

METHODS FOR IMPROVING CELL GROWTH WITH SPECIES-SPECIFIC OR GENUS-SPECIFIC PROTEINS AND THE APPLICATIONS THEREOF

Technical Field

5 [0001] Embodiments discussed herein generally relate to improved methods for meat production using in vitro cell culture. Embodiments discussed herein also generally relate to the improved methods for cell growth using growth factors.

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 agriculture or aquaculture may pose a public health risk as the production
15 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 scientists to develop techniques to produce meat by alternative processes.

20 [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 including soluble forms and solid forms. While still in an early stage of
25 development, in vitro meat and meat products may offer a number of advantages over traditional meat product 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 agriculture, or the production of agricultural products from cell cultures.

30 [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
35 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.

5 [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.

15 [0006] Generally, over 95% of the culture-medium cost is attributed to the protein components. Recombinant human growth factors (e.g. insulin, IGF-1), human serum albumin (HSA), or fetal bovine serum (FBS) are often supplemented in excess amounts to basal media. While human protein factors and FBS effectively promote growth and differentiation of human cells, they are less bioactive on cells from distant species (e.g. fish, bird). This causes a long culturing period and low cell quality. To compensate for the low bioactivity on non-human cells, excessively high levels of human protein factors or FBS are added to the growth medium, which leads to high costs.

25 [0007] 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.

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Summary

- [0008] The embodiments of the present disclosure apply methods for in vitro meat production for human consumption that provides a solution to the above challenges.
- 5 [0009] It is an objective of the present invention to provide an alternative method to cultivate cells using species-specific or genus-specific growth factors. This approach not only decreases medium-cost by lowering growth factor usage, but it also shortens the culturing time and improves cell quality by enhancing cellular responses. Using species-specific or genus-specific growth factors may help to
- 10 enhance cellular response (for example, growth, differentiation) and break the maximum cellular response encountered when using non-species-specific or non-genus-specific growth factors.
- [0010] It is also an objective of the present invention to provide a method to evaluate the efficacies of growth factors of different species origins on
- 15 stimulating cell growth.
- [0011] According to one embodiment of the present disclosure, a method for meat production by in vitro cell culture includes isolating tissue from an animal or plant source and making a cell suspension of cells. The method further includes introducing culture medium comprising growth factor of (i) genetically same or
- 20 similar species to the cells and/or (ii) genetically same genus to the cells. Additionally, the method further includes growing the cells on a food-grade scaffold in a culture medium, the cells growing into a solid or semi-solid structure that mimics an animal organ.
- [0012] According to another embodiment of the present disclosure, a method for meat production by in vitro cell culture includes isolating tissue from a plant or
- 25 animal source and making a cell suspension of cells, and growing the cells on a food-grade scaffold in a culture medium such that the cells grow into a solid or semi-solid structure that mimics an animal organ. The method further includes co-culturing the cells with bioengineered cells that secrete nutrients, growth
- 30 factors, and cytokines that support the growth of the cells, wherein the bioengineered cells are (i) genetically same or similar species to the cells and/or (ii) genetically same genus to the cells.
- [0013] In some embodiments, the species is genetically similar to the cells when they are more than 90% match in DNA sequence.

[0014] Embodiments disclosed herein apply methods for in vitro meat production for human consumption that provides a solution to the above challenges.

[0015] **Brief Description of the Drawings**

5 [0016] 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.

[0017] FIG. 1 is a flowchart of a method for meat production by in vitro cell culture, according to one embodiment of the present disclosure.

10 [0018] FIG. 2 is a schematic representation of a method for post-transcriptional enhancement of protein expression, according to one embodiment of the present disclosure.

[0019] 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.

15 [0020] 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.

[0021] 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.

[0022] 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.

25 [0023] FIG. 7 is a chart illustrating the respective cell numbers after treating MCF-7 cells by different concentrations (1 pg/ml to 100 ng/ml) of recombinant human IGF-1 (Oryzogen). Cells were harvested on day 10 for direct cell counting.

