

Method for producing milk like products

Field of the invention

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The present invention concerns a method for producing in vitro a mammalian milk like product, for example human milk like product, which comprises generating lactocytes derived from mammalian induced pluripotent stem cells (hiPSC), for example human induced pluripotent stem cells (hiPSC), through
10 culture and differentiation and/or mammary-like gland organoids comprising such lactocytes and expressing the mammalian milk like product, for example the human milk like product from such lactocytes and/or mammary-like gland organoids. The present invention also relates to the mammalian milk like product, for example the human milk like product obtainable from such method.

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Background of the invention

Mammalian and especially human milk is a complex fluid with a multitude of components, each of which may contribute substantially to infant and perhaps
20 maternal health. It is becoming increasingly clear that human breastmilk is the most appropriate source of nutrition at least up to the age of 6 months. Many components of human milk are simply not found or poorly found or less active in cow's milk upon which infant formula manufacture is based. This includes for instance protein lactoferrin, growth factors, long chain polyunsaturated fatty acids
25 or oligosaccharides. Human milk composition has been used as a gold standard to develop current infant formula, despite recent major development in infant formula composition, it is illusory to think that human milk replication will be achieved with current manufacturing processes.

Today the only source of human milk is human donors (breastfeeding mothers). Donation are reported for non-commercial use (human milk biobank) and commercial use. However, this is limited and has strong regulatory, safety and sometime ethical or religious constraints.

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Stem cells were found in mammalian and especially human milk called human breastmilk stem cells (hBSC). hBSCs were shown to be highly plastic and to differentiate in culture into multiple cell types and more importantly into the three lineages required to shape the lobulo-alveolar structure of the human mammary gland (Hassiotou F. et al. Stem Cells. 2012). However, the use of hBSC to produce human breast milk is neither practical nor sustainable, as it requires human donors.

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A technology based on a cell line with stem cell functionality - called induced pluripotent stem cells (iPSC) is known. A reliable two step protocol to generate human mammary like organoids from human iPSC (hiPSC) was developed (Ying Qu et al, Stem Cell Report vol 8, 205-215, February 14th 2017).

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Accordingly, it is an object of the present invention to reproduce expression of mammalian milk, for example human milk in cultured cells. It is also an object of the present invention to prepare customized mammalian milk like product, for example human milk like product, in cultured cells which could be adapted to specific needs of the recipient and/or to produce human milk bioactives to complement existing cow-based solutions for infant nutrition.

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Summary of the invention

The present invention solves the above-mentioned technical problem.

In one aspect, the present invention provides for a method for producing an edible mammalian milk like product comprising:

A') generating lactocytes derived from mammalian induced pluripotent stem cells (miPSC);

5 B') expressing the mammalian milk like product from such mammary-like gland organoids derived from mammalian induced pluripotent stem cells;

In another aspect, the present invention provides for a mammalian milk like product obtainable by a method comprising:

10 A') generating lactocytes derived from mammalian induced pluripotent stem cells (miPSC);

B') expressing the mammalian milk like product from such lactocytes derived from mammalian induced pluripotent stem cells.

15 In another aspect, the present invention provides for a mammalian milk like product obtainable by a method comprising:

A') generating lactocytes derived from mammalian induced pluripotent stem cells (miPSC);

20 B') expressing the mammalian milk like product from such lactocytes derived from mammalian induced pluripotent stem cells;
for use in therapy.

In another aspect, the present invention provides for the use of a mammalian milk like product obtainable by a method comprising:

25 A') generating lactocytes derived from mammalian induced pluripotent stem cells (miPSC);

B') expressing the mammalian milk like product from such lactocytes derived from mammalian induced pluripotent stem cells;

as a therapeutic agent in a subject in need thereof.

In one aspect, the present invention provides for a method for producing an edible human milk like product comprising:

- 5 A) generating lactocytes derived from human induced pluripotent stem cells (hiPSC);
- B) expressing the human milk like product from such mammary-like gland organoids derived from human induced pluripotent stem cells;

10 In another aspect, the present invention provides for a human milk like product obtainable by a method comprising:

- A) generating lactocytes derived from human induced pluripotent stem cells (hiPSC);
- B) expressing the human milk like product from such lactocytes derived from
- 15 human induced pluripotent stem cells.

In another aspect, the present invention provides for a human milk like product obtainable by a method comprising:

- A) generating lactocytes derived from human induced pluripotent stem cells
- 20 (hiPSC);
- B) expressing the human milk like product from such lactocytes derived from human induced pluripotent stem cells;

for use in therapy.

25 In another aspect, the present invention provides for the use of a human milk like product obtainable by a method comprising:

- A) generating lactocytes derived from human induced pluripotent stem cells (hiPSC);

B) expressing the human milk like product from such lactocytes derived from human induced pluripotent stem cells;
as a therapeutic agent in a subject in need thereof.

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Detailed description of the invention

Definitions

10 Within the context of the present invention, the term “in vitro” means performed or taking place in a test tube, culture dish, bioreactor or elsewhere outside a living organism.

Within the context of the present invention, the term “mammalian” identify an
15 animal belonging to the mammalian species, for example human, cow, monkey, camel, sheep, goat etc.

Within the context of the present invention, the terms “lactocytes” or “mammary-like cells” identify secretory epithelial cells expressing CK18 cell marker and derived
20 from mammalian induced pluripotent stem cells (miPSC) and in particular from human induced pluripotent stem cells (hiPSC). Human induced pluripotent stem cells (hiPSC) as used herein are commercially available and may be selected from any suitable hiPSC line. A suitable human induced pluripotent stem cell line in the context of the current invention is e.g., hiPSC line 603, commercially available from
25 Fujifilm Cellular Dynamics, Inc (FCDI), as used according to the invention. Further suitable hiPSCs may be selected as described e.g., in Ying Qu et al., (2017, supra). In one embodiment of the present invention, the hiPSC are not engineered. In one embodiment, they are not engineered to comprise an exogenous nucleic acid

and/or an inducible gene expression system which includes an exogenous nucleic acid, where the inducible gene expression system is configured to express a hormone or a signaling factor. In one embodiment, the exogenous nucleic acid and/or inducible gene expression system which includes an exogenous nucleic acid
5 is promoting the cell differentiation towards lactocytes.

Within the context of the present invention the terms “mammary gland like organoids” or “mammary like organoids” mean a miniaturized and simplified version of a mammary gland which develops into two or three dimensions (2D/3D) and
10 which comprises lactocytes as above defined.

Within the context of the present invention, the terms “human milk like product” and/or “human breast milk like product” indicate an edible product which is expressed by the lactocytes and/or mammary gland like organoids generated
15 according to the process of the present invention. The “human milk like product” according to the present invention is a “standard human milk like product” or a “non-standard human milk like product” as below defined. Non-limiting examples of human milk like products are selected from the group consisting of: supplement, fortifier, human breast milk substitute (or replacer) and ingredient enriched in only
20 one and/or a portion of bioactives, macro- and micronutrients which can be typically found in human breast milk of a well-nourished mother.

