal sources and/or plant sources to provide other types of in vitro meat and/or animal protein products, and vegetarian meat and/or protein products.

10 **[0032]** Many of the isolated cells are adult cells, and can be made to proliferate continuously using various established methods in medical research (block 14). For example, specific genes, such as Yamanaka factors, may be used to reprogram the adult cells into stem cells, such as induced pluripotent stem cells (iPSCs). Alternatively, the isolated adult cells may be
15 transformed into continuous cell lines by telomerase reverse transcriptase overexpression. In other embodiments, other types of cells may be isolated such as adult stem cells and embryonic stem cells. In this regard, it will be understood that the methods of the present disclosure include all sources of cell lines.

20 **[0033]** At a next block 16, the cells are grown into a solid or semi-solid structure mimicking an animal organ, such as a fish organ, by attaching/adhering to a food-grade biocompatible scaffold in a sterile chamber or container, such as a bioreactor. The sterile chamber or container may be temperature controlled, and may have inlets and outlets for introducing and
25 removing substances such as chemicals, nutrients, and cells. The food-grade biocompatible scaffold becomes part of the final edible product, and is made of plant-based or fungi-based materials such as, but not limited to, agarose, alginate, chitosan, mycelium, and konjac glucomannan. Alginate is a biopolymer naturally derived from brown algae and is biocompatible. In
30 addition, plant-based chitosan from fungi has antibacterial properties. In some embodiments, the block 16 is carried out in the absence of antibiotics or antimicrobial compounds in the sterile container. A block 18 involves supplying the culture medium to the bioreactor to support cell survival and

growth. The culture medium may be a buffered solution containing components such as, but not limited to, inorganic salts (e.g., calcium chloride (CaCl_2), potassium chloride (KCl), sodium chloride (NaCl), sodium bicarbonate (NaHCO_3), sodium dihydrogen phosphate (NaH_2PO_4),
5 magnesium sulfate (MgSO_4), etc.), amino acids, vitamins (e.g., thiamine, riboflavin, folic acid, etc.), and other components such as glucose, β -mercaptoethanol, ethylenediaminetetraacetic acid (EDTA), and sodium pyruvate. Non-limiting examples of growth media include, but are not limited to, Leibovitz's L-15 medium, Eagle's Minimum Essential Media (MEM),
10 Medium 199, Dulbecco's Modified Eagle Medium (DMEM), Ham's F12 Nutrient Mix, Ham's F10 Nutrient Mix, MacCoy's 5A Medium, Glasgow Modified Eagle Medium (GMEM), Iscove's Modified Dulbecco's Medium, and RPMI 1640.

[0034] According to a block 20, food-grade growth factors and cytokines are
15 introduced into the culture medium in the bioreactor to support cell growth and proliferation. The growth factors and cytokines may include, but are not limited to, insulin growth factor 1 (IGF-1), insulin, interleukin 6 (IL-6), interleukin 6 receptor (IL-6R), interleukin 11 (IL-11), fibroblast growth factor (FGF), epidermal growth factor (EGF), and transferrin. The block 20 may
20 involve co-culturing bioengineered cells with the isolated cells in the absence of fetal bovine serum (FBS). The bioengineered cells are engineered to secrete the above growth factors and cytokines, and supply these biomolecules to the isolated cells as needed for growth and proliferation.

[0035] As used herein, "bioengineered" cells are not equivalent to genetically-
25 modified cells. The bioengineered cells have a specific gene that overexpresses one or more specific proteins. The bioengineered cells may be fish cells, or other types of animal cells, such as cow cells. The bioengineered cells are not present in the final meat product. As non-limiting examples, bioengineered fish cells may be co-cultured with isolated fish cells,
30 or bioengineered cow cells may be co-cultured with isolated cow cells. The co-culturing method of the present disclosure eliminates the need for animal-derived fetal bovine serum (FBS) in the culture medium. Furthermore, the co-culturing method provides a continuous supply of food-grade specific growth

factors and cytokines to the growing isolated cells in situ, and simplifies and reduces the cost of the production process. However, in other embodiments, FBS or other serum may be used to supply growth factors, cytokines, and other nutrients to support cell growth during the block 16.

- 5 **[0036]** Additionally, according to a block 22, protein expression in the cells is increased to increase the biomass yield in the resulting meat product. As used herein, "biomass yield" refers to the amount of digestible material (e.g., proteins) in the resulting meat product that is available for energy production upon consumption. More specifically, the block 22 involves increasing protein
- 10 expression by altering micro RNA levels in the cells, with the manipulation of the cells being carried out prior to culturing. Micro RNAs are endogenous, short, non-encoding single-stranded RNA sequences involved in regulating post-transcriptional gene expression. The block 22 involves increasing the amount of up-regulating micro RNAs that increase protein expression by
- 15 promoting messenger RNA (mRNA) translation, and/or decreasing the amount of down-regulating micro RNAs that decrease protein expression by suppressing mRNA translation. The micro RNA levels may be increased or decreased by introducing micro RNAs, micro RNA mimics, or micro RNA
- 20 inhibitors into the cells. The micro RNA mimics have the same function as micro RNAs, but maybe more stable and efficient in modulating protein expression. In some embodiments, electroporation may be used to introduce episomal vectors into the cells that carry instructions to express specific micro
- 25 RNAs. Alternatively or in combination with this, an adeno-associated virus may be used as a vehicle carrying episomal instructions to express specific micro RNAs. Decreasing the amount of targeted down-regulating micro RNAs may be achieved by introducing inhibitors for the targeted micro RNAs into the cells by transfection. It is noted here that the methods of increasing protein expression/biomass yield according to the present disclosure is carried out without modifying the genome of the cells.
- 30 **[0037]** Turning to FIG. 2, a method for post-transcriptional enhancement of protein expression in the cell lines is schematically depicted. One or more up-regulating micro RNAs (miRNAs) may be increased to increase mRNA translation and protein production of selected proteins. Alternatively or in

combination with this, one or more down-regulating miRNAs may be blocked with inhibitors (anti-miRNAs) to increase mRNA translation and protein production of selected proteins.

[0038] Fish swim bladder primarily includes fibroblasts and collagen protein.

5 Collagen type 1 (collagen I) is a dominant protein in the fish swim bladder, and increased expression of collagen I in cultured fish swim bladder cells may increase biomass yield. Collagen I in the fish swim bladder cells includes collagen, type 1, alpha 1 (COL1A1) and collagen, type 1, alpha 2 (COL1A2). COL1A1 and COL1A2 expression is increased by up-regulating microRNA 21
10 (miR-21), such that increasing levels of miR-21 increase COL1A1 and COL1A2 production in fish swim bladder cells. Additionally, COL1A1 and COL1A2 expression are decreased by down-regulating microRNA 29a (miR-29a), such that decreasing levels of miR-29a or blocking the action of miR-29a increases COL1A1 and COL1A2 production in fish swim bladder cells.
15 FIGs. 3-4 show increasing COL1A1 (FIG. 3) and COL1A2 (FIG. 4) production by increasing miR-21 levels and by blocking the action of miR-29a with the use of inhibitors (anti-miR 29a). Increased COL1A1 and COL1A2 production results in increased biomass yield in the resulting meat product. Similar strategies may be applied to increase relevant protein levels in other types of
20 animal cells.