[0024] FIG. 8 is a chart illustrating the respective relative fluorescence after the treatment of MCF-7 cells by 1.5 nM of recombinant human IGF-1 (from 3 different suppliers), recombinant mouse IGF-1, and recombinant fish (tuna, bream) IGF-1. Cells were harvested on day 7 and subjected to CyQUANT Cell Proliferation Assay.

Detailed Description

[0025] 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 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. 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.

[0026] 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 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.

[0027] 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 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 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), 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), 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.

[0028] According to a block 20, food-grade growth factors and cytokines 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 growth factors of (i) genetically same or similar species to the isolated cells and/or (ii) genetically same genus to the isolated cells (i.e. cells growing at block 16) are used in the present invention. It is found that the use of growth factors of (i) genetically same or similar species to the isolated cells and/or (ii) genetically same genus to the isolated cells exert higher bioactivities to the isolated cells compared to the use of growth factors and serum of genetically distant species to the isolated cells. Due to the enhanced comparability, there is no need to supply a megadose of "suboptimal" growth factors when culturing isolated cells using growth factors of (i) genetically same or similar species and/or (ii) genetically same genus. Higher bioactivity could also help reducing the amount of growth factors needed in the culture medium, shortening the culture period and improving cell quality. The cost of the culture medium could be reduced due to the decrease in the levels of growth factors required in the culture medium for the stimulations of cell growth and differentiation. Furthermore, the use of suboptimal growth factors limits the

magnitude of the maximum cellular response (growth, differentiation). In some instances, certain responses can never be reached no matter how much of the suboptimal growth factor is supplied. Species-specific and/or genus-specific growth factors could help overcoming these limits.

5 **[0029]** In some embodiments, the species is genetically similar to the isolated cells when they are more than 90% match in DNA sequence.

10 **[0030]** Species-specific or genus-specific growth factors can effectively act on receptors of the isolated cells. Compared to the conventional growth medium, which is often supplemented by high levels of human protein growth factors and/or FBS irrespective of the species origin of the isolated cell, the use of species-specific or genus-specific growth factors is better optimized. Species-specific or genus-specific variations in amino acid sequence and post-translational modifications of the growth factor(s) and cell receptor(s) may account for this phenomenon.

15 **[0031]** As will be discussed in much greater detail below, to identify which species of a certain growth factor exerts the highest bioactivity on the target isolated cells, target cells are first seeded in complete medium (i.e. basal medium + FBS). Upon reaching the target confluence (around 20%-70%), target cells are treated by the growth factor of different species at a range of concentrations (e.g. 1 pM–1 μ M). Target cells are kept in the incubator until reaching the desired time point(s) for the studied parameter (e.g. cell growth, differentiation markers, cellular products). For example, when there are differences in cell confluence between the treatment groups (around 2-10 days), cell growth can be measured by trypan blue exclusion, the CyQUANT assay, or any other appropriate cell proliferation/death assays. The bioactivities of the growth factors of various species are compared based on their EC₅₀ values (half-maximal effective concentration). For cost-effectiveness, target cells should be cultured using the growth factor with the lowest EC₅₀ value. However, if the aim is to attain the shortest culturing time or the highest cell quality, select the growth factor which triggers the highest maximum cellular response. The optimal dose of the growth factor is defined as the lowest concentration required to elicit the maximum cellular response.

30 **[0032]** In some embodiments, the block 20 may 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

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factors and cytokines, and supply these biomolecules to the isolated cells as needed for growth and proliferation. As used herein, "bioengineered" cells are not equivalent to genetically-modified cells. The bioengineered cells have a specific gene that overexpresses one or more specific proteins. The

5 bioengineered cells may be fish cells, or other types of animal cells, such as cow cells. The bioengineering cells and the isolated cells may be genetically similar or identical species. Also the bioengineering cells and the isolated cells may belong in the same genus. As non-limiting examples, bioengineered fish cells may be co-cultured with isolated fish cells, or bioengineered cow cells may be

10 co-cultured with isolated cow cells. In some particular examples, the bioengineering cells may be chicken cells or bird cells if chicken cells are used as the isolated cells. In yet another particular example, the bioengineering cells may be yellow crocker cells or other fish cells if yellow crocker cells are used as the isolated cells. The bioengineered cells are not present in the final meat

15 product. 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, wherein the growth factors are

20 (i) of genetically same or similar species to the isolated cells and/or (ii) of the same genus to the isolated cells. 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.