In one embodiment of the present invention, the human milk like product’s composition resembles the composition of human breast milk of a well-nourished
25 mother (for example in terms of bioactives, macro and micronutrients and levels thereof). In such embodiment, the human milk like product may also be referred to as “standard human milk like product” and/or as “human breast milk replacer” or “human breast milk substitute”. In such embodiment, the standard human milk like

product according to the present invention comprises at least macro- and micronutrients which can be typically found in human breast milk of a well-nourished mother. In one embodiment, the standard human milk like product according to the present invention comprises: proteins, peptides, lipids (including
5 linoleic acid and alpha-linolenic acid), carbohydrates, Vitamins (including Vitamin A, Vitamin D3, Vitamin E, Vitamin K, Thiamin, Riboflavin, Niacin, Vitamin B6, Vitamin B12, Pantothenic acid, folic acid, Vitamin C and Biotin), minerals (including iron, calcium, phosphorus, magnesium, sodium, chloride, potassium, manganese, iodine, selenium, copper and zinc), choline, myoinositol and L-carnitine. In one
10 embodiment, the standard human milk like product according to the present invention also comprises at least one bioactive selected from the group consisting of: growth factors, cytokines, probiotics, extracellular vesicles (e.g. milk fat globules and or exosomes), bioactives from exosome (for example miRNA) and secretory IgA. In one embodiment, the standard human milk like product according to the present
15 invention is not the product of human breast lactation as occurring in nature.

In another embodiment, the human milk like product according to the present invention can be adapted to specific needs of the infant who will receive it. It may comprise only one and/or a portion of bioactives, macro and micro nutrients which
20 can be typically found in human breast milk of a well-nourished mother. In such embodiment, the human breast milk like product may also be referred to with the term "non-standard human milk like product". In one embodiment, the non-standard human milk like product according to the present invention comprises one or more of the nutrients or bioactives selected from the group consisting of proteins,
25 peptides, lipids (including linoleic acid and alpha-linolenic acid), carbohydrates (including human milk oligosaccharides), Vitamins (including Vitamin A, Vitamin D3, Vitamin E, Vitamin K, Thiamin, Riboflavin, Niacin, Vitamin B6, Vitamin B12, Pantothenic acid, folic acid, Vitamin C and Biotin), minerals (including iron, calcium,

phosphorus, magnesium, sodium, chloride, potassium, manganese, iodine, selenium, copper and zinc), choline, myoinositol, L-carnitine, growth factors, cytokines, probiotics, extracellular vesicles (e.g. milk fat globules and or exosomes), bioactives from exosome (for example miRNA) and secretory IgA.

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Within the context of the present invention, the term “non-modified human milk like product” indicates a human milk like product which is expressed by lactocytes and/or by the mammary gland like organoids generated according to steps A) and B) of the process of the present invention and which is not subject to the further treatment according to optional step C) of the process of present invention. Non-modified human milk like product may comprise both standard and non-standard human milk like products. Non limiting examples of non-standard human milk like products are selected from the group consisting of: supplement, fortifier, and ingredient enriched in only one and/or a portion of bioactives, macro and micro nutrients which can be typically found in human breast milk of a well-nourished mother.

Within the context of the present invention, the term “modified human milk like product” indicates a human milk like product which is expressed by lactocytes and/or by the mammary gland like organoids generated according to steps A) and B) of the process of the present invention and which is subject to the further treatment according to optional step C) of the process of present invention.

Modified human milk like products may comprise both standard and non-standard human milk like products.

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Within the context of the present invention the term “EBs” means “embryoid bodies”.

Within the context of the present invention the term “mEBs” means “MammoCult medium-cultured embryoid bodies”.

Within the context of the present invention the terms “embryoid bodies (EBs)”,
5 “MammoCult medium-cultured embryoid bodies (mEBs)”, “mammospheres”
and/or “spheroids” refer to three-dimensional aggregates formed in suspension by
pluripotent stem cells (PSC) under step A) of the process of the present invention.

The term “infant” in the context of the present invention identifies a child under the
10 age of 12 months, such as under the age of 9 months, particularly under the age of
6 months.

In the context of the present invention the infant may be any term infant or preterm
infant. In an embodiment of the invention, the infant is selected from the group of
15 preterm infants and term infants.

The term “term infant” refers to infants born at term or at a gestational age of 37
weeks or more.

20 The term “preterm infant” refers to infants who are born at a gestational age of less
than 37 weeks.

In the context of the present invention, the term “birth weight” means the first
weight of the fetus or newborn obtained after birth.

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Within the context of the present invention, the term “low birth weight” means a
birth weight of less than 2500 g (up to and including 2499 g).

Within the context of the present invention, the term “very low birth weight” means a birth weight of less than 1500 g (up to and including 1499 g).

Within the context of the present invention, the term “extremely low birth weight”
5 means a birth weight of less than 1000 g (up to and including 999 g).

The term “small for gestational age infant” refers to infants having a birth weight that is more than 2 standard deviations below the mean reference to a birth weight for gestational growth chart or having a birth weight that is less than the 10th
10 percentile of population-based weight data obtained from infants at the same gestational age. The term “small for gestational age infants” includes infants who are small at birth either from a constitutive or genetic origin or, as a consequence of intrauterine growth restriction.

15 Within the context of the present invention, the term “young children” or “toddler” indicates a child between the age of 1 and 3 years.

The term “infant formula” as used herein refers to a nutritional composition intended for infants and as defined in *Codex Alimentarius*, (Codex STAN 72-1981)
20 and Infant Specialities (incl. Food for Special Medical Purpose) as defined in *Codex Alimentarius*, (Codex STAN 72-1981). It also refers to a foodstuff intended for particular nutritional use by infants during the first months of life and satisfying by itself the nutritional requirements of this category of person (Article 2(c) of the European Commission Directive 91/321/EEC 2006/141/EC of 22 December 2006 on
25 infant formulae and follow-on formulae). The infant formulas encompass the starter infant formulas and the follow-up or follow-on formulas. Generally, a starter formula is for infants from birth as breast-milk substitute, and a follow-up or follow-on formula from the 6th month onwards.

The “growing-up milks” (or GUMs) are given from one year onwards. It is generally a milk-based beverage adapted for the specific nutritional needs of young children. They are nutritional compositions used for feeding children from 12 months to 2-3
5 years old in combination with other foods.

Within the context of the present invention, the term “fortifier” refers to a composition which comprises one or more nutrients having a nutritional benefit for infants or young children.

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By the term “milk fortifier”, it is meant any composition used to fortify or supplement either human breast milk, infant formula, growing-up milk or human breast milk fortified with other nutrients. Accordingly, the human milk fortifier of the present invention can be administered after dissolution in human breast milk,
15 infant formula, growing-up milk or human breast milk fortified with other nutrients or otherwise it can be administered as a standalone composition.