[0039] Turning to FIG. 5, an exemplary bioreactor 30 used for culturing the isolated cells is shown. The cells attach to and grow on a solid phase support
32 provided by a food-grade scaffold 34 which is held in a sterile chamber 36 in the bioreactor 30. The scaffold 34 may dictate the shape of the meat
25 product. The food-grade scaffold 34 is made of plant-based or fungi-based materials such as, but not limited to, agarose, alginate, chitosan, mycelium, and konjac glucomannan. The solid phase support 32 may be porous so that the cells may attach to and grow on the inner surfaces of the support 32. The culture medium supplying nutrients to the cells is introduced into the
30 bioreactor 30 through an inlet 38, and is emptied from the bioreactor 30 through an outlet 40.

[0040] FIG. 6 shows a bioreactor 50 similar to the bioreactor 30 of FIG. 5, but further includes a second solid phase 52 separated from the solid phase

support 32 by a fine mesh 54. The second solid phase 52 may contain or support the bioengineered cells that secrete nutrients, growth factors, and cytokines for the cells growing on the solid phase support 32 in situ, and may physically separate the bioengineered cells from the cells on the solid phase support 32. The second solid phase 52 is made of plant-based materials, similar to the solid phase support 32. The mesh 54 is permeable to nutrients, growth factors, and cytokines, but is impermeable to cells. The bioreactor 50 of FIG. 6 allows the co-culturing of the bioengineered cells with the growing cells. In some embodiments, the bioreactors 30 and 50 of FIGs. 5 and 6 may be arranged in tandem. In other embodiments, several of the bioreactors 30, several of the bioreactors 50, or mixtures of the bioreactors 30 and 50 may be arranged in series for scaling up the process. The bioreactor 30 may be used mainly for biomass production, whereas the bioreactor 50 may be used for providing nutrients, growth factors, and cytokines to the growing cells.

[0041] Turning to FIG.7, an exemplary cell culture system 100 comprises a cell culture unit 102, a fresh medium unit 104 and a waste removal unit 106. The cell culture unit 102 is connected to the fresh medium unit 104 through pumps 108a and 108b such that a first fluid may flow from cell culture unit 102 to the fresh medium unit 104 through pump 108a and the first fluid may flow from the fresh medium unit 104 to the cell culture unit 102 through pump 108b. Thereby the first fluid circulates between the cell culture unit 102 and the fresh medium unit 104.

[0042] The fresh medium unit 104 is further connected to the waste removal unit 106 through pumps 108c and 108d such that a second fluid may flow from the fresh medium unit 104 to the waste removal unit 106 through pump 108c and the second fluid may flow from the waste removal unit 106 to the fresh medium unit 104 through pump 108d. Thereby, the second fluid circulates between the fresh medium unit 104 and the waste removal unit 106. The first fluid may be cell culture medium which may include a basal medium supplemented with FBS, growth factors or cytokines. Growth factors or cytokines may include but are not limited to, insulin growth factor 1 (IGF-1), insulin, interleukin 6 (IL-6), interleukin 6 receptor (IL-6R), interleukin 11 (IL-11), fibroblast growth factor (FGF), epidermal growth factor (EGF), and

transferrin. The second fluid may be a fresh basal medium which may include a buffered solution containing components such as, but not limited to, inorganic salts (e.g., calcium chloride (CaCl₂), potassium chloride (KCl), sodium chloride (NaCl), sodium bicarbonate (NaHCO₃), sodium dihydrogen phosphate (NaH₂PO₄), magnesium sulfate (MgSO₄), etc.), amino acids, vitamins (e.g., thiamine, riboflavin, folic acid, etc.), and other components such as glucose, beta-mercaptoethanol, ethylenediaminetetraacetic acid (EDTA), and sodium pyruvate. Non-limiting examples of growth media include, but are not limited to, Leibovitz's L-15 medium, Eagle's Minimum Essential Media (MEM), Medium 199, Dulbecco's Modified Eagle Medium (DMEM), Ham's F12 Nutrient Mix, Ham's F10 Nutrient Mix, MacCoy's 5A Medium, Glasgow Modified Eagle Medium (GMEM), Iscove's Modified Dulbecco's Medium, and RPMI 1640.

[0043] The pumps 108 may be peristaltic pumps or any other similar suitable pumps.

[0044] The cell culture system 100 includes one or more vessels. Each cell culture unit 102, fresh medium unit 104 and waste removal unit 106 may be a vessel. In some embodiments, the cell culture system 100 may include more than one cell culture unit 102, fresh medium unit 104 and waste removal unit 106. The plurality of cell culture unit 102 may be connected in parallel or series and disposed in proximity to each other. The plurality of fresh medium unit 104 may be connected in parallel or series and disposed in proximity to each other. The plurality of waste removal unit 106 may be connected in parallel or series and disposed in proximity to each other.

[0045] In some embodiments, the cell culture system 100 may further include a gas source configured to supply gas to the cells in the cell culture unit 102. The gas source may be oxygen or carbon dioxide.

[0046] The cell culture unit 102 is configured to hold scaffold and culture medium such that cells may grow into a solid or semi solid structure. In some embodiments, the cells are grown into a solid or semi-solid structure mimicking an animal organ, such as a fish organ, by attaching/adhering to a food grade biocompatible scaffold to the interior of the cell culture unit 102. The cell culture unit 102 may be configured to contain at least one type of cell.

Suitable types of cells include but not are not limited to bone, cartilage, muscle, liver, skin, heart, lung and any combinations thereof. Other types of mammalian cells or fish cells may be used within the present invention. Cells from other plant and animal species can be used. Other starter cells may be stem cells of various origins such as mesenchymal stem cells, induced pluripotent stem cells and satellite cells. The starter cells may also be genetically modified cells or any cell lines. Bioengineered cells may be used as well.

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10 **[0047]** Different types of specialized cells to be expanded in the cell culture unit 102 may be obtained by biopsy from live animals.

[0048] The cell culture unit 102 may further include an inlet configured to receive the first fluid from the fresh medium unit 104 through pump 108b and an outlet configured to remove/release the first fluid to the fresh medium unit 104 through pump 108a. The cell culture unit 102 may further include a heating device configured to heat the interior of the cell culture unit 102 to a predetermined temperature and a temperature control unit to maintain the temperature within the cell culture unit 102 at such a predetermined temperature. The predetermined temperature can be approximately ranged from 25°C to 45°C.

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20 **[0049]** The cell culture may further include at least one stirrer configured to stir the first fluid within the cell culture unit 102 at a predetermined speed. The predetermined can be approximately from 10 rotations per minute (rpm) to 300 rpm.

[0050] The cell culture unit 102 may further include a gas outlet and a gas inlet connected to the gas source, which may be oxygen or carbon dioxide. Oxygen or carbon dioxide may be fed into the cell culture unit 102 through the gas inlet to optimize cell culture conditions. Wasted gas may be released through the gas outlet. The flow of the gas may be controlled by a valve.

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30 **[0051]** In some embodiments, the cell culture unit 102 could be the bioreactor 30 as shown in FIG. 5. Yet in some embodiments, the cell culture unit 102 could be the bioreactor 50 as shown in FIG. 6. In some embodiments, the cell culture unit 102 is a vessel. In some embodiments, the cell culture unit 102

may be any size. In some embodiments, the volume of the cell culture unit 102 may be ranged from 0.1L – 2000L..

