

ISOLATION AND CULTIVATION OF MUSCLE AND FAT CELLS FROM CRUSTACEANS

FIELD OF THE INVENTION

[001] The present disclosure is directed to the formation of renewable muscle and fat stem cell lines from shrimp, prawn, crab, crayfish, and/or lobster species.

BACKGROUND OF THE INVENTION

[002] The world population continues to increase while the amount of arable agricultural land decreases due to urban sprawl and changing weather conditions. This has increased commercial fishing and ocean “farming,” putting stress on the world’s seas and oceans. As a result, overfishing of particular popular species such as shrimp, prawn, crab, and lobster has significantly decreased those wild populations.

[003] In an attempt to ease the strain that animal meat production and wild fishing have on the environment, techniques are being developed for tissue engineered meats and fish. This type of cellular agriculture permits the production of food without the need to grow and sacrifice animals. Most of the research in cellular agriculture focuses on animals that have a wealth of information available from studies of embryonic muscle development, muscle stem cell, muscle repair, and muscle regeneration – in particular beef, pork, and chicken. Many challenges exist, however, in producing a meat or fish product that is a “like-for-like” replica of muscle extracted from a living animal.

[004] However, for seafood species, little research has been done, compounding the obstacles which must be overcome for successful cellular agricultural work (Rubio *et al.*, (2019) Front. Sustain. Food Syst. doi: 10.3389/fsufs.2019.00043). *Litopenaeus vannamei* (formerly *Penaeus vannamei*; a.k.a. whiteleg shrimp/Pacific white shrimp/king prawn) and *Penaeus monodon* (a.k.a. giant tiger prawn/ Asian tiger shrimp) are predicted to have 1.66 (*vannamei*)/2.6 (*monodon*) Gb genome size and 44 pseudochromosomes (*vannamei*)/88 chromosomes (Zhang *et al.* (2019) Nature Communications 10:356 and Yuan *et al.* (2017) J. Marine Genomics doi.org/10.1016/j.margen.2017.12.006). These large genome sizes and the presence of a

significant number of genomic repeats makes sequencing and assembly of prawn/shrimp genomes difficult (Yuan *et al.* (2017) J. Marine Genomics doi.org/10.1016/j.margen.2017.12.006 and Zhang *et al.* (2019) Nature Communications 10:356). In addition, the few genomic library search engines available for prawn/shrimp are not robust or well-annotated (Shrimp GPAT; Shrimp EST project; NCBI Taxonomy project). Furthermore, genome studies on shrimp/prawn have focused at genome evolution (Yuan *et al.* (2017) Mar Biotechnol 19(1):76-88; Yuan *et al.* (2017) Mar Drugs 15(7):213), genetic linkage (Yang *et al.* (2015) Scientific Reports 5: 15612), and developmental genes (Brown *et al.* (2018) Genome Biol Evol 10(1):143-156). This situation is further complicated by the inconsistent and/or interchangeable use of “shrimp” and “prawn” for a given species. Nonetheless, there are at least 2,000 recognized species, the most commercially important being those in the genera *Penaeus*, *Solenocera*, *Metapenaeus*, *Parapenaeus*, *Parapenaeopsis*, *Metapenaeopsis*, *Trachypenaeus*, *Protrachypene*, *Xiphopenaeus*, *Hymenopenaeus*, *Atypopenaeus*, *Eusicyonia*, *Sicyonia*, and *Litopenaeus*.

[005] Some studies have examined shrimp/prawn species in terms of physical animal growth by body size and weight. Shrimp growth was improved by transducing foreign Tilapia growth hormone genes into shrimp embryos (Arenal *et al.* (2008) Biotechnol Lett 30(5):845-51). Suppressive subtractive hybridization identified several growth genes related to body weight in *Penaeus monodon*, (Tangprasittipap *et al.* (2010) Aquaculture 307(1-2):150-156). Muscle growth genes have been identified in *Penaeus monodon* but no other species (Ngyuen *et al.* (2016) Aquaculture 464:545-553). However, genetic modification of genes to immortalize or reprogram muscle cells has either not been attempted or has not been successful.

[006] The situation for crab, crayfish, and/or lobster species is similar. For example, the marbled crayfish (*Procambarus virginalis* (previously *Procambarus fallax f. virginalis*)) has a genome size estimated to be larger than that of humans with 276 chromosomes (*i.e.* 3.5 Gbp; Gutekunst *et al.* (2018) Nature Ecology & Evolution 2:567-573), while the blue crab (*Callinectes sapidus*) genome is about 2 Gbp in size with an unknown number of chromosomes (see IMET Guardians of the Blue Crab website on the internet; 2.35 pg *see* Animal Genome Size Database on internet; and Dolezel *et al.* (Cytometry (2003) Part A 51A:127-128) who define genome size (bp) = (0.978 X 10⁹) X DNA content (pg). While no lobster genome has

been completely sequenced to date, estimates are that the American lobster (*Homarus americanus*) genome is about 4.40 pg/4.3 Gbp or greater (see Animal Genome Size Database website and Gloucester Marine Genomics Institute website, American Lobster Genome, on the internet).

[007] The lack of information regarding shrimp, prawn, crab, crayfish, and/or lobster is arguably largely due to challenges in cell culture. For example, shrimp/prawn cell lines have only been established from the lymphoid organ and ovaries (Tapay *et al.* (1995) Proc Soc Exp Biol Med 209(1):73-8; Hsu *et al.* (1995) Aquaculture 136:43-55; US patent number 6143547; George and Dhar (2010) In Vitro Cell Dev Biol Anim 46(9):801-10; Ma *et al.* (2017) Reviews in Aquaculture 9: 88-98). In addition, shrimp/prawn primary muscle cultures have lasted only up to 12 days (George and Dhar, 2010) and no known primary cultures or cell lines have been described for fat cells.

[008] Furthermore, attempts to culture fat cells from shrimp, prawn, crab, crayfish, and/or lobster have not been described.