When administered as a stand-alone composition, the human milk fortifier of the present invention can be also identified as being a “supplement”. In one
20 embodiment, the milk fortifier of the present invention is a supplement.

By the term “human milk fortifier”, it is meant any composition used to fortify or supplement human breast milk, or human breast milk fortified with other nutrients. The “human milk fortifier” according to the present invention may be
25 intended to be administered to infants who were born preterm, with very low birth weight (VLBW) or with extremely low birth weight (ELBW).

The milk fortifier according to the present invention may be in powder or liquid form.

Milk fortifier compositions having a liquid form presents some particular benefits. For example, liquid formulations might be more convenient if coupled with a packaging that delivers calibrated drops of a certain weight or volume.

In addition, liquid formulations are easier to mix with the compositions to be
5 fortified, whereas the powder ones can, in some cases, form lumps.

Method according to the present invention

The methods according to the invention as defined herein include any of steps A)
10 and B) as defined herein and optional step C) as defined herein.

Step A - Generating lactocytes and/or mammary like organoids from hiPSCs

According to the method of the present invention, mammary like cells and/or organoid structures are generated under step A).

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Such mammary like cells and/or organoid structures can be generated according to any reported method making use of iPSC.

In one embodiment, such mammary like cells and/or organoid structures may be
20 generated according to the procedure described in Ying Qu et al., Stem Cell Reports, Vol.8, 205-215 which is hereby incorporated in its entirety.

More precisely, the methodology described in the above-mentioned scientific publication (herein-below also referred to as "Ying Qu publication", or Ying Qu, et al. (2017)) represents a two-step protocol to generate human mammary like cells
25 and/or organoids from iPSCs.

It preferably includes as a first step (step 1) the differentiation and enrichment of non-neural ectoderm-cell-containing spheres (mEBs/mammospheres) from iPSCs and as a second step (step 2) the generation of mammary like organoids from 10-

day mEBs (mammospheres) using 3D floating mixed gel-culture of Matrigel and Collagen I.

In step 1 differentiation and enrichment of non-neural ectoderm-cell-containing spheres (mEBs/mammospheres) from hiPSCs occurs by culturing hiPSCs in complete MammoCult medium (StemCell Technologies). Complete MammoCult medium is preferably composed of the basal medium, proliferation supplements, heparin (typically 4µg/mL), and hydrocortisone (typically 0.48µg/mL). Medium is usually changed every three days. mEBs (mammospheres) obtained in said step are then enriched for non-neural ectoderm cells.

- 10 In step 2, following the protocol of Ying Qu, et al. (2017), mammary-like organoids are generated by firstly preparing a 3D culture on basis of a floating mixed gel (e.g. Matrigel and Collagen I). 10 day mEBs (mammospheres) are then grown for 5 days in the mixed gel floated in complete EpiCultB medium supplemented with Parathyroid hormone (pTHrP). For induction of branch and alveolar differentiation
- 15 for preparation of mammary like organoids/lactocytes, cells are then cultured in complete EpiCultB medium supplemented with hydrocortisone, insulin, FGF10 and HGF. Milk protein expression is typically induced at day 35 by adding prolactin, hydrocortisone and insulin to complete EpiCultB medium supplemented with BSA (lactogenic medium) and culturing for 5 days. The process of Ying Qu, et al. (2017)
- 20 is typically completed at day 40.

In one embodiment of the present invention, a method for producing a human milk like product is provided comprising generating lactocytes under step A) from human induced pluripotent stem cells (hiPSC), where such step A) comprises:

- 25 i) directing iPSCs to differentiate towards non-neural ectoderm cells by culturing them in an appropriate culture medium (for example MammoCult medium) and after 10 days collecting mammospheres formed thereof; and

ii) growing such mammospheres in an appropriate system (for example a floating mixed gel culture system as described in Hassiotou F. et al. Stem Cells. 2012) for at least 10 days to generate lactocytes.

5 In another embodiment, a method for producing a human milk like product is provided comprising generating lactocytes under step A) from human induced pluripotent stem cells (hiPSC), where such step A) comprises:

- i) directing iPSCs to differentiate towards non-neural ectoderm cells by culturing them in an appropriate culture medium (for example MammoCult medium) in non-
10 adherent conditions for mammospheres formation; and
- ii) growing such mammospheres in a 3D appropriate system (for example a mixed floating gel composed of matrix protein such as Matrigel and/or Collagen or in suspension cultures in non-adherent plates) for at least 10 days to generate lactocytes.

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In one embodiment, mammary commitment under step A) is obtained by applying a conditioned medium (for example EpiCultB) supplemented with specific factors (for example Parathyroid hormone (pThrP), hydrocortisone, insulin, FGF10, and HGF).

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In one embodiment of the present invention, the method comprises generating mammary - like organoids under step A).

In one embodiment of the present invention, the method to generate mammary -
25 like organoids under step A) includes culturing the cells under conditions selected from the group consisting of: 2D monolayers of cells, 2D with attached EBs, in suspension in non-adherent plates and in mixed floating gel.

In a preferred embodiment, mixed floating gel comprises Matrigel and Collagen I.

In another preferred embodiment, mammospheres (mEBs) in step A) are grown in an appropriate system (for example a floating mixed gel culture system as described
5 in Hassiotou F. et al. Stem Cells. 2012) for at least 15 days.

In a more preferred embodiment, mammospheres (mEBs) in step A) are grown in an appropriate system (for example a floating mixed gel culture system as described
10 in Hassiotou F. et al. Stem Cells. 2012) for 20 days.

In one embodiment, the method according to the present invention provides for culture conditions according to step A) [for example under step A)i) and /or under step A)ii)], which are adapted to generate lactocytes derived from human induced pluripotent stem cells (hiPSC) capable to secrete a standard human milk like product.
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In one embodiment, the method according to the present invention provides for culture conditions according to step A) [for example under step A)i) and /or under step A)ii)] which are adapted to generate lactocytes derived from human induced
20 pluripotent stem cells (hiPSC) capable to secrete a non-standard human milk like product.

In a preferred embodiment, a method for producing a human milk like product is provided comprising generating lactocytes under step A) from human induced
25 pluripotent stem cells (hiPSC), where such step A) comprises directing hiPSCs to differentiate towards mammary gland cells (for example lactocytes) in an appropriate 3D culture system (for example 3D-suspension condition) for at least 42 days.

In another preferred embodiment, a method for producing a human milk like product is provided comprising generating lactocytes under step A) from human induced pluripotent stem cells (hiPSC), where such step A) comprises:

- 5 i) directing hiPSCs to differentiate towards non-neural ectoderm cells by culturing them in an appropriate culture medium (for example MammoCult medium) in an appropriate 3D culture system (for example 3D-suspension condition) for at least 12 days (day -2 to day 10), and
- ii) growing the formed mEBs (mammospheres) in an appropriate 3D embedding
10 system (for example a mixed floating gel composed of matrix protein such as Matrigel and/or Collagen I) for at least 30 days, preferably for 32 days, to generate lactocytes.