5 **[0052]** Turning to FIG. 8, the fresh medium unit 104 may comprise a first fluid medium inlet 110 and a first fluid outlet 112 configured to connect to pump 108a and pump 108b respectively. In addition, the fresh medium unit 104 may further comprise a second fluid inlet 114 and a second fluid outlet 116 configured to connect to pump 108d and pump 108c respectively. In addition, the fresh medium unit 104 may comprise at least one first dialysis unit 118 having a first fluid compartment 120 and a second fluid compartment 122 separated by a first dialysis membrane 123. The first fluid inlet 110 and the first fluid outlet 112 are connected to the first fluid compartment 120. The second fluid inlet 114 and the second fluid outlet 116 are connected to the second fluid compartment 122. Different types of dialysis membrane 123 including Cellulose Ester (CE), Regenerated Cellulose (RC) or Polyvinylidene fluoride (PVDF) may be used. Dialysis membrane 123 of different molecular weight cut-off (MWCO) may be used to retain desirable macromolecules in the first fluid (e.g. growth factors secreted by cells in culture vessel) and allow waste to be removed from the first fluid in the first dialysis unit 118. For example, 100Da – 1,000,000Da MWCO membrane, preferably 500 Da MWCO membrane, can be used to retain insulin-like growth factor (IGF, 7.5kDa for a recombinant form) and transforming growth factor-beta (TGF beta, 44kDa for pro TGF-beta) in the first fluid and allow lactate (89 Da) and ammonia (17 Da) to be removed from the first fluid. Such membrane may also be used to allow nutrients of the second fluid such as glucose (180 Da) to get across the membrane into the first fluid for replenishment.

25 **[0053]** The fresh medium unit 104 may comprise at least one stirrer in either or both compartments configured to stir the fluid within the dialysis unit at a predetermined speed. The predetermined can be approximately from 10 rpm to 500 rpm.

30 **[0054]** The fresh medium unit 104 may further include a separated inlet and outlet connected to either or both compartments to add, replenish and remove desired fluid. The desired fluid may be cell culture medium, fresh basal medium and/or differentiation medium.

[0055] Turning to FIG. 9, the waste removal unit 106 may comprise a waste inlet 124 and a refresh outlet 126 configured to connect to pump 108c and pump 108d respectively. In addition, the waste removal unit 106 may further comprise a waste removal inlet 128 and a waste removal outlet 130. In addition, the waste removal unit 106 may comprise at least one second dialysis unit 132 having a waste compartment 134 and a waste removal compartment 136 separated by a second dialysis membrane 137. The waste inlet 124 and the fresh medium outlet 116 are connected to the waste compartment 134. The waste removal inlet 128 and the waste outlet 130 are connected to the waste removal compartment 136. The waste removal unit 106 may use other waste removal technique to remove wastes from the second fluid. For example, waste removal may be carried out by passing the second fluid through zeolite as adsorbents. Zeolites are microporous, aluminosilicate minerals. Examples are analcime, chabazite, clinoptilolite, heulandite, natrolite, phillipsite, and stilbite. The waste removal unit 106 may include a column of packed-bed zeolite. The second fluid flows into the waste removal unit 106 from one end, passing through the zeolite and exiting the waste removal unit 106 from another end. The zeolite absorbs toxic chemicals or chemicals inhibiting cell growth in the second fluid, e.g. ammonia and lactate.

[0056] The waste removal unit 106 is configured to remove metabolic wastes such as ammonia and lactate in second fluid and allow retention of maximum amount of nutrients such as glucose in the waste medium. Dialysis membrane of 100Da – 1,000,000Da MWCO, preferably 100Da MWCO, may be used to allow ammonia and lactate to get into the dialysate with glucose retained in the second fluid. Dialysate, which enters the waste removal compartment 136 can be phosphate-buffered saline (PBS) or any other possible buffers.

[0057] In some embodiments, each of the cell culture unit 102, fresh medium unit 104 and the waste removal unit 106 may be a removable plug-in module. Each of the inlets and outlets of the foregoing units may connect and/or disconnect with the inlets and outlets of the pumps of the cell culture system 100. For example, a fresh medium unit 104 or the waste removal unit 106 may be quickly replaced by unplugging it from the system and plugging a new

unit into the cell culture system 100. The present embodiment could reduce the downtime if one of the unit is malfunctioned. Furthermore, each of the units can be unplugged from the cell culture system 100 and operates on its own for independent use.

- 5 **[0058]** Now, turning to the method of utilizing the cell culture system 100 to grow cell culture. The method describes herein may be used to culture cells such as skin, muscle, adipose and bone cells to produce cultured meat. The method includes providing the cell culture system 100, transferring of cultured medium with metabolic wastes or growth inhibitors into the first dialysis unit
- 10 118 of the fresh medium unit 104, replenishing nutrient and removing growth inhibitors in the first dialysis unit 118 of the fresh medium unit 104, transferring of basal medium with metabolic waste or growth inhibitors from the first dialysis unit 118 of the fresh medium unit 104 to the waste removal unit 106, and removing growth inhibitors in the waste removal unit 106.
- 15 **[0059]** At least one type of cell is added to the cell culture unit 102. Suitable types of cells include but not are not limited to bone, cartilage, muscle, liver, skin, heart, lung and any combinations thereof. Other types of mammalian cells or fish cells may be used within the present invention. Cells from other plant and animal species can be used.
- 20 **[0060]** Different types of specialized cells to be expanded in the culture vessel may be obtained by biopsy from live animals. Other starter cells may be stem cells of various origins such as mesenchymal stem cells, induced pluripotent stem cells and satellite cells. The starter cells may also be genetically modified cells or any cell lines.
- 25 **[0061]** The cells are grown into a solid or semi-solid structure mimicking an animal organ, such as a fish organ, by attaching/adhering to a food-grade biocompatible scaffold in a sterile chamber or container, such as the cell culture unit 102. The temperature of the cell culture unit 102 is controlled and the culture medium is introduced into the cell culture unit 102 at its inlet and
- 30 released from the cell culture unit 102 outlet to remove substances such as chemicals, nutrients, and cells. The food-grade biocompatible scaffold becomes part of the final edible product.

[0062] Growth factors and cytokines from fetal bovine serum (FBS)

supplements or from recombinant sources are introduced into the culture medium in the bioreactor to support cell growth and proliferation. The growth factors and cytokines may include, but are not limited to, insulin growth factor 1 (IGF-1), insulin, interleukin 6 (IL-6), interleukin 6 receptor (IL-6R), interleukin 11 (IL-11), fibroblast growth factor (FGF), epidermal growth factor (EGF), and transferrin. The application may involve co-culturing bioengineered cells with the cultured cells in the cell culture unit 102 in the absence of FBS. The bioengineered cells are engineered to secrete the above growth factors and cytokines and supply these biomolecules to the cultured cells as needed for growth and proliferation. The co-culturing method of the present disclosure eliminates the need for animal-derived fetal bovine serum (FBS) in the culture medium. Furthermore, the co-culturing method provides a continuous supply of food-grade specific growth factors and cytokines to the growing isolated cells in situ and simplifies and reduces the cost of the production process. However, in other embodiments, FBS, other serum or proteins from recombinant sources may be used to supply growth factors, cytokines, and other nutrients to support cell growth.