[009] The availability of immortalized cell lines from shrimp, prawn, crab, crayfish, and/or lobster would allow cellular agriculture companies to progress to the next steps of cellular agriculture (Rubio *et al.*, (2019) Front. Sustain. Food Syst. doi: 10.3389/fsufs.2019.00043). Rather than the time consuming process of isolating primary cells, food regulatory and research groups would then have a ready source of cell lines. This would facilitate understanding shrimp, prawn, crab, crayfish, and/or lobster viral diseases, testing for the presence of those viruses, and developing treatments (Ma *et al.* (2017) Reviews in Aquaculture 9: 88-98). Consequently, there remains a need for immortalized muscle and fat cell lines from shrimp, prawn, crab, crayfish, and/or lobster. The disclosure presented herein addresses this need.

SUMMARY OF THE INVENTION

[010] The present disclosure is directed to tissue engineered shrimp, prawn, crab, crayfish, and/or lobster meat products and methods for producing such products. In one aspect the meat product comprises muscle cells that are grown *ex vivo* and/or *in vitro*. In a further aspect, the

meat product comprises muscle and/or fat cells grown *ex vivo* and/or *in vitro*. In yet another aspect the meat product comprises muscle cells, fat cells, other cells, and combinations thereof grown *ex vivo* and/or *in vitro*. The meat product is essentially free of any harmful microbial or parasitic contamination.

[011] Accordingly, the present disclosure also provides methods of producing shrimp, prawn, crab, crayfish, and/or lobster meat products for consumption by developing renewable muscle and/or fat stem cell lines.

[012] One method for producing a shrimp, prawn, crab, crayfish, and/or lobster immortalized muscle and/or fat cell line includes isolating shrimp, prawn, crab, crayfish, and/or lobster muscle and/or fat cells, integrating at least one muscle-specific and/or fat-specific growth gene into an adenoviral vector or non-integrative lentiviral vector to form a recombinant adenoviral construct or non-integrative lentiviral vector and transducing the isolated shrimp, prawn, crab, crayfish, and/or lobster muscle cells with the recombinant adenoviral construct or non-integrative lentiviral vector. However, the method for producing a shrimp, prawn, crab, crayfish, and/or lobster immortalized muscle and/or fat cell line also includes inducing activity of telomerase after isolation of shrimp, prawn, crab, crayfish, and/or lobster muscle and/or fat cells by chemical activators such as Cycloastragenol, Genistein, and/or Resveratrol.

[013] Also presented is a method for producing shrimp, prawn, crab, crayfish, and/or lobster induced pluripotent stem (iPS) cells. This method includes isolating muscle and/or fat cells from shrimp, prawn, crab, crayfish, and/or lobster, transfecting the cells with an episomal plasmid comprising a targeting factor and then culturing the transfected cells to produce iPS cells.

[014] In addition, the present disclosure provides a method for producing shrimp, prawn, crab, crayfish, and/or lobster induced pluripotent stem (iPS) cells via isolating shrimp, prawn, crab, crayfish, and/or lobster muscle and/or fat cells, transfecting the cells with microRNA containing reprogramming microRNAs and mRNAs such as mir302a-d, mir367, Oct4, Sox2, Klf4, c-Myc, and Lin28, and culturing the transfected cells to produce iPS cells.

[015] The present disclosure further includes a method of producing shrimp, prawn, crab, crayfish, and/or lobster meat products for consumption by isolating shrimp, prawn, crab, crayfish, and/or lobster muscle cells, reprogramming the isolated shrimp, prawn, crab, crayfish, and/or lobster muscle cells to express pluripotent genes, isolating induced pluripotent stem (iPS) cells, inducing the iPS cells to produce muscle fibers and/or fat, and growing the muscle fibers and/or fat to produce a shrimp, prawn, crab, crayfish, and/or lobster meat product.

BRIEF DESCRIPTION OF THE DRAWINGS

[016] **Figure 1** depicts shrimp explants in culture media immediately after sectioning. **A**: explants in T75 flask; **B**: explants in petri dish.

[017] **Figure 2** shows explant cell growth on day 7.

[018] **Figure 3** shows brightfield imaging on Ti-2 Nikon microscope of cells diluted at a 1:3 ratio after reaching 80-90% confluence. **A**: x20 magnification; **B**: x20 magnification.

[019] **Figure 4** shows myofibers beginning to form 3 days after generation of a suspension culture, x40 magnification.

[020] **Figure 5** shows myofibers ready for harvesting after 7 days growth, x40 magnification.

[021] **Figure 6** shows histology of tail segments where muscle stem cells are isolated from; **A**: the final body segment; **B**: increased magnification view of the final body segment; **C**: the middle wing of the tail; **D** increased magnification view of the middle wing; **E**: the side wing of the tail; **F**: increased magnification view of the side wing; **G**: histology of midline juvenile shrimp; muscle regions are outlined in black.

DETAILED DESCRIPTION OF THE INVENTION

[022] Throughout this disclosure, the term "a" or "an" entity refers to one or more of that entity; for example, "a polynucleotide," is understood to represent one or more polynucleotides.

As such, the terms "a" (or "an"), "one or more," and "at least one" can be used interchangeably herein.

[023] Furthermore, "and/or" where used herein is to be taken as specific disclosure of each of the two specified features or components with or without the other. Thus, the term "and/or" as used in a phrase such as "A and/or B" is intended to include "A and B," "A or B," "A" (alone), and "B" (alone). Likewise, the term "and/or" as used in a phrase such as "A, B, and/or C" is intended to encompass each of the following aspects: A, B, and C; A, B, or C; A or C; A or B; B or C; A and C; A and B; B and C; A (alone); B (alone); and C (alone).

[024] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to whom this disclosure is directed. For example, the Concise Dictionary of Biomedicine and Molecular Biology, Juo, Pei-Show, 2nd ed., 2002, CRC Press; The Dictionary of Cell and Molecular Biology, 3rd ed., 1999, Academic Press; The Oxford Dictionary Of Biochemistry And Molecular Biology, Revised, 2000, Oxford University Press; Takahashi *et al.* (2007) Cell 131:861-872; and Yu *et al.* (2007) Science 21; 318(5858):1917-20 provide one of skill with a general dictionary of many of the terms used in this disclosure.