In a particularly preferred embodiment of the present invention, a method of
15 producing a human milk like product is provided comprising generating lactocytes under step A) from human induced pluripotent stem cells (hiPSCs), wherein step A)i) is defined as follows:

- i) generation of embryoid bodies (EBs) from hiPSCs by incubation in standard iPSC medium E8 (comprising DMEM/F12, L-ascorbic acid-2-phosphate magnesium,
20 sodium selenium, FGF2, insulin, NaHCO₃ and transferrin, TGFβ1 or NODAL as described in Chen et al., Nat Methods, 2011) or mTeSR™ for two days (day -2-day 0), and producing mEBs (mammospheres) highly enriched in non-neural ectodermal cells by incubation of EBs in complete MammoCult medium (StemCell Technologies) comprising the basal medium, proliferation supplement and supplemented with
25 heparin (typically 4μg/mL), and hydrocortisone (typically 0.48μg/mL) for 10 days (day 0-day 10), and wherein
step A)ii) is distinguished into further substeps and comprises the following steps:
ii), iii) and iv):

- ii) incubation of mEBs (mammospheres) in complete EpiCultB medium supplemented with EpiCult proliferation supplement and Parathyroid hormone (pThrP) for 5 days (day 10-day 15),
- iii) promotion of branch and alveolar differentiation and mammary cell specification
- 5 by incubating mEBs (mammospheres) in EpiCultB medium supplemented with EpiCult proliferation supplement, hydrocortisone, insulin, FGF10 and HGF for 20 days (day 15-day 35), and
- iv) induction of milk protein expression by incubating mEBs (mammospheres) in EpiCultB medium supplemented with EpiCult proliferation supplement,
- 10 hydrocortisone, insulin, FBS, prolactin, progesterone and β -estradiol for 7 days (day 35-day 42).

Step iv) preferably leads to differentiation into milk protein expressing cells, particularly lactocytes, and/or mammary like gland organoids.

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In a further particularly preferred embodiment of the present invention, a method of producing a human milk like product is provided comprising generating lactocytes under step A) from human induced pluripotent stem cells (hiPSCs), wherein step A)i) is defined as follows:

- 20 i) generation of embryoid bodies (EBs) from hiPSCs by incubation in standard iPSC medium E8 (comprising DMEM/F12, L-ascorbic acid-2-phosphate magnesium, sodium selenium, FGF2, insulin, NaHCO_3 and transferrin, TGF β 1 or NODAL as described in Chen et al., Nat Methods, 2011) mTeSRTM for two days (day-2-day 0), and producing mEBs (mammospheres) highly enriched in non-neural ectodermal
- 25 cells by incubation of EBs in MammoCultB medium supplemented with MammoCult proliferation supplement, hydrocortisone and heparin for 10 days (day 0-day 10), and wherein step A)ii) is distinguished into further substeps and comprises the following steps ii), iii) and iv):

- ii) embedding the formed mEBs (mammospheres) in a mixture of Matrigel and Collagen I floated in EpiCultB medium supplemented with EpiCult proliferation supplement and Parathyroid hormone (pThrP) for 5 days (day 10-day 15),
- iii) promotion of branch and alveolar differentiation and mammary cell specification
- 5 by incubating embedded mEBs (mammospheres) in EpiCultB medium supplemented with EpiCult proliferation supplement, hydrocortisone, insulin, FGF10 and HGF for 20 days (day 15 to day 35), and
- iv) induction of milk protein expression by incubating mEBs (mammospheres) in EpiCultB medium supplemented with EpiCult proliferation supplement,
- 10 hydrocortisone, insulin, FBS, prolactin, progesterone and β -estradiol for 7 days (day 35 to day 42).

Step iv) preferably leads to differentiation into milk protein expressing cells, particularly lactocytes, and/or mammary like gland organoids.

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Standard iPSC medium E8 (comprising DMEM/F12, L-ascorbic acid-2-phosphate magnesium, sodium selenium, FGF2, insulin, NaHCO₃ and transferrin, TGF β 1 or NODAL as described in Chen et al., Nat Methods, 2011) as mentioned herein is commercially available, e.g., as "Essential 8™ Medium" from ThermoFischer

20 Scientific, catalogue number A1517001 (see also <https://www.thermofisher.com/order/catalog/product/A1517001#/A1517001>).

mTeSR™ medium is commercially available from STEMCELL Technologies, catalogue number 85850 (see also <https://www.stemcell.com/mtesr1.html>). Suchc

25 medium is also described in "Defined, Feeder- Independent medium for human embryonic stem cell culture", Current protocol in Stem Cell Biology, Volume 2, Issue 1, Sept 2007.

In one embodiment, steps iii) and/or iv) as defined above for the particularly preferred embodiments, preferably lead to formation of/differentiation into at least breast cells, luminal cells, and basal cells. In this context, breast cells preferably express one or more, preferably all of markers selected from the group consisting of: β -Casein, milk protein, and hormone receptors. Moreover, luminal cells
5 of: β -Casein, milk protein, and hormone receptors. Moreover, luminal cells preferably express one or more, preferably all markers selected from the group consisting of: EpCAM, MUC1, CD49F, GATA3, CK8, and CK18. Moreover, basal cells preferably express one or more markers selected from the group consisting of: CK14, α -smooth muscle actin and P63.

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In one further embodiment, after induction of mEBs (mammospheres) in step ii) and/or iv) as defined above for the particularly preferred embodiments, mammary like gland organoids may be obtained, that express one or more markers selected from the group consisting of: β -Casein, milk protein, and hormone receptors,
15 luminal cells that express one or more markers selected from the group consisting of: EpCAM, MUC1, CD49F, GATA3, CK8, CK18, and basal cells that express one or more markers selected from the group consisting of: CK14, α -smooth muscle actin and P63.

20 In one embodiment of the invention, the methods above described are provided for producing a standard human milk like product.

In another embodiment, the methods above described are provided for producing a non-standard human milk like product.

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In one embodiment (of step A), delivery of nutrients and biomimetic stimuli is controlled to influence cell growth, differentiation and tissue formation. In one embodiment (of step A), such control is performed in a bioreactor.

Step B - Expressing a human breast milk like product

In one embodiment of the present invention, the methods comprise expressing the human milk like product from mammary like organoids derived from human induced pluripotent stem cells (hiPSCs), preferably prepared according to step A). Expressing human milk like products preferably occurs upon induction of expression of the human milk like product from such lactocytes and/or mammary-like gland organoids.

In one embodiment, lactating lactocytes are induced by applying a specific medium (for example EpiCultB) supplemented with lactogenic factors (for example prolactin, hydrocortisone, and insulin).