[0063] In some embodiments, gases may be introduced to optimize cell

culture conditions. Oxygen and carbon dioxide may be used. The cell culture unit 102 may contain 0-10% of carbon dioxide. The cell culture unit 102 may contain 15-30% of oxygen.

[0064] In some embodiments, the temperature may be controlled to optimize

the cell culture conditions. Different types of cells may have a different optimal culture temperature. The temperature may be ranged from 25-45°C.

[0065] In some embodiments, the culture medium in the cell culture unit 102 is

stirred. The stirring speed in the culture vessel may be optimized to enhance the expansion of cells as it is known that cells react differently to shear stress. Stirring speed may also be optimized to enhance the mixing of inflow culture medium and culture medium inside the cell culture unit 102. Stirring speed may be ranged from 10 rpm – 300 rpm.

[0066] The pump 108a may transfer the culture medium from the cell culture unit 102 to the first dialysis unit 118 of the fresh medium unit 104.

[0067] In the first dialysis unit 118, different types of dialysis membrane including CE, RC or PVDF may be used. Dialysis membrane of different molecular weight cut-off (MWCO) may be used to retain desirable macromolecules in culture medium (e.g. growth factors secreted by cells in culture vessel) and allow waste to be removed from the culture medium in the first dialysis unit 118. For example, 100Da-1,000,000Da MWCO membrane, preferably 500 Da MWCO membrane, can be used to retain insulin-like growth factor (IGF, 7.5kDa for a recombinant form) and transforming growth factor-beta (TGF beta, 44kDa for pro TGF-beta) in the culture medium and allow lactate (89 Da) and ammonia (17 Da) to be removed from the culture medium. Such membrane may also be used to allow nutrients of fresh basal medium such as glucose (180 Da) to get across the membrane into the culture medium for replenishment.

[0068] The fresh basal medium fills the second fluid compartment 122 while the culture medium fills the first fluid compartment 120 to perform dialysis. For example, lactate, ammonia and other wastes are transferred from the culture medium to the fresh basal medium through the first dialysis membrane 123 and the glucose and other growth-enhancing compounds are transferred from the fresh basal medium to the culture medium through the first dialysis membrane 123. The rate of dialysis can be controlled by changing the volume of the fresh basal medium, the volume of the culture medium contained within the dialysis membrane, membrane surface area, temperature, and agitation by stirring in the dialysis unit. Rate of nutrient replenishment and waste removal of culture medium in the dialysis unit may also be controlled by changing pumping speed ranged from 1ml/min – 10L/min for fluid inflow from the cell culture unit 102 into the first fluid compartment 120 of the first dialysis unit 118.

[0069] Metabolic wastes or growth inhibitors move from the culture medium into the fresh basal medium inside the first dialysis unit 118. Growth inhibitors accumulate in the fresh basal medium over time. Basal medium with accumulated growth inhibitors (including but not limited to lactate, ammonia) is referred to as waste medium herein. The waste medium may be transferred to a waste removal unit 106 by the pump 108c. Metabolic wastes such as

ammonia and lactate in the waste medium may be removed by using the dialysis principle at the second dialysis unit 132 of the waste removal unit 106. Waste removal methods should allow the retention of the maximum amount of nutrients such as glucose in the waste medium. A dialysis membrane of 100 Da – 1,000,000 Da WMCO membrane, preferably 100 Da MWCO membrane, may be used to allow ammonia and lactate to get into the dialysate with glucose retained in the waste medium. The dialysate can be phosphate-buffered saline (PBS) or any other possible buffers. Rate of dialysis can be controlled by changing the volume of the dialysate, the volume of the waste medium contained within dialysis membrane 137, membrane surface area, temperature, and agitation by stirring in the dialysis unit 132. Stirring speed may be ranged from 10 rpm – 500 rpm. The rate of waste removal in the waste removal unit 106 may also be controlled by changing pumping speed for fluid inflow from the first dialysis unit 118 of the fresh medium unit 104 into the waste removal unit 106. Waste medium cleaned by dialysis can be transferred back to the first dialysis unit 118 of the fresh medium unit 104. The pumping speed may be ranged from 1ml/min – 10L/min.

[0070] The separation of the culture from the fresh basal medium unit 104 and waste medium unit 106 allows the cell culture unit 102 to be kept closed securely throughout production. Contaminations in any of the units will be confined to the affected unit and will not affect other units.

[0071] Furthermore, the waste removal unit 106 helps centralizing, extracting and/or collecting the metabolic wastes from the whole cell culture system 100 by extracting such wastes from the second fluid. The wastes of the whole cell culture system 100 can then be easily collected and discharged. It reduces the wastes discharged and the overall running cost of cell culturing compared to the conventional cell culturing technique. As discussed in the background section, the conventional cell culturing technique discards spent culture medium containing both nutrients and wastes and replaces it with a new culture medium. Therefore, the amount/volume of waste (i.e. the spent culture medium) created in the conventional method is more than the amount/volume of waste created in the present invention.

[0072] Yet, the fresh medium unit 104 and waste removal unit 106 help lower the running cost and optimize medium use for growing cell cultures. Cell culture medium is generally more expensive than basal medium as the cell culture medium contains expensive FBS, growth factors or cytokines. With the help of the fresh medium unit 104, the cell culture medium does not need to be prematurely discarded and can be refreshed (i.e. removing waste thereof and obtaining nutrients from the basal medium).

[0073] Different types of cells include but are not limited to skin, bone, cartilage, heart, and liver can be cultured in suitable scaffolds to form engineered tissues in the cell culture unit 102. Stem cells of various origins such as mesenchymal stem cells, induced pluripotent stem cells and satellite cells can also be cultured in suitable scaffolds for tissue engineering applications. Components of differentiation medium can be added into the basal medium in the first dialysis unit 118 or second dialysis unit 132 for subsequent differentiation after an expansion of stem cells in scaffolds to form functional engineered tissues. For example, osteoinduction medium components such as dexamethasone, ascorbic acid and β -Glycerophosphate can be added to the basal medium for osteogenic differentiation of stem cells in scaffolds. The first dialysis membrane 123 of may be ranged from 100Da – 1,000,000Da MWCO, preferably 500Da MWCO, should be selected to allow the components of osteoinduction medium to get into the culture medium.

[0074] Alternatively, stem cells can be expanded on a 2D culture surface in cell culture unit 102. Stem cells can be expanded and trypsinized to obtain high-density cell suspensions for regenerative medicine applications. For example, human mesenchymal stem cells can be isolated from patients and expanded on a culture dish in cell culture unit 102. Upon confluence, cells are trypsinized to form a high-density cell suspension. The cell suspension is then injected into the injury sites of patients for healing.