[025] Units, prefixes, and symbols are denoted in their Systeme International de Unites (SI) accepted form. Numeric ranges are inclusive of the numbers defining the range. The headings provided herein are not limitations of the various aspects of the disclosure, which can be had by reference to the specification as a whole.

[026] Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. Where a specific range of values is provided, it is understood that each intervening value is intended to be included therein, and all smaller subranges are also included.

[027] Disclosed herein are comestible shrimp, prawn, crab, crayfish, and/or lobster products as well as new and improved methods for generating muscle and/or fat tissue *in vitro* from a cell source for use in the comestible shrimp, prawn, crab, crayfish, and/or lobster products. Such methods are useful for production of shrimp, prawn, crab, crayfish, and lobster muscle tissue, or “meat,” without reliance upon extensive farming or wild fishing. Currently, meat from shrimps, prawns, crab, crayfish, and/or lobster is typically only taken from the tail, leg, or claw muscles. The tail, leg, or claw meat is generally sold whole or in large pieces after removal of the exoskeleton; however, shrimp, prawn, crab, crayfish, and/or lobster meat products also include derivatives such as ground meat for inclusion in “meatballs”/“fishballs” and products containing smoked, pureed, seasoned, and/or dried meat.

[028] Also disclosed are *in vitro* produced shrimp, prawn, crab, crayfish, and/or lobster tissues, such as muscle and/or fat tissue, made by any of the methods provided herein. Accordingly, the cell source can be an adult stem cell, an induced pluripotent stem (iPS) cell, and/or an immortalized cell line. The shrimp and/or prawn cell source is preferably isolated from a species from the genus *Penaeus*, *Solenocera*, *Metapenaeus*, *Parapenaeus*, *Parapenaeopsis*, *Metapenaeopsis*, *Trachypenaeus*, *Protrachypene*, *Xiphopenaeus*, *Hymenopenaeus*, *Atypopenaeus*, *Eusicyonia*, *Sicyonia*, and/or *Litopenaeus*. The crab cell source is preferably isolated from a species from the genus *Chionoecetes*, *Callinectes*, *Charybdis*, *Cancer*, *Scylla* and/or *Metacarcinus*. The crayfish cell source is preferably isolated from the genus *Cambarus*, *Jasus*, *Thenus*, *Cambarellus*, *Cambaroides*, *Atacopsis*, *Austropotamobius*, *Astacus*, *Procambarus*, *Orconectes*, *Faxonella*, and *Pacifastacus*. The lobster cell source is preferably isolated from a species from the genus *Acanthacaris*, *Eunephrops*, *Hominarinus*, *Homarus*, *Metanephrops*, *Nephropides*, *Nephrops*, *Nephropsis*, *Thaumastocheles*, *Thaumastochelopsis*, *Thymopides*, *Thymops*, or *Thymopsis*.

Isolation of primary muscle and fat cells from crustaceans

[029] One aspect of the present disclosure is directed to a method of producing shrimp, prawn, crab, crayfish, and/or lobster meat products for consumption by developing renewable muscle and/or fat stem cell lines. This can be accomplished by isolating muscle and/or fat cells from various shrimp, prawn, crab, crayfish, and/or lobster species and culturing them *in vitro*. For

example, Figure 6 illustrates the histology of shrimp tail segments from which stem cells are isolated.

[030] Preferably, explants from freshly sacrificed shrimps, prawns, crabs, crayfish, and/or lobsters are separated into small pieces. Typically the pieces are at least 0.1 mm in size and can be 0.25 mm, 0.5 mm, 0.75 mm, 1 mm, 1.25 mm, 1.5 mm, 1.75 mm, 2 mm, 2.25 mm, 2.5 mm, 2.75 mm, 3 mm, 3.25 mm, 3.5 mm, 3.75 mm, 4 mm, 4.25 mm, 4.55 mm, 4.75 mm, 5 mm or any number between 0.1 mm and 5 mm. Explants are used to seed cell culture media for cell culturing.

[031] When used, explants are incubated in cell culture media, such as Grace's insect media, DMEM high glucose (HyClone SH30022.01, Fischer Scientific, Waltham MA), NUTRISTEM® MSC XF supplement mix (Biological Industries, Cromwell, CT), NUTRISTEM® MSC XF basal media (Biological Industries, Cromwell, CT), Myocult™ SF expansion human 10x (Stemcell Technologies™, Cambridge, MA) HyClone™ media for undifferentiated mesenchymal stem cells (SH30879.01, GE Lifescience, Boston, MA), Hyclone stem cell 10x Supplement (HyClone™ SH30878.02, Fischer Scientific, Waltham MA), Leibovitz's-15 (L-15) media (ThermoFisher, Waltham, MA), M199 media (ThermoFisher, Waltham, MA), MPS media (ThermoFisher, Waltham, MA), Pj-2 media, NCTC 135 media (ThermoFisher, Waltham, MA), MM insect media (Sigma-Aldrich®, St. Louis, MO), or TC 100 media ((ThermoFisher, Waltham, MA), and combinations thereof. The cell culture media may additionally comprise 10% fetal bovine serum (FBS) and/or 5% Penicillin/Streptomycin (PS). Fresh media is added periodically, preferably every 2-3 days.

[032] Optionally, explants are periodically rinsed with about 5% penicillin-streptomycin filtered Phosphate-Buffered Saline (PBS; 0.137 M NaCl, 0.0027 M KCl, 0.01 M Na₂HPO₄, 0.0018 M KH₂HPO₄, pH 7.4) without further removal of any cells shed from the explants during the rinsing process. Preferably, the rinsing occurs every 2-3 days until explants are removed from the culture media.

[033] The explant/cell cultures are incubated at 28°C without carbon dioxide. About 7 days after culturing has begun, explants are removed by centrifugation, filtration, magnetic beads,

and/or other means known to those of skill in the art, leaving behind a large population of single cells. The identity of muscle stem cells in the population is determined by morphological identification and/or PCR amplification of muscle genes. The identity of fat cells is also determined by morphological identification and/or PCR amplification of adipose-specific genes. Cultures are routinely tested for mycoplasma and/or other pathogens using standard techniques such as direct growth on broth/agar, specific DNA staining, PCR amplification, ELISA, RNA labeling and enzymatic procedures (e.g. enzymatic conversion of ADP to ATP, *etc.*), Plasmotest™ (InvivoGen, San Diego, CA), BAM 4/LST-MUG (Feng *et al.* (2002) available from the [fda.gov/food/laboratory-methods-food](https://www.fda.gov/food/laboratory-methods-food) website, chapters 4 and 5), and combinations thereof.