Particularly, the human milk like product obtained from mammary like organoids derived from human induced pluripotent stem cells (hiPSCs), preferably prepared according to step A), contains bioactives of human milk, selected from the group comprising or consisting of proteins, lipids or oligosaccharides, preferably human milk oligosaccharides, etc.. Inventors particularly managed to identify with the particularly preferred protocol according to steps A i) to iv) as carried out above inter alia oligosaccharides (including lactose and some HMOs), lipids (including 4 fatty acids), proteins (7 detected including caseins), and miRNA (75 detected, including 11 typically detected in HBM).

In one embodiment, the human milk like product obtained from mammary like organoids derived from human induced pluripotent stem cells (hiPSCs), preferably prepared according to step A), contains bioactives of human milk, selected from the group comprising or consisting of: oligosaccharides, lipids, proteins, exosomes and miRNA.

In another embodiment, human milk like product obtained from mammary like organoids derived from human induced pluripotent stem cells (hiPSCs), preferably prepared according to step A), contains bioactives of human milk, selected from the group comprising or consisting of: lactose, 6'SL, C-4:0 fatty acid, C-8:0 fatty acid, C-10:0 fatty acid, C-14:0 fatty acid, C-15:0 fatty acid, C-16:0 fatty acid, C-16:1n7 fatty acid, C-17:0 fatty acid, C-18:0 fatty acid, C-18:1 n9 fatty acid, C-18:1 fatty acid, C-18:2 n6 fatty acid, C-20:0 fatty acid, C-20:1 n9 fatty acid, C-18:3 n3 fatty acid, C-22:0 fatty acid, lactoferrin, albumin, prolactin, Alpha S1-casein, Hemoglobin subunit beta, Hemoglobin subunit alpha, α -lactalbumin, Alpha-2-macroglobulin, β -casein, bile salt-activated lipase, κ -casein, lactadherin, CD14, fatty acid synthase, IgA, plgR, Serum albumin, Xanthine dehydrogenase, exosomes, miR-21-5p, miR-181a-5p, miR-30d-5p, miR-30b-5p, miR-22-3p, miR-146b-3p, miR-30c-5p, miR-30a-5p, miR-30e-5p and miR-148b-3p.

In one embodiment of the present invention, the human milk like product obtained from mammary like organoids derived human induced pluripotent stem cells (hiPSCs) is a standard human milk product. In another embodiment of the present invention, the human milk like product obtained from mammary like organoids derived from human induced pluripotent stem cells (hiPSCs), is a non -standard human milk product.

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Step C - Further treatments to produce modified human breast milk like product

In one optional embodiment of the present invention, the herein-described methods comprise an additional step C) which is performed on the human milk like product obtainable from step B) and which comprises performing an additional treatment on such product to provide a modified human milk like product.

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In one particular embodiment, the additional treatment step C) performed on the inventive human breast milk like product may be selected from the group consisting

of: a purification step, an isolation process, an extraction process, a fractionation step, an enrichment process, an enzymatic treatment, the addition of further components (for example which can't be expressed by the human mammary gland organoid (such as for example Immunoglobulins, probiotic and/or minerals) or combinations thereof.

Human milk like products

Standard human breast milk like product

10 In one embodiment of the present invention, the human breast milk like product is a standard human breast milk like product.

The benefits of breast feeding are well known in the scientific literature and the possibility to have access to a standard human breast milk like product allows its use
15 for a number of equally well-known health benefits.

In such an embodiment, the standard human breast milk like product can be used as a substitute of breastfeeding under circumstances where real breastfeeding is not possible.

20

In such embodiment, the standard human breast milk like product is intended to be used for example to support longer breastfeeding experience for women who have less milk or who stop to produce milk after 6 months from birth.

Similarly, the standard human breast milk like product is intended to be used for
25 example to allow breastfeeding even under circumstances where sicknesses compromise real breastfeeding from the mother.

In another embodiment, the standard human breast milk like product is intended to be used under circumstances whereby breastmilk production would not naturally be initiated, for example if an infant is adopted.

- 5 In one embodiment, the standard human milk like product according to the present invention is not the product of human breast milk lactation as occurring in nature.

In one embodiment, the standard human breast milk like product is for use in providing optimal nutrition for infant.

- 10 In one embodiment, the standard human breast milk like product is for use in providing healthy growth in infants.

In one embodiment, the standard human breast milk like product is for use in preventing infection, obesity and promoting immunity development in infants.

- 15 In one embodiment, the standard human breast milk like product is a non-modified human breast milk like product.

In another embodiment, the standard human breast milk like product is a modified human breast milk like product.

- 20 In one embodiment, the standard human milk like product according to the present invention comprises: proteins, lipids, carbohydrates, vitamins and minerals.

In another embodiment, the standard human milk like product according to the present invention comprises: proteins, lipids, carbohydrates, vitamins, minerals and bioactives.

- 25 In one embodiment, the standard human milk like product according to the present invention comprises: proteins, lipids (including linoleic acid and alpha-linolenic acid), carbohydrates, Vitamins (including Vitamin A, Vitamin D3, Vitamin E, Vitamin K, Thiamin, Riboflavin, Niacin, Vitamin B6, Vitamin B12, Pantothenic acid, folic acid,

Vitamin C and Biotin), minerals (including iron, calcium, phosphorus, magnesium, sodium, chloride, potassium, manganese, iodine, selenium, copper and zinc), choline, myoinositol and L-carnitine.

In a further embodiment, the standard human milk like product according to the
5 present invention also comprises at least one bioactive selected in the group consisting of: growth factors, cytokines, probiotics, extracellular vesicles (e.g. milk fat globules and or exosomes), bioactives from exosomes (for example miRNA) and secretory IgA.

10 Such standard human breast milk like product may be prepared according to the method of the present invention for example by including a step C) of addition of growth factors, cytokines, probiotics, extracellular vesicles (e.g. milk fat globules and or exosomes), bioactives from exosomes (for example miRNA) and secretory IgA.

15 In one embodiment, the standard human breast milk like product contains probiotics.

Such standard human breast milk like product may be prepared according to the method of the present invention for example by including a step C) of addition of probiotics (for example *B.Lactis*, *B.Infantis*, *L. Ramnhosus*) which can be obtained
20 from several commercially available sources.

In such embodiment, the standard human breast milk like product may be used for optimizing gastro intestinal function and/or promoting Immunity.

In one embodiment, the standard human breast milk like product contains secretory
25 IgA and probiotics.

Such standard human breast milk like product may be prepared according to the method of the present invention for example by including a step C) of addition of a combination of probiotics and secretory IgA which may be prepared as described

for example in patent applications WO2009/156301 and WO2009/156367 which are hereby incorporated by reference. In such embodiment, the standard human breast milk like product may be used for preventing Immunoglobulin deficiency and/or in the prevention of recurrent infection in infants and young children.

5

Non-standard human breast milk like product

In one embodiment, the non-standard human milk like product according to the present invention may be selected from the group consisting of a milk fortifier, a
10 supplement, and/or a human breast milk replacer adapted for special purposes.