[0075] Example 1

[0076] A culture of HEK293 cells was washed in PBS and trypsinized to form a cell suspension with a cell density of 2.5×10^6 cell/ml. A 200 μ l cell suspension from 2.5×10^6 cell/ml suspension was loaded onto a pre-cut square collagen-based scaffold (1 cm x 1cm) to make a tissue construct with a total cell

number of $5e^5$ cells. Tissue constructs were incubated for 4 hours at 37°C and 5% CO₂. 800 µl medium was added along the side of each well gently. Tissue constructs were then transferred into the bioreactor of cell culture unit 102 and a 6-well culture plate for control. At day 0, 4 and 11, images of cells inside scaffolds were captured by a brightfield microscope. At day 0, 4 and 11, LIVE/DEAD staining was done according to the following protocol. At day 0, 4 and 11, media from the tissue culture bottle, control and the dialysis unit were collected for measurement of glucose.

[0077] LIVE/DEAD staining of cells in scaffolds

[0078] 1. Calcein AM and Ethidium homodimer-1 (LIVE/DEAD kit from thermofisher) were added to DPBS at 1:1000 to obtain the staining reagent.

[0079] 2. Samples were washed in DPBS once.

[0080] 3. Samples were stained in 200-250 µl staining reagent for 30 minutes.

[0081] 4. Samples were washed in DPBS once and viewed under a fluorescent microscope.

[0082] Results

[0083] Observable microtissues were formed in the bioreactor but not in control

[0084] FIGs. 10A-C shows the growth of tissue constructs and cell viability in the bioreactor prototype and control. Bioreactor rows show cells cultured in the working cell culture system prototype. The control row shows cells cultured in a 6-well plate. On day 0, cells attached to the scaffolds as cell aggregates as shown in FIG. 10A. On day 4, cell aggregates grew to larger spheroids in the bioreactor as shown in FIG. 10B. On day 11, spheroids clumped together to form microtissues in the bioreactor while spheroids in control seemed to have no obvious growth as shown in FIG. 10C. LIVE/DEAD staining showed that microtissues in the bioreactor are formed by connecting viable spheroids and they were much larger than those in control.

[0085] FIG. 11 shows the microtissues formed on day 11. In the bioreactor, clumps of observable microtissues were formed in the scaffolds and these microtissues were not observed in control. After trypsinization to digest the scaffolds, microtissues were released and it is remarkable that microtissues in the bioreactor had a much higher volume than those in control.

[0086] FIG. 12 shows the glucose level was maintained in the culture vessel of the cell culture unit 102 of the bioreactor.

[0087] At day 0, Glucose concentration in the culture vessel cell culture unit 102 of the present invention = 18.8 mmol/L. Glucose concentration in the control culture plate = 18.7 mmol/L. Glucose concentration in the first dialysis unit 118 = 20.9 mmol/L

[0088] At day 4, Glucose concentration in the culture vessel cell culture unit 102 of the present invention = 19.1 mmol/L. Glucose concentration in the control culture plate = 4.9 mmol/L. Glucose concentration in the first dialysis unit 118 = 20.0mmol/L.

[0089] At day 11, Glucose concentration in the culture vessel cell culture unit 102 of the present invention = 10.4 mmol/L. Glucose concentration in the control culture plate = not detectable. Glucose concentration in the first dialysis unit 118 = 12.5 mmol/L.

[0090] As shown above, the present invention also keeps nutrients such as glucose at an optimal level and growth inhibitors such as ammonia and lactate at a low level for optimal cell viability and cell growth.

[0091] In addition, the present invention enhances the cell expansion process and reduces the production costs of the cultured meat industry and tissue engineering by retaining growth factors secreted by cells and reducing the use of animal-derived serum in the culture medium. The present invention can be used for large-scale meat production.

[0092] The in vitro meat production method of the present disclosure provides meat products with a simple tissue organization of one cell type. The meat product with one cell type is easier to make, develop, and commercialize compared to other cultured meats having multiple cell types. Alternative embodiments of the present disclosure provide meat products with multiple cell types. Furthermore, Applicant has discovered a strategy to increase biomass/protein production by altering micro RNA levels or activity in the growing cells. In one example, two key micro RNAs (miR-21 and miR-29a) are targeted to increase the levels of the dominant protein (collagen I) found in fish swim bladder cells. As far as the Applicant is aware, alteration of micro RNA levels or activity to achieve an increased protein/biomass yield in

cultured meat products has not been used by others in the field of cultured meat development. Targeting micro RNAs for increased protein production may cause less stress to the cells than known knock-in or knock-out methods. Bio-engineered cells are co-cultured with the growing animal cells to supply the growing fish cells with food-grade growth factors and cytokines for cell growth and proliferation in situ, reducing or eliminating the need for animal-derived FBS in the culture medium. The co-culturing technique simplifies the production process and reduces production costs.

[0093] Furthermore, the nutrients of the cultivated meat product may be customized to generate a healthier food product. For example, the cultured meat product may be customized according to diet recommendations from a dietician to from a personal genomic test. Healthy nutrients such as high-density cholesterol, polyunsaturated fatty acids, and monounsaturated fatty acids in the meat product may be enriched by culturing the cells in specific conditions. Alternatively, or in combination with this, nutrients known to be damaging to health such as low-density cholesterol and saturated fatty acids may be reduced by culturing the cells in specific conditions. Micronutrients, such as vitamins and minerals, may also be enhanced. Nutrient customization of the cultivated meat products may be achieved in various ways such as, but not limited to, 1) tailoring the nutrients fed to the growing cells during cell culture, and/or 2) controlling the proportions of layering scaffolds with different cells.

[0094] The production of the cultivated food product is under a clean, sterile and highly controlled process. Thus, undesirable degradation by microorganisms such as bacterial or fungi of the nutrients in the food product is minimized. Undesirable taste and smell from the breakdown of nutrients by microorganisms are also minimized. This property of cultivated food enables new uses in cooking and helps creates novel recipes. One such application of cultivated food is cultivated fish maw derived from fish swim bladders. Traditional fish maw has an undesirable fishy taste and smell due to the degradation of amine by bacteria in the production process. This undesirable property limits the food ingredient to savory dishes served hot or warm. Cultivated fish maw produced from cell culture technology does not have an

undesirable fishy taste and smell. In addition to hot and savory dishes, cultivated fish maw can be used in sweet dishes, as a dessert or in a ready-to-eat format served at chilled or at ambient temperature.

Exemplary Protocols

5 **A. Development of a fish bladder cell line**

1. Obtain a healthy yellow crocker, sea bass or fish of a similar category from a local fish market.
2. Keep the fish on ice until cell isolation.
3. Immerse the fish in 10% bleach.
- 10 4. Remove swim bladder from the fish under aseptic condition.
5. Wash the organ one or more times in hypochlorous acid.
6. Wash the organ one or more times in antibiotic medium (Leibovitz's L-15 or DMEM or EMEM with 400 IU/ml, penicillin, 400 pg/ml streptomycin).
7. After washing, cut the organ into small pieces (2-3 mm³).
- 15 8. Transfer the cut organ to a centrifuge tube containing 0.25% trypsin-EDTA in PBS.
9. Incubate at room temperature with continuous shaking for 1 hour.
10. Filter the supernatant with a 100 pm mesh to remove undigested tissue.
11. Centrifuge the filtrate at 200g for 5 minutes.
- 20 12. Resuspend the cell pellet with complete medium (Leibovitz's L-15 or DMEM or EMEM with 200 IU/ml, penicillin, 200 pg/ml streptomycin, 10% fetal bovine serum).
13. Seed the cell into a T25 flask.
14. Incubate at 24-28°C.
15. Remove cells that are not attached to the tissue culture flask the next day.
- 25 16. Replace half of the medium with fresh medium every 2-3 days.
17. The cells are considered established when a complete monolayer is formed and the established cells are ready for subculture.