[034] After removal of explants, the remaining cell population is divided, fresh cell culture media is added and incubation continued. Fresh media is added periodically, preferably about every 2-3 days. When the cells are nearing confluence, preferably about 70%, 80%, 90%, 95%, 99%, or any value in between, or the cell population is about 8 million cells in 10 ml media, the cells are again divided and diluted at a 1:3 ratio. The culturing process can include slowly reducing the amount of penicillin-streptomycin from 5% to 2% and then to 0% prior to cryopreservation of the cells.

[035] Cells can be cryopreserved after passage 2 using standard cryopreservation conditions and standard cryopreservation media (see, for example, protocols and reagents available on the internet from Thermo Fisher Scientific, Nippon Genetics, and ATCC). For example, cells can be briefly centrifuged (e.g. about 5 minutes) at 1000 x g, supernatant removed, and cells resuspended in 1 ml of Bambanker freezing media in cryotubes prior to storage at -80°C.

Differentiation into Myofibers

[036] Approximately 2-3 million primary muscle cells, immortalized muscle stem cells, and/or reprogramed cells are added to appropriate culture media. Appropriate culture media includes DMEM high glucose (HYCLONE™, SH30022.01; Fisher Scientific, Waltham, MA), Grace's insect media, DMEM high glucose (HYCLONE™ SH30022.01, Fischer Scientific, Waltham MA), NUTRISTEM® MSC XF supplement mix (Biological Industries, Cromwell, CT), NUTRISTEM® MSC XF basal media (Biological Industries, Cromwell, CT),

MYOCULT™ SF expansion human 10x (STEMCELL TECHNOLOGIES™, Cambridge, MA) HYCLONE™ media for undifferentiated mesenchymal stem cells (SH30879.01, GE Lifescience, Boston, MA), HYCLONE™ stem cell 10x Supplement (HYCLONE™ SH30878.02, Fischer Scientific, Waltham MA), and combinations thereof. The culture media can include augmentation with inactivated fetal bovine serum and/or penicillin-streptomycin. Cell cultures are preferably incubated at 28°C with stirring. Typically, myofibers begin to form 3 days after initiation of the suspension culture. Media is changed periodically (e.g. every 3 days) by centrifuging the cells/developing myofibers, replacing the media supernatant, and resuspending the cells/developing myofibers. Myofibers are generally harvested 7 days after the suspension culture is initiated.

Immortalization of isolated primary muscle and/or fat cells

[037] Growth genes specific to muscle and/or fat cells identified from sequencing are used to immortalize isolated muscle and/or fat cells. RNA sequencing is performed and analyzed using standard techniques (see, for example, Kukurba and Montgonery (2015) Cold Spring Harb Protoc 2015(11):951-969). These genes include, for example, MyoD and SMARCD3, Pax3, Pax7 (reviewed in Chal and Pourquié (2017) Development 144:2104-2122), myosin-1, integrin alpha-7, cadherin-15, myogenin, growth hormone and insulin-like growth factor, myostatin and growth differentiation factor, crustacean hyperglycemic hormone, and myosin heavy chain (Jung *et al.* (2013) Reviews in Aquaculture 5, 77-110; Ngyuen *et al.* (2016) Aquaculture 464: 545-553; Okita *et al.* (2011) Nat Methods 8: 409-412). Non-integrating immortalization methods such as recombinant adenoviral vectors (adm); non-integrative lentiviral vectors like lentiflash particles (vectalys), inducible lentiviral vectors (Bar-Nur *et al.* (2018) Stem Cell Reports 10:1505-1521), and integrase-deficient lentiviral vectors (Chick *et al.* (2012) Human Gene Therapy 23:1247-1257); and/or mini circles (Kim *et al.* (2017) Stem Cell Research 23:87-94) are used to integrate and overexpress the growth genes of interest. Adenoviral vectors include an Ad5-derived, E1A-deleted adenoviral vector expressing the full-length murine *MyoD* cDNA under the transcriptional control of Rous sarcoma virus LTR (Lattanzi *et al.* (1998) J Clin. Invest. 101(10):2119-2128); Ad-MyoD, Ad-m-MYOD1 (Vector Biolabs, Cat. No 1492, ADV-265351); human type-5 adenovirus (Suehiro *et al.* (2010) FEBS Letters 584:3545-3549) and white sturgeon adenovirus (WSAdV-1 (Hendrick *et al.* (1985) Can J Fish Aquat Sci

42:1321-1325; Hendrick *et al.* (1990) *Dis Aquat Org* 8:39-44). An example of non-integrative lentiviral vectors is commercially available lentiflash particles (Vectalys); this includes the customizable pRLP-MS2 plasmid (Vectalys, Toulouse, France), where a muscle growth gene, as mentioned above, is cloned into the vector and is transduced along with pRLP-MCP and VSVG plasmids. Alternatively, inducible lentiviral vectors like tetOP-MyoD and M2rtTA are co-expressed in the isolated muscle cells (Bar-Nur *et al.* (2018)). Similarly, suitable integrase-deficient lentiviral plasmids can be constructed according to Chick *et al.* (2012), including a combination of pHR'SIN-cPPT-SFFV-eGFP-WPRE, pHR'SIN-cPPT-SFFV-NogoB-WPRE, and pCMV delta R8.74 D64V plasmids. Finally, DNA minicircles expressing hPax7 are cloned according to Kim *et al.* (2017).

[038] Otherwise, chemical immortalization of cells is stimulated by use of telomerase activators such as Cycloastragenol (Sigma, SMB00372-20MG; Fauze *et al.* (2008) *J Immunol.* 181(10): 7400–7406), Genistein from Glycine Max (soybean) (Sigma, G6776-5MG; Chau *et al.* (2007) *Carcinogenesis* 28(11): 2282–2290), or Resveratrol (Sigma, R5010-100MG; Xia *et al.* (2008) *British Journal of Pharmacology* 155: 387–394; Zhai *et al.* (2016) *Oncology Letters* 11: 3015-3018).