Human Milk fortifiers and Human milk bioactive supplements

In one embodiment, the method of the present invention provides for a non-
15 standard human breast milk like product which may be used to fortify human breast milk naturally obtained from a nursing mother or to fortify infant formulas.

In another embodiment, the method of the present invention provides for a non-
standard human breast milk like product which may be used as a supplement for
20 infants or young children in need thereof.

In such embodiments the non-standard human breast milk like products may be used for providing healthy growth and/or to reduce the risk of developing a disease typically associated to specific conditions in an infant or young child (such as for
25 example asthma, allergy, cognitive alterations) and /or to promote catch up growth, development of immunity, protection from infections.

Remarkably, the human origin of the constituents (especially bioactive constituents) in such fortifiers or supplements combined with the fact that they are according to the method of the invention, is supposed to provide to such constituents an intact or higher functionality.

5

The non-standard human breast milk like product is preferably intended to be used as a fortifier. Such non-standard human breast milk like product intended to be used as a fortifier and may be prepared according to the method of the present invention for example by including a step C) of isolation and/or enrichment of (certain) bioactives from the non-modified human breast milk like product obtainable from step B). Such isolation step may be performed via classical fractionation, enrichment and/or purification of the non-modified human breast milk like product obtainable from step B).

15 The non-standard human breast milk like product intended to be used as a supplement may comprise one or more bioactives selected from the group consisting of: human milk oligosaccharides (for example 2FL, 3FL, LNT, LnNT, DiFI, 6SL and /or 3SL), lipids, growth factors (for example epidermal growth factor (EGF), heparin binding epidermal growth factor), cytokines (for example transforming growth factor -beta 2 (TGFbeta-2), IL-1, IL-2, IL-6, IL-10, IL-18, interferon gamma (INF-gamma), TNF-alpha), extracellular vesicles (e.g. milk fat globules and or exosomes), exosome comprising microRNAs and antimicrobial/protecting bioactives (for example IgA, lactoferrin, lysozyme, lactadherine). Such non-standard human breast milk like product intended to be used as a supplement may be prepared according to the method of the present invention for example by including a step C) of isolation of the bioactives from the non-modified human breast milk like product obtainable from step B). Such isolation step may be performed via classical

fractionation, enrichment and/or purification of the non -modified human breast milk like product obtainable from step B).

In one embodiment, the non-standard human breast milk like product is a
5 supplement or milk fortifier which contains fucosylated human milk oligosaccharides, for example 2FL and/or 3FL. Such supplement or milk fortifier is for use in completing the profile of human breast milk of women who do not secrete fucosylated oligosaccharides because of the inactivity of their FUT2 gene.

Such non-standard human breast milk like product intended to be used as a fortifier
10 or supplement may be prepared according to the method of the present invention for example by including a step C) of isolation and/or enrichment of fucosylated oligosaccharides (for example 2FL and or 3FL) from the non-modified human breast milk like product obtainable from step B).

15 In such an embodiment, the non-standard human breast milk like product may be used for optimizing gastro intestinal function and/or promoting Immunity.

Human breast milk like product for infants with genetic diseases

20 In one embodiment, the non-standard human breast milk like product according to the present invention may be adapted to address the specific need of infants who are born with a genetic disease.

Galactossemia

25

In such embodiment, the non-standard human breast milk like product may be adapted to the needs on infants suffering from Galactossemia. Galactossemia is a rare genetic disease that affects babies' ability to metabolize galactose.

In such embodiment, the non-standard human breast milk like product should be deprived of lactose and/or lactose containing saccharides. In such embodiment non-standard human breast milk like product may be used for providing healthy growth
5 to the infants affected by galactossemia.

In one embodiment, a non-standard human breast milk like product deprived of lactose and/or lactose containing saccharides may be obtained according to the method of the present invention by including a step C) of enzymatic treatment
10 (lactase treatment), or of membrane fractionation and ultrafiltration of the non-modified human breast milk like product obtainable from step B).

In another embodiment, a non-standard human breast milk like product deprived of lactose and/or lactose containing saccharides may be obtained according to the
15 method of the present invention by using under step A) GMO alpha-lactalbumin deficient human cells to generate hiPSCs.

Phenyl Keturonia

20 In such an embodiment, the non-standard human breast milk like product may be adapted to the needs on infants suffering from Phenyl Keturonia (PKU). PKU is due to absent or dysfunctional phenylalanine hydroxylase, which converts phenylalanine to tyrosine. Untreated, it leads to severe mental retardation due to brain toxicity.

25 In such an embodiment, the non-standard human breast milk like product should be deprived or depleted of phenylalanine.

In such an embodiment, the non-standard human breast milk like product may be used for providing healthy growth to the infants affected by PKU.

In one embodiment, the non-standard human breast milk like product is depleted of phenyl alanine in such a way that phenylalanine content is kept below 20 mg/kg body weight of the subject receiving it.

- 5 In one embodiment, a non-standard human breast milk like product depleted or deprived of phenylalanine may be obtained according to the method of the present invention by including a step C) of enzymatic treatment (protein hydrolysis) or of filtration of the non -modified human breast milk like product obtainable from step B).

10

In one embodiment, a non-standard human breast milk like product depleted of phenylalanine may be obtained according to the method of the present invention by including a step C) of enzymatic treatment (protein hydrolysis) or of filtration of the non -modified human breast milk like product obtainable from step B).

15

In another embodiment, a non-standard human breast milk like product depleted of phenylalanine may be obtained according to the method of the present invention by providing in step B) a culture medium providing limited or zero amounts of phenylalanine, such as for example a culture medium containing
20 Glycomacropeptide (GMP) from whey.

- It should be appreciated that the various aspects and embodiments of the detailed description as disclosed herein are illustrative of the specific ways to make and use the invention and do not limit the scope of invention when taken into consideration
25 with the claims and the detailed description. It will also be appreciated that features from aspects and embodiments of the invention may be combined with further features from the same or different aspects and embodiments of the invention.

As used in this detailed description and the appended claims, the singular forms “a,” “an” and “the” include plural referents unless the context clearly dictates otherwise.

Additional embodiments of the invention

- 5 i) A method for producing a non standard human milk like product in vitro comprising:
- A) Generating lactocytes mammary-like gland organoids derived from human induced pluripotent stem cells (hiPSC);
- B) Secreting the human milk like product from such lactocytes from human
10 induces pluripotent stem cells.
- ii) A method according to embodiment i) which optionally comprises a step C) whereby the human milk like product of claim 1 is further treated to generate a modified human milk like product.
- 15
- iii) A method for producing a human milk like product according to embodiment i) or ii) wherein culture conditions according to step A) are adapted to generate lactocytes derived from human induced pluripotent stem cells (hiPSC) capable to secrete a non-standard human milk like
20 product.
- iv) A method according to anyone of embodiment ii) or iii) wherein a modified human milk like product is obtained under step C) by adding to the human milk like product of step B) one or more human breast milk
25 components that are not secreted by the lactocytes in step B).