B. Development of a fish bladder cell line by tissue explant

- 30 1. Obtain a healthy yellow crocker, sea bass, or fish of a similar category from a local fish market.
2. Keep the fish on ice until cell isolation.
3. Immerse the fish in 10% bleach.
4. Remove swim bladder from the fish under aseptic condition.
- 35 5. Wash the organ one or more times in hypochlorous acid.
6. Wash the organ one or more times in antibiotic medium (Leibovitz's L-15 or DMEM or EMEM with 400 IU/ml, penicillin, 400 pg/ml streptomycin).
7. After washing, cut the organ into small pieces (1-2 mm³).
8. Place organ pieces into a 24 well plate individually containing complete medium
- 40 (Leibovitz's L-15 or DMEM or EMEM with 200 IU/ml, penicillin, 200 pg/ml streptomycin, 10% fetal bovine serum).
9. Incubate at 24-28°C.
10. Replace half of the medium with fresh medium every 2-3 days without disturbing the tissue explant.
- 45 11. Incubate the tissue explant until adherent cells are observed.

12. Remove tissue explant.
13. The cells are considered established when a complete monolayer is formed and the established cells are ready for subculture.

C. Development of a fish muscle cell line

- 5 1. Obtain a healthy grouper, cod, sole, halibut, flounder, or fish of a similar category from a local fish market.
2. Keep the fish on ice until cell isolation.
3. Immerse the fish in 10% bleach.
4. Remove muscle from the fish under aseptic condition.
- 10 5. Wash the tissue one or more times in hypochlorous acid.
6. Wash the tissue one or more times in antibiotic medium (Leibovitz's L-15 or DMEM or EMEM with 400 IU/ml, penicillin, 400 pg/ml streptomycin).
7. After washing, cut the tissue into small pieces (2-3 mm³).
8. Transfer the cut tissue to a centrifuge tube containing collagenase and dispase in
15 PBS.
9. Incubate at room temperature with continuous shaking for 1 hour.
10. Filter the supernatant with a 100 pm mesh to remove undigested tissue.
11. Centrifuge the filtrate at 200g for 5 minutes.
12. Resuspend the cell pellet with complete medium (Leibovitz's L-15 or DMEM or
20 EMEM with 200 IU/ml, penicillin, 200 pg/ml streptomycin, 10% fetal bovine serum).
13. Seed the cell into a T25 flask.
14. Incubate at 24-28°C.
15. Remove cells that are not attached to the tissue culture flask the next day.
16. Replace half of the medium with fresh medium every 2-3 days.
- 25 17. The cells are considered established when a complete monolayer is formed and the established cells are ready for subculture.

D. Development of a fish muscle cell line from tissue explant

1. Obtain a healthy grouper, cod, sole, halibut, flounder, or fish of a similar category from a local fish market.
- 30 2. Keep the fish on ice until cell isolation.
3. Immerse the fish in 10% bleach.
4. Remove muscle from the fish under aseptic condition.
5. Wash the tissue one or more times in hypochlorous acid.
6. Wash the tissue one or more times in antibiotic medium (Leibovitz's L-15 or
35 DMEM or EMEM with 400 IU/ml, penicillin, 400 pg/ml streptomycin).
7. After washing, cut the muscle into small pieces (1-2 mm³).
8. Place muscle pieces into a 24 well plate individually containing complete medium (Leibovitz's L-15 or DMEM or EMEM with 200 IU/ml, penicillin, 200 pg/ml streptomycin, 10% fetal bovine serum).
- 40 9. Incubate at 24-28°C.
10. Replace half of the medium with fresh medium every 2-3 days without disturbing the tissue explant.
11. Incubate the tissue explant until adherent cells are observed.

12. Remove tissue explant.
13. The cells are considered established when a complete monolayer is formed and the established cells are ready for subculture.

E. Adult Stem cell isolation and culture

- 5 1. Obtain a healthy grouper, cod, sole, halibut, flounder or fish 6 months or younger of similar category from a local fish market.
2. Keep the fish on ice until cell isolation.
3. Immerse the fish in 10% bleach.
4. Remove muscle from the fish under aseptic conditions.
- 10 5. Wash the tissue one or more times in hypochlorous acid.
6. Wash the tissue one or more times in antibiotic medium (Leibovitz's L-15 or DMEM or EMEM with 400 IU/ml, penicillin, 400 pg/ml streptomycin).
7. After washing, cut the tissue into small pieces (2-3 mm³).
8. Transfer the cut tissue to a centrifuge tube containing collagenase and dispase in
15 PBS.
9. Incubate at room temperature with continuous shaking for 1 hour.
10. Filter the supernatant with a 100 pm mesh to remove undigested tissue.
11. Centrifuge the filtrate at 200g for 5 minutes.
12. Resuspend the cell pellet with complete medium (Leibovitz's L-15 or DMEM or
20 EMEM with 200 IU/ml, penicillin, 200 pg/ml streptomycin, 10% fetal bovine serum, 100ng/ml basic fibroblast growth factor).
13. Plate the cells on an uncoated plate for 1 hour at 24-28°C.
14. Harvest the supernatant and place it on a plate coated with laminin, gelatin, Matrigel or a similar matrix.
- 25 15. Incubate at 24-28°C.
16. After 24 hours, wash away any loosely attached and non-adherent cells.
17. Replace medium every day with complete medium (Leibovitz's L-15 or DMEM or EMEM with 200 IU/ml, penicillin, 200 pg/ml streptomycin, 10% fetal bovine serum, 100ng/ml basic fibroblast growth factor).

30

F. Generating and culturing iPSC

1. 2-4 days before transfection, plate cells in complete medium (L15 with 10% FBS) in a tissue culture flask. Cells should be approximately 75-90% confluent on the day of transfection (Day 0).
- 35 2. Aspirate the medium from gelatin-coated 6-well plates and replace them with 2 mL of fresh complete medium per well. Place the coated plates at 37°C until ready for use.
3. Thaw the Epi5™ vectors at 37°C and place them on wet ice until ready for use. Before use, briefly centrifuge the thawed vectors to collect them at the bottom of
40 the tube.
4. Wash the cells in PBS.
5. Add 3 mL of 0.05% Trypsin/EDTA to the culture flask containing the cells.
6. Incubate the flask at room temperature for 3 minutes.
7. Add 5-8 mL of complete medium to each flask. Carefully transfer cells into an
45 empty, sterile 15mL conical tube.