[039] Isolated primary cells at about 70% confluence are used for transduction. Cells are incubated with adenoviral vector constructs containing the gene of interest are incubated at or around 28°C for an hour and evaluated for transduction by PCR, quantitative PCR and/or FACS after 48-72 hours. Lentiflash particles are incubated at or around 28°C with primary cells for about 12 hours before removal. Optionally, the transduction process is repeated approximately 30 hours after the first transduction (Prel *et al.* (2015) *Mol Ther Methods Clin Dev* 21(2): 15039). Inducible lentiviral vectors are incubated with primary cells at or around 28°C for 24 hours before removal and then supplemented at 48 and 72 hours with 4-8 µg/ml polybrene transfection reagent (Sigma-Aldrich) (Bar-Nur *et al.* (2018)). Integrase-deficient lentiviral plasmids are incubated with primary cells for 18 hours at or around 28°C in the presence of 8 µg/ml polybrene (Chick *et al.* (2012) *Human Gene Therapy* 23(12):1247-1257). Alternatively, mini circles are transduced into primary cells with GENEIN™ transfection kit (MTI-Global Stem, Gaithersburg, Maryland) for a total of 3 times every 3 days as reported by Kim *et al.*

(Stem Cell Research (2017) 23:87-94).

[040] When chemical immortalization is relied upon, the cells are incubated with Cycloastragenol (0.01-1 μ M, Sigma; Fauce *et al.* (2008) J Immunol. 181(10): 7400–7406); Genistein (0.5-1 μ M, Sigma; Chau *et al.* (2007) Carcinogenesis 28(11): 2282–2290) or Resveratrol (10-50 μ M, Sigma; Xia *et al.* (2008) British Journal of Pharmacology 155: 387–394; Zhai *et al.* (2016) Oncology Letters 11: 3015-3018) for a total of 72 hours or 144 hours (supplemented at 72 hours).

[041] The immortality of primary cells is verified by PCR expression of muscle stem cell and/or growth genes or by the ability of cells to be passaged beyond 10 times. Telomerase activity is assessed by telomerase repeated amplification ELISA.

Generating iPS cells from isolated primary muscle and fat cells

[042] Primary muscle and/or fat cells, and/or immortalized muscle and/or fat cells are reprogrammed by transfection with episomal plasmids (see, for example, Chandrobose *et al.* (2018) Stem Cell Research & Therapy 9:68; Slamecka *et al.* (2016) Cell Cycle 15(2):234-249) or by integration- and xeno-free mRNA transfection (Lee *et al.* (2016) Stem Cells International 2016: 6853081).

[043] Episomal plasmids from the Yamanaka cocktail are used: pCXLEhOct3/4-shp53-F, pCXLE-hSK, pCXLE-hUL, and pCXLE-EGFP (Addgene, Watertown, MA) as well as the Yamanaka cocktail of Oct4, Sox2, Klf4 and Myc (Takahashi *et al.* (2007) Cell 131:861-872; Okita *et al.* (2011) Nat Methods 8: 409-412; Rosello *et al.* (2013) Elife 2: e00036).

[044] Immediately following transfection, cells are placed in culture media. Appropriate culture media include Grace's Insect Media supplemented with 10% FBS and 2% PS (Ma *et al.*, 2017; George and Dhar, 2010) and/or mesenchymal stem cell media. Media is changed daily and on day 7, cells are seeded onto MATRIGel® (Corning, Tewksbury, MA) for feeder- free induced pluripotent stem (iPS) cell derivation. Subsequently, the media is changed to mTeSR1 (STEMCELL TECHNOLOGIES™, Cambridge, MA), preferably supplemented with sodium

butyrate. Media is then preferably supplemented at a later date with small molecules SMC4 cocktail (containing small molecules: PD0325901, CHIR99021, Thiazovivin, and SB431542 (FOCUS Biomolecules, Plymouth meeting, PA)) until initial colonies are formed.

[045] Alternatively, microRNA containing reprogramming microRNAs and mRNAs (mir302a-d, mir367, Oct4, Sox2, Klf4, c-Myc, Lin28; Stemgent Inc., Cambridge, MA) are transfected as previously described (Lee *et al.*, 2016). Isolated primary muscle cells are seeded in vitronectin XF coated wells a day prior to transfection. mRNA transfection is repeated daily for a period of time, for example for 11 days, while microRNA transfection is repeated on two occasions, such as day 1 and 5, after seeding.

[046] To confirm that the primary muscle and/or fat cells have been reprogrammed to induced pluripotent cells, known pluripotent genes are analyzed via quantitative PCR and FACS and the results verified by sequencing. Exemplary pluripotent genes include, but are not limited to, PL10 family genes (Mochizuki *et al.* (2001) Dev Genes Evol 211(6):299-308); Lv-Vasa (Aflalo *et al.* (2007) Mol Repro Dev 74:172-177), DEAD family genes (Shukalyuk *et al.* (2007) Cell Biol Int 31(2):97-108); Pou5f1, klf, sall4, hsp60 (Robles *et al.* (2011) Zebrafish 8(2): 57-63); VVL, SoxN, dmyc, Luna family genes (Rosello *et al.* (2013) Elife 2: e00036); and piwi (Alie *et al.* (2011) Dev Biol 350(1):183-97). Functional tests are then carried out to verify cell potency. Examples of suitable tests include, but are not limited to, *in vitro* differentiation assays, teratoma formation, karyotype analysis and bisulfite sequencing.

Generation of Muscle and Fat cells from pluripotent embryonic stem cells

[047] In other embodiments the muscle and/or fat cells are derived from pluripotent embryonic stem cells, such as cells from the blastocyst stage and fertilized eggs.