[0033] In some embodiments, the block 20 contains recombinant growth factors

25 of genetically same or similar species to the isolated cells. In yet some embodiments, the recombinant growth factors of the same genus to the isolated cells are used. The recombinant growth factors are introduced into the growth medium. The use of such recombinant growth factors exerts higher bioactivities to the isolated cells than growth factors and serum of distant species to the

30 isolated cells. Bacterial, yeast, insect, mammalian, or any other appropriate protein expression systems may be used to produce such recombinant growth factors. Protein purification is performed by (but not limited to) affinity chromatography, ion-exchange chromatography, size exclusion chromatography, or a combination of these strategies.

[0034] In some embodiments, recombinant growth factors of *Epinephelus akaara* (fish), which is genetically similar species to the fish muscle cells or swim bladder cells of *Epinephelus awoara* (fish), are used. The recombinant growth factors for culturing *Epinephelus awoara* used are *Epinephelus akaara*'s IGF-1, insulin and/or transferrin. The concentration of such IGF-1 is ranged from 10ng/ml to 100ng/ml. The concentration of such insulin is ranged from 1µg/ml – 10µg/ml. The concentration of such transferrin is ranged from 0.5µg/ml – 5µg/ml.

[0035] 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 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 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 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 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.

[0036] 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.

5 **[0037]** Fish swim bladder primarily includes fibroblasts and collagen protein. Collagen type 1 (collagen I) is a dominant protein in 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).
10 COL1A1 and COL1A2 expression is increased by up-regulating microRNA 21 (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
15 COL1A1 and COL1A2 production in fish swim bladder cells. 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
20 increase relevant protein levels in other types of animal cells.

[0038] 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 product. The
25 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 inner surfaces of the support 32. The culture medium supplying nutrients to the cells is introduced into the bioreactor 30 through an
30 inlet 38, and is emptied from the bioreactor 30 through an outlet 40.

[0039] 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
35 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.

[0040] 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.

[0041] 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.

[0042] 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 create 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.

[0043] Identification Of Species-Specific Or Genus-Specific Growth Factor

[0044] The method of identifying species-specific or genus-specific growth factors will be discussed in detail here. It includes two major steps, which are the cell growth stimulation step and measuring cell growth step.

[0045] Cell growth stimulation step

[0046] The MCF-7 human epithelial cell line is cultured in DMEM/F12 complete medium DMEM/F12 (ThermoFisher Scientific), 10% FBS (ThermoFisher Scientific), 1% Glutamax (ThermoFisher Scientific), 0.2% Primocin (Invivogen) inside a humidified incubator (34°C; 5% CO₂; 95% air). Split cells at a ratio of 1:4 to 1:8 for routine maintenance.

[0047] Upon reaching about 80% confluence, detach cells by trypsin/EDTA (ThermoFisher Scientific). To study the effect of growth factors on cell growth,

cells are seeded at a density of 3×10^4 cells/cm² in complete medium onto 24-well (if cell growth is measured by cell counting) or 96-well plates (if cell growth is measured by the CyQUANT Cell Proliferation Assay Kit). Return the cells to the incubator.

5 **[0048]** After 24 hours, remove the medium. Pre-adapt cells to serum-free conditions by adding serum-free medium DMEM/F12, 0.1% human serum albumin (Sigma Aldrich), 0.2% Primocin. Keep the cells in this medium for at least 16 hours inside the incubator.