8. Check the viability by trypan blue dye exclusion cell viability assay
9. Centrifuge the cells at 200g for 2 min.
10. Carefully aspirate most of the supernatant and resuspend with complete medium.
- 5 11. Seed cells on gelatin-coated dishes plate 50,000 to 100,000 cells per well into a 6-well plate at 30-60% confluence in 2 mL complete medium and Incubate overnight at 24-28°C.
12. Prewarm Opti-MEM/Reduced-Serum Medium to room temperature and prepare Tube A and Tube B as described below.
- 10 13. Add 1.2 µL each of the two Epi5™ Reprogramming Vector mixes (2.4 µL total) to 118 µL Opti- MEM medium in a 1.5 mL microcentrifuge tube labeled Tube A. Add 4.8 µL of P3000™ Reagent and mix well.
14. Dilute 3.6 µL Lipofectamine 3000 reagent in 121 µL prewarmed Opti-MEM medium in a 1.5 mL microcentrifuge tube labeled Tube B.
- 15 15. To prepare a transfection master mix, add the contents of Tube A to Tube B and mix well.
16. Incubate the transfection master mix for 5 minutes at room temperature.
17. Mix one more time and add the entire 250 µL of transfection master mix to each well.
- 20 18. Incubate overnight at 24-28°C.
19. 24 hours post-transfection, aspirate the medium from the plates. Add 2 mL N2B27 Medium (L15 with IX N-2 supplement, IX B27 supplement, 100 ng/mL bFGF to each well.
20. Change the N2B27 Medium every day for a total of 14 days by replacing the spent medium with 2 mL N2B27 Medium.
- 25 21. Aspirate the spent N2B27 Medium on Day 14 and replace it with a complete medium. Resume medium changes every day at 2 mL per well.
22. Observe the plates every other day under a microscope for the emergence of cell clumps, indicative of transformed cells. Within 15 to 21 days post-transfection,
- 30 the iPSC colonies will grow to an appropriate size for transfer.
23. Colonies are distinct by Day 21 and can be picked for further culture and expansion.

G. Method for subculturing cells

1. Remove and discard the culture medium.
- 35 2. Briefly rinse the cell PBS to remove all traces of serum which contains trypsin inhibitor.
3. Add 2-3 mL of 0.25% Trypsin-EDTA solution to the flask.
4. Incubate at room temperature for 1 min.
5. Add 5-8 mL of complete growth medium.
- 40 6. Aspirate cells by gently pipetting.
7. Add appropriate aliquots of the cell suspension to new culture flasks at a subcultivation ratio of 1:2 to 1:3.
8. Incubate at 24-28°C.

H. Adaption to suspension culture

1. Passage monolayer culture at a frequency appropriate for the cell in question by trypsinization.
2. At each passage, wash cell monolayer with PBS and overlay with 0.25% trypsin.
- 5 3. Incubate at room temperature for 5 min.
4. Inactivate the enzyme with a complete medium.
5. Harvest the cell suspension and check the viability by trypan blue dye exclusion cell viability assay.
6. Seed the cell suspension into another culture flask.
- 10 7. Repeat passaging until the viability of the suspended cells is equal or more than 90%.
8. Establish a suspension culture with 50 ml complete medium in a spinner or shaker flask at a cell density of 0.1-0.5 million/ml.
9. Incubate the spinner or shaker flask suspension cultures in a CO₂ incubator
- 15 under the same conditions of temperature, humidity, and atmosphere optimal for monolayer cultures.
10. Adjust the cell density to 0.1-0.5 million/ml with fresh medium every 2-3 days.
11. Check the viability by trypan blue dye exclusion cell viability assay.
12. Establish multiple parallel cultures at cell density that promote healthy cell
- 20 growth.
13. Increase cell density gradually to 1 million/ml using part of the culture.
14. If increasing cell density leads to cell death, discard the high-density culture.
15. Restart high-density adaption using cell from step 12.
16. Scale up to a 3L bioreactor when cells are adapted to grow in suspension.

25 **I. Adaption to serum-free medium (plant hydrolysate)**

1. Culture cells in DMEM/F12 complete medium (1:1 mixture of DMEM medium and Ham's F12 medium, 2-4mM glutamine, 10% FBS).
2. Prepare serum-free medium (1:1 mixture of DMEM medium and Ham's F12 medium, 2-4mM glutamine, 20% plant hydrolysate e.g. soy, cottonseed, rapeseed,
- 30 wheat, yeast or equivalent).
3. When cells reach confluence, replace medium with adaption medium I (40% fresh complete medium, 40% conditioned media from the passage before, 20% serum-free medium).
4. Check the viability by trypan blue dye exclusion cell viability assay every 2-3
- 35 days.
5. If adaption leads to cell death, discard the culture and repeat step 3.
6. When cells reach confluence, replace medium with adaption medium II (30% fresh complete medium, 30% conditioned media from the cells in step 1, 40% serum-free medium).
- 40 7. Check the viability by trypan blue dye exclusion cell viability assay every 2-3 days.
8. If adaption leads to cell death, discard the culture and repeat step 6.
9. When cells reach confluence, replace medium with adaption medium III (20% fresh complete medium, 20% conditioned media from the cells in step 1, 60% serum-free medium).
- 45 10. Check the viability by trypan blue dye exclusion cell viability assay every 2-3

days

11. If adaption leads to cell death, discard the culture and repeat step 9.
12. When cells reach confluence, replace medium with adaption medium IV (10% fresh complete medium, 10% conditioned media from the cells in step 1, 80% serum-free medium).
13. Check the viability by trypan blue dye exclusion cell viability assay every 2-3 days
14. If adaption leads to cell death, discard the culture and repeat step 12.
15. When cells reach confluence, replace medium with serum-free medium.
16. Check the viability by trypan blue dye exclusion cell viability assay every 2-3 days.
17. If adaption leads to cell death, discard the culture and repeat step 15.
18. The serum-free medium usage can be increased more gradually in each step, i.e. an increase of 20% or less in each step.

15 J. Adaption to serum-free medium (chemically defined)

1. Culture cells in DMEM/F12 complete medium (1:1 mixture of DMEM medium and Ham's F12 medium, 2-4mM glutamine, 10% FBS).
2. Prepare serum free medium (1:1 mixture of DMEM medium and Ham's F12 medium, 2-4 mM glutamine, ascorbic acid 2-phosphate 65-130 ug/ml, NaHCO₃ 550-1100 ug/ml, sodium selenite 14-28 ng/ml, insulin 19-38 ug/ml, transferrin 11-22 ug/ml, FGF-2 100-200 ng/ml, TGF-beta 2-4 ng/ml).
3. When cells reach confluence, replace medium with adaption medium I (40% fresh complete medium, 40% conditioned media from the passage before, 20% serum-free medium).
4. Check the viability by trypan blue dye exclusion cell viability assay every 2-3 days
5. If adaption leads to cell death, discard the culture and repeat step 3
6. When cells reach confluence, replace medium with adaption medium II (30% fresh complete medium, 30% conditioned media from the cells in step 1, 40% serum-free medium).
7. Check the viability by trypan blue dye exclusion cell viability assay every 2-3 days..
8. If adaption leads to cell death, discard the culture and repeat step 6
9. When cells reach confluence, replace medium with adaption medium III (20% fresh complete medium, 20% conditioned media from the cells in step 1, 60% serum-free medium).
10. Check the viability by trypan blue dye exclusion cell viability assay every 2-3 days.
11. If adaption leads to cell death, discard the culture and repeat step 9.
12. When cells reach confluence, replace medium with adaption medium IV (10% fresh complete medium, 10% conditioned media from the cells in step 1, 80% serum-free medium).
13. Check the viability by trypan blue dye exclusion cell viability assay every 2-3 days.
14. If adaption leads to cell death, discard the culture and repeat step 12.