- v) A method according to anyone of embodiments i) to iv) wherein the lactocytes are part of a mammary gland-like organoid structure generated under step A).
- 5 vi) A human milk like product which is obtainable according to the method described in anyone of embodiments i) to v).
- vii) A human milk like product according to embodiment vi) for use in therapy.
- 10 viii) Use of a human breast milk like product according to embodiment vi) as a breast feeding substitute.

Figures

15

Fig. 1: shows the differentiation of human induced pluripotent stem cells (hiPSCs) according to the protocol outlined in Ying Qu and as applied in one alternative in step A) of the inventive methods.

20 **Fig. 2:** shows the differentiation of human induced pluripotent stem cells (hiPSCs) according to the preferred and particularly preferred embodiments for step A) of the inventive methods.

25 **Fig. 3:** shows that three-dimensional organotypic cultures of hiPSCs as produced according to the invention are highly permissive for mammary glands specification. mRNA expression of Nanog, TUBB3, FOXA2, TP63, KR-14, EpCAM, KRT8 and CSN2 for 3D-differentiation (42 days) protocol are shown. Markers from left to right: Stages of Pluripotency (Nanog), Lineage (ectoderm

& endoderm) (TUBB3, FOXA2), Basal-cell/myoepithelial markers (TP63, KR-14), Luminal epithelial markers (EpCAM, KRT8), and milk proteins (CSN2 (Casein Beta)).

- 5 **Fig. 4:** shows the two-dimensional organotypic culture of hiPSCs produced as a comparative example. mRNA expression of Nanog, TUBB3, FOXA2, TP63, KR-14, EpCAM, KRT8 and CSN2 for 2D-differentiation (31 days) protocols are shown. Markers from left to right: Sages of Pluripotency (Nanog), Lineage (ectoderm & endoderm) (TUBB3, FOXA2), Basal-cell/myoepithelial markers (TP63, KR-14), Luminal epithelial markers (EpCAM, KRT8), and milk proteins (CSN2 (Casein Beta)).
- 10

Experimental section**Example 1****Cultivation and differentiation of hiPSCs into lactocytes to obtain a human milk
5 like product**

Lactocytes are cultured starting from ihPSCs according to the procedure described in Ying Qu et al, Stem Cell Report vol 8, 205-215 February 14th 2017 and the human milk like product thereby secreted is collected and can be used in therapy and/or as
10 a breastfeeding substitute according to the present invention.

Example 2**Cultivation and differentiation of hiPSCs into 3D-lactocytes to obtain a human milk
15 like product**

Lactocytes are cultured starting from hiPSCs according to the method of the present invention following steps A) and B) as described above) and the human milk like product thereby secreted is collected and can be used in therapy and/or as a breastfeeding substitute according to the present invention.

20

Example 3**Alternative methods of cultivation and differentiation of hiPSCs into lactocytes to
obtain a human milk like product**

25 Efficient lactocytes differentiation from hiPSCs can be obtained from alternative culture conditions including conditions 1 to 4 as below described:

1. 2D culture on vitronectin coated plates as monolayer of cells derived from the EBs and cultured for at least 28 days in a medium containing (RPMI 1640 with L-glutamine; Fetal bovine serum (FBS); Insulin; Epidermal growth factor (EGF); hydrocortisone; Pen-Strep (penicillin/streptomycin : antibiotic-
30 antimycotic solution).

2. 2D culture on vitronectin coated plates of attached aggregates (EBs) of cells derived from the EBs and cultured for at least 28 days in a medium containing (RPMI 1640 with L-glutamine; Fetal bovine serum (FBS); Insulin; Epidermal growth factor (EGF); hydrocortisone; Pen-Strep (antibiotic-antimycotic solution).
3. 3D culture in suspension in MammoCult medium for at least 10 days and then culture in mixed floating gels (for example Matrigel and Collagen 1) for another 5 days in a specific medium (for example EpiCultB) in presence of Parathyroid hormone followed by 25 days in presence of insulin, HGF, hydrocortisone and FGF10;
4. 3D culture of EBs in suspension (ultra low adherent plate) in MammoCult medium for at least 10 days and then in suspension culture for another 5 days in a specific medium (for example EpiCultB) in presence of Parathyroid hormone followed by 25 days in presence of insulin, HGF, hydrocortisone and FGF10.

Example 4

2D- and 3D-lactocyte differentiation based on human-induced pluripotent stem cell (hiPSC) line 603

(a) 3D-lactocyte differentiation based on human-induced pluripotent stem cell (hiPSC) line 603:

The human-induced pluripotent stem cell (hiPSC) line 603 was used for 3D-lactocyte differentiation. The human-induced pluripotent stem cell (hiPSC) line 603 was purchased from Fujifilm Cellular Dynamics, Inc (FCDI).

(i) For the 3D differentiation protocol (according to the invention), EBs (spheroids) were formed by incubating single cells of hiPSC in E8 medium with 10uM rock inhibitor at 37°C, 5% CO₂ in rotation at 95 rpm overnight.

Second day, medium was replaced with E8 (day -2-day 0).

Next day, medium was replaced with Mammo1 medium (MammoCult - medium with proliferation supplements, heparin (4µg/mL), and hydrocortisone (0.48µg/mL) with penicillin/streptomycin) for 10 days (day 0-day 10).

5 Medium was changed every second day.

(ii) The differentiation was followed by 5 days in Mammo2 medium (EpiCultB + supplements, pThrP 100ng/ml plus penicillin/streptomycin). Culture medium was changed every 3 days (day 10-day 15).

(iii) In order to induce branching epithelial structure, alveolar
10 differentiation and mammary cell specification, mEBs (spheroids/mammospheres) were fed with Mammo3 medium (complete EpiCultB, hydrocortisone (1 µg/ml), insulin (10 µg/ml), FGF10 (50 ng/ml), HGF (50 ng/ml) and penicillin/streptomycin) for 20 days. Medium was changed every 3 days (day 15-day 35).

(iv) Finally, to induce the milk bioactive production (3D), we used
15 the Mammo4 medium (complete EpiCultB, 10% FBS, prolactin (10 µg/ml), hydrocortisone (1 µg/ml), insulin (10 µg/ml), progesterone, β-estradiol and penicillin/streptomycin for 7 days and medium was changed every 3 days (day 35-day 42). During all the differentiation procedure, spheroids were maintained in the suspension culture (rotating at 95 rpm). The differentiation procedure ended at day
20 42. Results are displayed in Figure 3.

(b) 2D-lactocyte differentiation based on human-induced pluripotent stem cell (hiPSC) line 603

The human-induced pluripotent stem cell (hiPSC) line 603 was
25 used also for 2D-lactocyte differentiation. The human-induced pluripotent stem cell (hiPSC) line 603 was purchased from Fujifilm Cellular Dynamics, Inc (FCDI).