10 **[0049]** Prepare growth factors (e.g. IGF-1) at 10x working concentrations in serum-free medium for each species/genus, which growth-stimulating effect to be examined (e.g. recombinant human IGF-1, human IGF-1-LR3, mouse IGF-1, bream IGF-1 and tuna IGF-1). Add the 10x growth factors into the wells such that cells will be treated by 1x growth factors (e.g. add 50 µl 10x growth factor to well containing 450 µl serum-free medium) (e.g. 1 pM–1 µM). Return the cells to
15 the incubator.

20 **[0050]** Observe the cells daily under a microscope for signs of cell growth. When there are obvious differences in terms of cell confluence between the treatment groups (usually detected between day 2 – day 10), quantify cell growth either by cell counting, the CyQUANT Cell Proliferation Assay, or any other cell proliferation/death assays.

25 **[0051]** Measuring cell growth step

30 **[0052]** There are two ways to measure cell growth, namely, trypan blue exclusion and CyQUANT Cell Proliferation Assay Kit.

35 **[0053]** Trypan blue exclusion

40 **[0054]** Cells should have been treated in 24-well plates. Aspirate the culture medium and detach cells by trypsin-EDTA.

45 **[0055]** Stop trypsin activity by adding 1 volume of the complete medium into the well. Ensure that all cells are detached by pipetting 3-5 times inside the well.

50 **[0056]** Collect the cell suspension into 1.5 ml tubes. Pellet the cells by centrifuging the tubes at 400 x g for 5 minutes.

55 **[0057]** Remove the supernatant without disturbing the cell pellet. Resuspend the cell pellet in 200 µl DMEM/F12 basal medium.

60 **[0058]** Mix 10 µl of the cell suspension with 10 µl of 0.4% Trypan Blue solution (Thermofisher Scientific).

[0059] After 2 minutes, add 10 μ l of the cell/trypan blue mixture to each chamber of a Countess II FL Disposable Slide (Thermofisher Scientific) or a hemocytometer. If using the Countess II FL system, insert the slide into the slide holder of a Countess II FL Automated Cell Counter (Thermofisher Scientific) and determine the cell concentration and % viability. If using a hemocytometer, count cells with a microscope.

[0060] Calculate the number of cells in each treatment group. The number of viable cells per well equals to viable cell concentration (cells/ml).

[0061] CyQUANT Cell Proliferation Assay Kit

[0062] The CyQUANT Cell Proliferation Assay Kit (Thermofisher Scientific) quantifies cell growth by measuring the nucleic acid content in samples. Cells should have been seeded onto 96-well plates, preferably in triplicate wells per treatment group.

[0063] Remove the culture medium as much as possible by a multichannel pipette. Avoid scratching the well bottom with the pipette tip.

[0064] Freeze the plate in a -80°C freezer. The plate may be stored at -80°C for up to 4 weeks.

[0065] Thaw the plate and assay kit reagents at room temperature.

[0066] Mix the kit reagents according to Table 1 for each well.

Table 1

Components	Volume per well (μl)
Cell lysis buffer stock solution	10 μ l
Autoclaved MilliQ water	189.5 μ l
CyQUANT GR stock solution	0.5 μ l
Total= 200 μl	

[0067] When the plate has completely thawed, add 200 μ l of the CyQUANT GR dye/lysis buffer mixture to each sample well and to three empty wells (blank). Incubate the plate at room temperature for 5 minutes, protected from light.