[048] Pluripotent embryonic stem cells are initially cultured essentially as described above. Muscle cells are differentiated from embryonic stem cells or induced pluripotent stem cells as described in Salani *et al.* (J Cell Mol Med (2012) 16(7):1353-1364) and Chai and Pourquié (Development (2017) 144:2104-2122). Adipocytes are differentiated from embryonic stem cells and induced pluripotent stem cells as described in Barberi *et al.* (PLoS Med (2005) 2(6):e161),

Mohsen-Kanson *et al.* (Stem Cells (2014) 32:1459-1467) and Hafner *et al.* (Scientific Reports (2016) 6: Article Number 32490).

EXAMPLES

Example 1- Isolation of primary muscle stem cells from shrimp/prawns

[049] Live Whiteleg shrimps and Tiger prawns (including adult or postlarval stages) are held on ice for 10 minutes prior to sacrificing. Whole shrimps/prawns are rinsed in 11% sodium hypochlorite for 10 seconds and the last body segment and tail are dissected. The remaining body parts are kept on ice.

[050] The dissected tail and attached segment are transferred to 2% potassium permanganate and sterilized for 10 minutes. The tail and attached segment are then rinsed once in PBS buffer (0.137 M NaCl, 0.0027 M KCl, 0.01 M Na₂HPO₄, 0.0018 M KH₂HPO₄, pH 7.4), once in 2% penicillin-streptomycin filtered PBS, and held in 70% ethanol for 5-10 minutes prior to washing in 5% or 10% penicillin-streptomycin filtered PBS for 5-10 minutes.

[051] Cleaned tails and attached segment is kept on ice along with a separate 10 cm dish containing cell culture media. Five different media are used: (1) Grace's insect media with 10% heat-inactivated fetal bovine serum (HYCLONE™ SH30071.03, Fischer Scientific, Waltham MA) and 5% penicillin-streptomycin, (2) DMEM high glucose (HYCLONE™ SH30022.01, Fischer Scientific, Waltham MA) with 10% heat-inactivated bovine serum and 5% penicillin-streptomycin, (3) NUTRISTEM® MSC XF supplement mix (Biological Industries, Cromwell, CT):NUTRISTEM® MSC XF basal media (Biological Industries, Cromwell, CT) with 5% penicillin-streptomycin, (4) DMEM high glucose (HYCLONE™ SH30022.01, Fischer Scientific, Waltham MA):MYOCULT™ SF expansion human 10x (Stemcell Technologies™, Cambridge, MA):5% penicillin-streptomycin, and (5) HYCLONE™ media for undifferentiated mesenchymal stem cells (SH30879.01, GE Lifescience, Boston, MA): HYCLONE™ stem cell 10x Supplement (HYCLONE™ SH30878.02, Fischer Scientific, Waltham MA): 5% penicillin-streptomycin.

[052] For adult muscle isolations, the tail is removed and reserved while the opaque muscle is

dissected from the body segment. The layer of fat and epidermal tissue is removed.

[053] For postlarval muscle isolation, the head is separated from the body. Legs are removed as much as possible and the body segments are minced initially with an electrical mincer or handheld blender.

[054] The adult or partially-minced postlarval muscle are manually minced into approximately 1 mm pieces prior to transfer to the prepared 10 cm media containing dish where a second round of mincing is performed. For the adults, muscle is then removed from the tail, minced, transferred to the prepared media dish and minced again. This process is then repeated for the adult winged ends. Upon completion of dissection, the explants and media are transferred to cell culture flasks and/or dishes and incubated at 28°C without CO₂ (*see* Figure 1).

[055] Cultures are maintained by adding fresh media every 2-3 days. Between 3 to 7 days after isolation, explant tissues are removed by gravity separation or centrifugation for 10-30 seconds. The remaining cell population is split according to cell density, usually into 2 T75 flasks, and media added to a final volume of 10-12 ml in each flask (*see* Figure 2). Incubation at 28°C is continued and fresh media added every 2-3 days. When cells number about 8 million and/or are about 80-90% confluent in about 10 ml media, the cell population is again diluted at a 1:3 ratio and incubated under the same conditions continued until passage 2 (*see* Figure 3). At that time, some cells are centrifuged for 5 minutes at 1000 x g, supernatant removed and cells resuspended in cryotubes in 1 ml of Bambanker freezing media prior to storage at -80°C. Other cells are further cultured in media until at least passage 4. In those cases, the antibiotics present in the culture media is reduced to 2% after passage 2 and further reduced to 0% after passage 4.

[056] To confirm the identity of muscle stem cells, expression of muscle genes is analyzed via quantitative PCR. Here, muscle cell genes such as myostatin and growth differentiation factor, Muscle LIM protein, Alpha skeletal muscle, Myosin heavy chain, myosin-1, Pax3, Pax7, integrin alpha-7, cadherin-15, and myogenin (Jung *et al.* (2013) *Reviews in Aquaculture* 5, 77-110; Ngyuen *et al.* (2016) *Aquaculture* 464: 545-553; Okita *et al.* (2011) *Nat Methods* 8: 409-412) are targeted. Fat cells are identified by the presence of fatty acid-binding protein (Ngyuen *et al.* (2016) *Aquaculture* 464), serum amyloid A (SAA), transmembrane 4 L six family

member 1 (TM4SF1) (Jernas *et al.* (2006) FASEB 20(9):1540-1542), or a combination of surface antigens CD44, CD45, and CD105 (Wang *et al.* (2017) Exp Ther Med 13(3):1039-1043).

[057] Sterility of cell lines is determined by the absence of Mycoplasma. This is tested using commercially available kits such as Cell Culture Contamination Kit, MycoFluor™ Mycoplasma Detection Kit, MycoSEQ™ Mycoplasma Detection Kit (ThermoFisher Scientific), MycoAlert PLUS detection kit, PyroGene™ Recombinant Factor C Endpoint Fluorescent Assay (Lonza) and/or FTA Sample Collection Kit for PCR-based Mycoplasma Detection Service (ATCC).