15. When cells reach confluence, replace medium with serum-free medium.
16. Check the viability by trypan blue dye exclusion cell viability assay every 2-3 days.
17. If adaption leads to cell death, discard the culture and repeat step 15.
- 5 18. The serum-free medium usage can be increased more gradually in each step. For example, an increase of 20% or less in each step.

K. Post-transcriptional enhancement of protein expression

1. Culture cells in complete medium (Leibovitz's L-15 or DMEM or EMEM with 200 IU/ml, penicillin, 200 pg/ml streptomycin, 10% fetal bovine serum), or serum-free medium (DMEM/F12 with plant hydrolysate or chemically defined compounds).
2. Remove and discard the culture medium.
3. Briefly rinse the cell PBS to remove all traces of serum which contains trypsin inhibitor.
- 15 4. Add 2-3 mL of 0.25% Trypsin-EDTA solution to the flask.
5. Incubate at room temperature for 1 min.
6. Aspirate cells by gently pipetting.
7. Centrifuge cell at 200g for 2 min.
8. Resuspend cells in complete medium or serum-free medium.
- 20 9. Add 0.5 million cells to each well of a 6-well plate.
10. Incubate at 24-28°C overnight.
11. Transfect micro RNA oligonucleotides (miR-21, miR-29a, miR-21 mimic, miR-29a mimic, anti-miR-21, anti-miR-29a, or equivalent) into the cell using polyethylenimine, liposome, electroporation, or other methods.
- 25 12. Incubate at 24-28°C overnight.
13. Transfer the cells to a multi-layer flask, spinner flask or shaker flask in a CO₂ incubator under the same conditions of temperature, humidity, and atmosphere optimal culture

L. Scaffolding for cell culture (Konjac + gum)

- 30 1. Boil water with a few pieces of saffron until the color becomes pale yellow.
2. Remove the saffron and rest the solution until warm.
3. Prepare all dry ingredient
 - a. Konjac-0.5-5%, preferably 3 %
 - b. Baking soda - 0.3-3%, preferably 2 %
 - 35 c. Perfected Xanthan Gum - 0.2-2%, preferably 1.5%
4. Measure 100ml of saffron solution.
5. Add Baking soda, Locust Bean Gum, Xanthan Gum sequentially. Stir the mixture well after adding each ingredient.
6. Add Konjac by sprinkling little by little on top of the solution. Keep stirring. The solution should become mushy.
- 40 7. Spread the konjac mixture into mold with approximately 1-15mm thickness.
8. Cover the mold with the lid and rest under room temperature for more than 30 min.
9. Put the mold in 4°C fridge for 4 hours.

10. Steam the mold under low heat for 40 minutes.
11. Rest the mold under room temperature for 2 hours.
12. Dehydrate the scaffold at 45-55 °C for 15 minutes.

M. Scaffolding for cell culture (Alginate + Glutinous Rice Flour)

- 5 1. Weigh 0.1-2 g (0.1-2%), preferably about 1g (1%) Sodium Alginate.
2. Add 100ml water into the blender.
3. Add the Alginate powder into the blender and blend the mixture until dissolved.
4. Cover the container with plastic film and put the Alginate solution into the refrigerator overnight to eliminate the gas bubbles.
- 10 5. Weigh 1-10 g (1-10%), preferably about 5g (5%) Glutinous Rice Flour and put in a mold.
6. Add Alginate solution into the mold with approximately 1-15mm thickness.
7. Stir the mixture until all flour dissolves.
8. Steam the mixture under low heat for 30 min until the shape is set.
- 15 9. Cover the mold with the lid and rest under room temperature for 30 min.
10. Weigh 1% Calcium Lactate and stir to dissolve in water.
11. Immerse the scaffold with 1% Calcium Lactate solution for at least 2.5 hours to allow the formation of the membrane around the scaffold.

CLAIMS

What is claimed is:

1. A cell culture system comprising:
 - a cell culture unit configured to hold at least one type of cell;
 - 5 a fresh medium unit configured to supply and receive the first fluid to the cell culture unit;
 - a waste removal unit configured to supply and receive a second fluid from the fresh medium unit;
 - a first pump connected between the cell culture unit and the fresh medium
 - 10 unit configured to circulate the first fluid;
 - a second pump connected between the fresh medium unit and the waste removal unit configured to circulate the second fluid,
 - wherein the cell culture unit, the fresh medium unit and the waste removal unit are separated from each other to avoid cross contaminations.
- 15 2. The system of claim 1, wherein the cells are obtained from skin, muscle, adipose, bone or any other tissues from mammals, fish, animals or plants.
3. The system of claim 1, wherein the fresh medium unit further comprises a first dialysis unit configured to extract the waste from the first fluid to the second fluid and to supply nutrients from the second fluid to the first fluid.
- 20 4. The system of claim 3, wherein the first dialysis unit further comprises at least one 500Da molecular weight cut-off (MWCO) membrane, wherein the membrane is selected from Cellulose Ester (CE), Regenerated Cellulose (RC), and Polyvinylidene fluoride (PVDF).
5. The system of claim 1, wherein the waste removal unit further comprises a second
- 25 dialysis unit configured to extract the waste from the second fluid.
6. The system of claim 5, wherein the second dialysis unit further comprises at least one 100 Da molecular weight cut-off (MWCO) membrane, wherein the membrane is selected from Cellulose Ester (CE), Regenerated Cellulose (RC), and Polyvinylidene fluoride (PVDF).
- 30 7. A method of growing cell culture comprising the steps of
 - providing a cell culture system of claim 1;
 - placing at least one type of cell into the cell culture unit;
 - circulating the first fluid between the cell culture unit and the fresh medium unit;

circulating the second fluid between the fresh medium unit and the waste removal unit;

extracting waste from the first fluid to the second fluid at the fresh medium unit; and

5 replenishing nutrients from the second fluid to the first fluid at the fresh medium unit.

8. The method of claim 7, wherein the cells are obtained from skin, muscle, adipose, bone or any other tissues from mammals, fish, animals or plants.

9. The method of claim 7 further comprises the step of extracting waste from the
10 second fluid at the waste removal unit.

10. The method of claim 7 further comprising the steps of:

heating the culture vessel to a predetermined temperature for culturing;

agitating the first fluid in the cell culture unit;

growing the cell culture to a predetermined density; and

15 isolating cell culture from the first fluid.

11. The method of claim 10, wherein the first fluid is a culture medium and the second fluid is a fresh basal medium.

12. The method of claim 10 further comprising the step of forming the cells into the meat.

20 13. The method of claim 10 further comprising the step of forming the cells into tissue constructs.

14. The method of claim 7, wherein the cells are grown with or without microcarriers.

15. The method of claim 7, wherein the cells are grown with or without scaffolds.

16. The method of claim 7, wherein the cells can be stem cells of various origins
25 such as mesenchymal stem cells, induced pluripotent stem cells and satellite cells or genetically modified cells.

17. The method of claim 16 further comprising the step of supplying at least one component of differentiation medium to the second fluid at the first dialysis unit for
30 subsequent differentiation after the expansion of stem cells in order to form functional engineered tissues.