- [0068] Using a multichannel pipette, transfer 160 μ l from each well of the 96-well plate to the corresponding well of a black 96-well plate.
- [0069] Measure the sample fluorescence using a fluorescence microplate reader (e.g. Molecular Devices SpectraMax iD5). Set the excitation and emission wavelengths at 480 nm and 520 nm respectively.
- [0070] Average the blank wells fluorescence readings. Subtract this average reading from all sample fluorescence readings to correct for background fluorescence.
- [0071] Calculate the mean corrected fluorescence for the vehicle group. Express the treatment group fluorescence readings as fold of control (FOC) by dividing the sample readings (i.e. IGF-1 groups) by the mean vehicle reading.
- [0072] Example 1
- [0073] IGF-1 stimulated the growth of MCF-7 cells in a dose-dependent manner
- [0074] To verify that IGF-1 stimulates the growth of human MCF-7 cells, cells were treated with increasing doses of human IGF-1 (0 pg/ml – 100 ng/ml) for 10 days and processed for cell counting. As seen in FIG. 7, while 1-100 pg/ml IGF-1 did not enhance the cell number, further increase of IGF-1 concentration (1-100 ng/ml) promoted cell growth in a dose-dependent manner. Hence, MCF-7 cells are suitable for evaluating the growth-stimulating activity of IGF-1.
- [0075] Example 2
- [0076] Human IGF-1, but not the mouse or fish IGF-1, promoted the growth of human MCF-7 cells
- [0077] To investigate whether the growth-stimulating activity of IGF-1 depends on its species origin, we treated MCF-7 cells with 1.5 nM recombinant IGF-1 of various species, i.e. human, mouse, and fish (bream, tuna). After 7 days, cell growth was assessed by the CyQUANT Assay (FIG. 8). While human IGF-1 obtained from multiple sources consistently increased MCF-7 cell growth (~50 - 100% increase), mouse and fish IGF-1 did not (FIG. 8). These findings suggest that human IGF-1, being the same species as the human MCF-7 cells, is more effective than fish and mouse IGF-1 in promoting cell growth.
- [0078] The present invention shows that it is more effective to apply growth factors and albumin of (i) genetically same or similar species or (ii) same genus as the cultured cell type. The usage of these growth factors or protein factors may be decreased while achieving the same growth rate. Species-specific

growth factors and/or genus-specific growth factors represent a promising direction to reduce media cost especially during large-scale cell production for cultivated meat and other applications using the cultivated cell mass. Using more bioactive growth factors can also decrease processing times and improve the quality (e.g. texture, taste, nutritional value) of cultivated meat or cell mass.

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[0079] The above description is illustrative and is not restrictive. Many variations of embodiments may become apparent to those skilled in the art upon review of the disclosure. The scope embodiments should, therefore, be determined not with reference to the above description, but instead should be determined with reference to the pending claims along with their full scope or equivalents.

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[0080] One or more features from any embodiment may be combined with one or more features of any other embodiment without departing from the scope embodiments. A recitation of "a", "an" or "the" is intended to mean "one or more" unless specifically indicated to the contrary. Recitation of "and/or" is intended to represent the most inclusive sense of the term unless specifically indicated to the contrary.

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[0081] While the present disclosure may be embodied in many different forms, the drawings and discussion are presented with the understanding that the present disclosure is an exemplification of the principles of one or more inventions and is not intended to limit any one embodiment to the embodiments illustrated.

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[0082] The disclosure, in its broader aspects, is therefore not limited to the specific details, representative system and methods, and illustrative examples shown and described above. Various modifications and variations may be made to the above specification without departing from the scope or spirit of the present disclosure, and it is intended that the present disclosure covers all such modifications and variations provided they come within the scope of the following claims and their equivalents.

25

Exemplary Protocols

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.
- 5 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.
5. Wash the organ one or more times in hypochlorous acid.
- 10 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).
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.
- 15 11. Centrifuge the filtrate at 200g for 5 minutes.
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.
- 20 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.
17. The cells are considered established when a complete monolayer is formed and the established cells are ready for subculture.

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

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.
- 30 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 (1-2 mm³).
- 35 8. Place organ 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).
9. Incubate at 24-28°C.
10. Replace half of the medium with fresh medium every 2-3 days without disturbing the tissue explant.
- 40 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.

45 C. Development of a fish muscle cell line

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.
5. Wash the tissue one or more times in hypochlorous acid.
- 5 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 PBS.
- 10 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 EMEM with 200 IU/ml, penicillin, 200 pg/ml streptomycin, 10% fetal bovine serum).
- 15 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.
17. The cells are considered established when a complete monolayer is formed and the
- 20 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.
2. Keep the fish on ice until cell isolation.
- 25 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 DMEM or EMEM with 400 IU/ml, penicillin, 400 pg/ml streptomycin).
- 30 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).
9. Incubate at 24-28°C.
- 35 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
- 40 established cells are ready for subculture.