Example 2 – Differentiation into myofibers

[058] Myofiber differentiation is accomplished by adding approximately 2-3 million primary or immortalized adult muscle stem cells to DMEM high glucose (HYCLONE™ SH30022.01, Fischer Scientific, Waltham MA) media with 10% heat-inactivated bovine serum and 5% penicillin-streptomycin. Cells are incubated at 28°C with stirring on a magnetic stirrer set at speed 3 (IKA RCT basic). Media is changed every 3 days by centrifuging fibers at 1000 x g for 5 minutes, removing the used media and replacing it with fresh media. Myofibers begin to form about 3 days after initiation of a suspension culture (*see* Figure 4) and are ready for harvest after 7 days (*see* Figure 5).

Example 3 - Immortalization of isolated primary muscle and fat cells

[059] Non-integrating immortalization methods can be used to integrate and overexpress growth genes of interest. These genes include for example, MyoD and SMARCD3, Pax3, Pax7 (reviewed in Chal and Pourquié (2017) Development 144:2104-2122), myosin-1, myogenin, integrin alpha-7, cadherin-15, growth hormone and insulin-like growth factor, myostatin and growth differentiation factor, crustacean hyperglycemic hormone, and myosin heavy chain (Jung *et al.* (2013) doi.org/10.1111/raq.12005; Ngyuen *et al.* (2016) Aquaculture 464:545-553; Okita *et al.* (2011) Nat Methods 8: 409-412). However, chemical immortalization can also be accomplished using activators such as Cycloastragenol (Fauce *et al.* (2008) J Immunol. 181(10): 7400–7406), Genistein from Glycine Max (soybean) (Chau *et al.* (2007) Carcinogenesis 28(11): 2282–2290), or Resveratrol (Xia *et al.* (2008) British Journal of

Pharmacology 155: 387–394; Zhai *et al.* (2016) *Oncology Letters* 11: 3015-3018) to increase telomerase activity.

[060] Immortality of primary cells is verified by PCR expression of muscle stem cell and/or growth genes or the ability of cells to be passaged beyond 10 times. When chemical immortalization is used, the telomerase repeated amplification ELISA assay is used to assess the increase in activity and length of telomerase.

Example 4 - Generating iPS cells from isolated primary muscle and fat cells

[061] Primary muscle and fat cells are reprogrammed by episomal plasmids essentially as described by Chandrabose *et al.*, 2018 or by integration- and xeno-free mRNA transfection as described by Lee *et al.*, 2016.

[062] Episomal plasmids (Addgene) targeting factors from the sequencing results for shrimp are used. In addition, the Yamanaka cocktail of Oct4, Sox2, Klf4 and Myc is also used. Grace's Insect Media supplemented with 10% FBS and 2% PS (Ma *et al.*, 2017; George and Dhar, 2010) or mesenchymal stem cell media is used immediately following transfection. Media is changed daily. On day 7, cells are seeded onto Matrigel (Corning) for feeder- free iPS derivation. On the next day, the media is changed to mTeSR1 (Stem Cell Technologies), supplemented with sodium butyrate. On day 12, sodium butyrate is no longer added, and instead replaced with small molecules SMC4 cocktail (containing small molecules: PD0325901, CHIR99021, Thiazovivin, and SB431542 (FOCUS Biomolecules)) until initial colonies are formed.

[063] Alternatively, microRNA containing reprogramming microRNAs and mRNAs (mir302a-d, mir367, Oct4, Sox2, Klf4, c-Myc, Lin28; Stemgent Inc., MA) are transfected as described previously (Lee *et al.*, 2015). Isolated primary muscle cells are seeded in a vitronectin XF coated well a day prior to transfection. mRNA transfection is repeated for 11 days from day 2 while microRNA transfection is repeated on day 1 and 5 after seeding.

[064] To confirm that the primary muscle and fat cells are reprogrammed to induced pluripotent cells, known pluripotent genes, verified by sequencing, are analyzed via quantitative

PCR and FACS. Exemplary pluripotent genes include, but are not limited to, PL10 family genes (Mochizuki *et al.* (2001) *Dev Genes Evol* 211(6):299-308); Lv-Vasa (Aflalo *et al.* (2007) *Mol Repro Dev* 74:172-177), DEAD family genes (Shukalyuk *et al.* (2007) *Cell Biol Int* 31(2):97-108); Pou5f1, klf, sall4, hsp60 (Robles *et al.* (2011) *Zebrafish* 8(2): 57-63); VVL, SoxN, dmyc, Luna family genes (Rosello *et al.* (2013) *Elife* 2: e00036); and piwi (Alie *et al.* (2011) *Dev Biol* 350(1):183-97). Functional tests such as *in vitro* differentiation assays, teratoma formation, karyotype analysis and bisulfite sequencing are carried out to verify the potency of the cells.

[065] Upon confirmation of the presence of iPS cells, these cells can be frozen upon 80% confluence.

Example 5 – Isolation and expansion of adipose stem cells from shrimp, crab, crayfish, and lobster

[066] Following sacrifice and cleaning of shrimps/prawns, crab, crayfish, or lobster as described above in paragraphs [045], [046] and/or [049], the head of the shrimp/prawn, crab, crayfish, or lobster is separated from the body and tail segments. The legs of are also separated from the body of the shrimp/prawns, crab, crayfish, or lobster. The shell is removed from the segments; and the adipose and epidermal tissue is peeled off the tail and body segments with a disposable scalpel and forceps. The adipose and epidermal tissues are washed extensively in PBS containing 5% Penicillin/Streptomycin. The tissue is then digested in Collagenase Type I and minced with scalpels as described in Bunnell *et al.* (Methods 2008; 45(2):115-120). The adipose stem cells are separated from epidermal or stromal cells as described by Bunnell *et al.* (Methods 2008; 45(2):115-120).

[067] The isolated cells are resuspended in alpha-MEM (Mediatech, Herndon, VA) supplemented with 20% FBS, 1% L-glutamine (Mediatech) and 1% penicillin/streptomycin. The cell suspension is filtered through a 70 µm cell strainer as described in Bunnell *et al.* (Methods 2008; 45(2):115-120). The cells are cultured in a lysine coated culture plate (Bunnell *et al.* Methods 2008; 45(2):115-120) and incubated at 28°C (George and Dhar (2010) *In Vitro Cell Dev. Biol. – Animal* 46:801-810).