E. Adult Stem cell isolation and culture

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.
- 45 3. Immerse the fish in 10% bleach.

4. Remove muscle from the fish under aseptic conditions.
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).
- 5 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 PBS.
9. Incubate at room temperature with continuous shaking for 1 hour.
10. Filter the supernatant with a 100 µm mesh to remove undigested tissue.
- 10 11. Centrifuge the filtrate at 200g for 5 minutes.
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, 100ng/ml basic fibroblast growth factor).
13. Plate the cells on an uncoated plate for 1 hour at 24-28°C.
- 15 14. Harvest the supernatant and place on a plate coated with laminin, gelatin, Matrigel or similar matrix.
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
- 20 EMEM with 200 IU/ml, penicillin, 200 pg/ml streptomycin, 10% fetal bovine serum, 100ng/ml basic fibroblast growth factor).

F. Generating and culturing iPSC

1. 2-4 days before transfection, plate cells in complete medium (L15 with 10% FBS)
- 25 in a tissue culture flask. Cells should be approximately 75-90% confluent on the day of transfection (Day 0).
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.
- 30 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 the tube.
4. Wash the cells in PBS.
5. Add 3 mL of 0.05% Trypsin/EDTA to the culture flask containing the cells.
- 35 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 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.
- 40 10. Carefully aspirate most of the supernatant and resuspend with complete medium.
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
- 45 Tube A and Tube B as described below.
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. 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.
- 5 17. Mix one more time and add the entire 250 μ L of transfection master mix to each well.
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
- 10 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.
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.
- 15 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, 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.

20 **G. Method for subculturing cells**

1. Remove and discard the culture medium.
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.
- 25 4. Incubate at room temperature for 1 min.
5. Add 5-8 mL of complete growth medium.
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.
- 30 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.
- 35 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.
- 40 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
- 45 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 health cell growth.
- 5 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.

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

- 10 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, wheat, yeast or equivalent).
- 15 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.
- 20 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.
- 25 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.
- 30 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.
- 35 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.

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

- 40 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).
- 45

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.
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.
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.
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.
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)

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 %
 - 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.
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)

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.
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.
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 method for meat production by in vitro cell culture, comprising:
isolating tissue from an animal or plant source and making a cell
5 suspension of cells;
introducing culture medium comprising growth factor of (i) genetically
same or similar species to the cells and/or (ii) genetically same genus to the
cells; and
growing the cells on a food-grade scaffold in a culture medium, the cells
10 growing into a solid or semi-solid structure that mimics an animal organ.
2. The method of claim 1, wherein isolating the tissue comprises isolating organ
tissue from a fish.
3. The method of claim 1, further comprising the step of increasing expression of a
protein in the growing cells by altering a level of one or more micro RNAs that regulate
15 the expression of the protein.
4. The method of claim 2, wherein the organ tissue is derived from fish swim
bladder of a fish from the Osteichthyes class.
5. The method of claim 3, wherein the protein is collagen.
6. The method of claim 3, wherein the micro RNAs are one or both of micro RNA21
20 (miR-21) and micro RNA 29a (miR-29a).
7. A method for meat production by in vitro cell culture, comprising:
isolating tissue from an animal or plant source and making a cell
suspension of cells;
growing the cells on a food-grade scaffold in a culture medium, the cells
25 growing into a solid or semi-solid structure that mimics an animal organ; and
co-culturing the cells with bioengineered cells that secrete nutrients,
growth factors, and cytokines that support the growth of the cells, wherein the
bioengineered cells is (i) genetically same or similar species to the cells and/or
(ii) genetically same genus to the cells.
- 30 8. The method of claim 7, wherein isolating the tissue comprises isolating organ
tissue from a fish.
9. The method of claim 8, wherein the organ tissue is derived from fish swim
bladder of a fish from the Osteichthyes class.