[068] Culture media is changed and cells are washed 72 hours after plating as described in Bunnell *et al.* (Methods 2008; 45(2):115-120). To maintain cells afterwards, culture media is changed every second day until cells reach 80-90% confluence. The cells can be harvested or differentiated once they reach 80-90% confluence, as described in Bunnell *et al.* (Methods 2008; 45(2):115-120).

Example 6 – Isolation of primary muscle stem cells from crab, crayfish, and lobster

[069] Following sacrifice and cleaning of crabs or lobster on ice as described in [045] and [046], muscle of crab claws, legs and main body are isolated and processed as described in [045] – [047]. The crab muscle stem cells are cultured at 20-24°C as described in Sashikumar and Desai (Cytotechnology (2008) 56:161-169).

[070] For crayfish, and lobsters, muscle from the claws, legs and tail is isolated, processed and cultured as described in [045] – [047]. Crayfish muscle stem cells are cultured at 27°C as described in Neumann *et al.* (In Vivo (2000) 14(5): 691-8). Lobster muscle stem cells are cultured at saturated humidity at 5°C as described in Stepanyan *et al.* (Chemical Senses (2004) 29(3):179-187) or the temperature at which the lobsters were kept while alive.

CLAIMS

We claim

1. A meat product comprising shrimp, prawn, crab, crayfish, and/or lobster muscle cells that are grown *in vitro*.
2. The meat product according to claim 1, further comprising shrimp, prawn, crab, crayfish, and/or lobster fat cells grown *in vitro*.
3. The meat product according to claim 1 or claim 2, further comprising other shrimp, prawn, crab, crayfish, and/or lobster cells grown *in vitro*.
4. The meat product according to any one of claims 1-3, wherein it is essentially free of any harmful microbial and/or parasitic contamination.
5. The meat product according to any one of claims 1-4, wherein the shrimp, and/or prawn muscle cells are from a species belonging to the genus *Penaeus*, *Solenocera*, *Metapenaeus*, *Parapenaeus*, *Parapenaeopsis*, *Metapenaeopsis*, *Trachypenaeus*, *Protrachypene*, *Xiphopenaeus*, *Hymenopenaeus*, *Atypopenaeus*, *Eusicyonia*, *Sicyonia*, or *Litopenaeus*.
6. The meat product according to any one of claims 1-5, wherein the shrimp, and/or prawn muscle cells are from *Penaeus monodon* and/or *Litopenaeus vannamei*.
7. A method for producing a shrimp, prawn, crab, crayfish, and/or lobster immortalized muscle and/or fat cell line comprising
 - (a) isolating shrimp, prawn, crab, crayfish, and/or lobster cells muscle and/or fat cells;
 - (b) integrating at least one muscle-specific and/or fat-specific growth gene into an adenoviral vector or lentiflash particle to form a recombinant adenoviral construct or lentiflash particle;

(c) transducing the isolated shrimp, prawn, crab, crayfish, and/or lobster cells muscle cells with the recombinant adenoviral construct or lentiflash particle of (b); and

(d) producing an immortalized muscle and/or fat cell line.

8. A method for producing shrimp, prawn, crab, crayfish, and/or lobster cells induced pluripotent stem (iPS) cells comprising

(a) isolating muscle and/or fat cells from shrimp, prawn, crab, crayfish, and/or lobster cells;

(b) transfecting the cells of step (a) with an episomal plasmid comprising a targeting factor; and

(c) culturing the transfected cells of step (b) to produce iPS cells.

9. A method of producing shrimp, prawn, crab, crayfish, and/or lobster cell meat products for consumption comprising

(a) isolating shrimp, prawn, crab, crayfish, and/or lobster muscle cells;

(b) reprogramming the isolated shrimp, prawn, crab, crayfish, and/or lobster muscle cells to express pluripotent genes;

(c) isolating induced pluripotent stem (iPS) cells;

(d) inducing the iPS cells to produce muscle fibers and/or fat; and

(e) growing the muscle fibers and/or fat to produce a shrimp, prawn, crab, crayfish, and/or lobster cell meat product.

10. A stably transduced immortalized shrimp, prawn, crab, crayfish, and/or lobster muscle cell, wherein the immortalized muscle cell comprises at least exogenous MyoD, SMARCD3, Pax3, Pax7, myosin-1, integrin alpha-7, cadherin-15, myogenin, growth hormone and insulin-like growth factor, myostatin and growth differentiation factor, crustacean hyperglycemic hormone and/or myosin heavy chain.

11. A stably immortalized shrimp, prawn, crab, crayfish, and/or lobster muscle cell, wherein the immortalized muscle cell exhibits increased telomerase activity.

12. A cell culture comprising the immortalized muscle cell of claim 10.
13. A cell culture comprising the immortalized muscle cell of claim 11.
14. A stably transduced immortalized shrimp, prawn, crab, crayfish, and/or lobster fat cell, wherein the immortalized fat cell comprises at least exogenous MyoD, SMARCD3, Pax3, Pax7, myosin-1, integrin alpha-7, cadherin-15, myogenin, growth hormone and insulin-like growth factor, myostatin and growth differentiation factor, crustacean hyperglycemic hormone and/or myosin heavy chain.
15. A stably immortalized shrimp, prawn, crab, crayfish, and/or lobster fat cell, wherein the immortalized fat cell exhibits increased telomerase activity.
16. A cell culture comprising the immortalized fat cell of claim 14.
17. A cell culture comprising the immortalized fat cell of claim 15.
18. A stably transfected shrimp, prawn, crab, crayfish, and/or lobster induced pluripotent cell, wherein the induced pluripotent cell originates from a primary shrimp, prawn, crab, crayfish, and/or lobster muscle and/or fat cell and/or an immortalized shrimp, prawn, crab, crayfish, and/or lobster muscle and/or fat cell.
19. A stably transfected shrimp, prawn, crab, crayfish, and/or lobster induced pluripotent cell, wherein the induced pluripotent cell originates from a pluripotent shrimp, prawn, crab, crayfish, and/or lobster embryonic stem cell, such as a blastocyst stage cell and/or a fertilized egg cell.
20. A cell culture comprising the induced pluripotent cell of claim 18.
21. A cell culture comprising the induced pluripotent cell of claim 19.