For the 2D-differentiation protocol (used for comparison), we used the Lacto medium during all the differentiation stages (RPMI 1640, 20% FBS,

1mM glutamine, 4 µg/ml insulin, 20 ng/ml EGF, 0.5µg/ml hydrocortisone with penicillin/streptomycin). Cells were incubated at 37°C, 5% CO₂. Medium was replaced every second day. Results are displayed in Figure 4.

5 (c) Results

The different differentiation stages during lactocyte derivation were captured using quantitative RT-PCR (Figure 3, 3D-differentiation, Figure 4, 2D-differentiation). In both 2D- and 3D-settings, Nanog expression as a marker for pluripotency is decreased while cells are passing towards the maturation and differentiation. The neuroectodermal and endodermal markers, TUBB3 (Tubulin Beta 3 Class III) and Forkhead box protein A2 (FOXA2) were not expressed significantly in 3D-format and TUBB3 elevation is only captured in 2D-setting. This demonstrates that hiPSCs are patterned towards the non-neural ectodermal lineage, thus enriching mammary progenitors in 3D-format. We investigated the expression pattern of commonly used basal cell/myoepithelial markers, such as p63 (a p53-homologous nuclear protein) and cytokeratin 14 (KRT-14). Both markers are detectable significantly in both systems. Additionally, the epithelial cell adhesion molecule (EpCAM) and cytokeratin 8 (KRT8) were tracked only in the 3D-system and KRT8 was only partially expressed in the 2D-format. Consequently, 3D-platform in an organotypic setting expressed common breast tissue, luminal, and basal markers. Such mammary like organoids express human breast specific proteins including CSN2 (casein beta), milk protein peptides, and hormone receptors. The luminal cells specifically express EpCAM, MUC1, CD49F, GATA3, CK8, and CK18 while basal cells will specifically express CK14, α-smooth muscle actin and P63. Eventually EpCAM and CD49F double positive cells can be detected at an earlier progenitor stage between D10 and D35. Interestingly, CSN2 expression is only captured at the last time point (D42) of the 3D-organotypic system and not in the 2D-directed differentiation platform.

Analysis of the mammary like organoids secretome showed secretion of human milk specific bioactives including oligosaccharides (including lactose and some HMOs), lipids (including 4 fatty acids), proteins (7 detected including caseins), and miRNA (75 detected, including 11 typically detected in HBM) as below described.

Primary cell supernatant was analyzed for presence of lactose or human milk oligosaccharides following the procedure described in "Austin and Benet, Quantitative determination of non-lactose milk oligosaccharides, *Analytica Chimica Acta* 2018, 1010, 86-96" with minor modification. The samples were analysed with UHPLC and detected lactose or human milk oligosaccharides (HMOs) were quantified against a calibration curve of lactose and a mix of 7 HMOs (2'FL, 3FL, DFL, LNT, LNnT, 3'SL and 6'SL). The method had an estimated limit of 0.1mg/L. In the primary cell supernatants, Lactose (0.22 mg/l) and 6'SL (0.32 mg/l) were detected at day 42.

Fatty acids were analysed in media and cell supernatants by gas chromatography coupled with flame ionization detector. Briefly, the supernatants obtained at day 42 is analysed to investigate the presence of fatty acids contained in several lipid classes. A 7890A gas-chromatograph with a 7693 autosampler with preparative station module equipped with a fused-silica CP-Sil 88 capillary column (100% cyanopropylpolysiloxane; 100 m, 0.25 mm id, 0.25 mm film thickness is used with a split injector (1:25 ratio) heated at 250°C and a flame-ionization detector operated at 300°C. Preparation of FAMES (fatty acids methyl esters) is performed by direct transesterification of sample with methanolic chloridric acid. Separation of FAMES is performed using capillary gas chromatography-FID (GC). Identification of FAMES is done by retention time (RT) and comparison with an external standard. Quantification of fatty acids is done by calculation using methyl C11:0 as internal

standard. Transesterification performance of the method is controlled with TAG C13:0 as second internal standard. After addition of internal standards, the solution was mixed with 2 mL of methanol, 2 mL of Methanol/HCl (3N) and 1 mL of hexane. After heating at 100°C/60min, the sample is cooled down to room temperature

5 (about 15 min) and the reaction is stopped by adding 2mL of water. After centrifugation the organic phase is directly injected into the GC.

Fatty acid results from protocol of Example 4a at time day 42 are reported in table 1 (differences observed between media and supernatant).

10 The table 1 below lists the expressed fatty acids in cell supernatant sample.

Fatty acid	Detected amount in cell supernatant (mg/100 mL)
C-4:0	2.53
C-8:0	0.49
C-10:0	0.38
C-14:0	0.44
C-15:0	0.41
C-16:0	1.85
C-16:1n7	0.08
C-17:0	0.09
C-18:0	0.97
C-18:1 n9	18.82
C-18:1	0.28
C-18:2 n6	2.16
C-20:0	0.13
C-20:1 n9	0.11

C-18:3 n3	0.08
C-22:0	0.29
Other fatty acids	1.38

- Proteins in the cell supernatant were analysed using SDS-PAGE profiling and then band isolation for identity confirmation by LC-MSMS. For SDS-PAGE analysis, the total volume of the prepared sample was loaded on the gel. A human milk sample was added for comparison as control. Selected gel regions (bands) were cut to look for human proteins by LC-MSMS. Eventually, bands were submitted to in-gel trypsin digestion and analyzed by LC-MSMS. LC-MSMS data were analyzed with Peaks Studio and matched against the UniProt database for human proteins.
- 10 The table 2 below lists the best candidates for all the excised bands.

Name of expressed proteins in the cell supernatant
Lactoferrin
Albumin
Prolactin
Alpha S1-casein
Hemoglobin subunit beta
Hemoglobin subunit alpha
α -lactalbumin
Alpha-2-macroglobulin
β -casein

bile salt-activated lipase
κ -casein
lactadherin
CD14
fatty acid synthase
IgA
pIgR
Serum albumin
Xanthine dehydrogenase

Exosome isolation and miRNA profiling was performed using ExoQuick polymer nets. ExoQuick polymer works to precipitate exosomes by forming a network and collects all exosomes of a certain size. Once the ExoQuick mesh is formed, a simple, low-speed centrifugation easily precipitates the exosomes as a pellet. The exosomes are intact, ready for protein or RNA analysis and are bioactive for functional studies. Precipitation buffer was added in a ration 0.25X to the sample then vortex. The mix was incubated overnight at 4°C. After incubation, samples were centrifuged 30 min at 1,500xg. The exosome pellet was re-suspended by vertexing in initial volume with Buffer XE (QIAGEN) for QC or Lysis Buffer from HTG EdgeSeq miRNA Whole Transcriptome Assay for miRNA profiling. In order to assess the extracellular vesicles (EVs) isolation, the supernatant was first centrifuged at 3000g for 15 min to remove cell pellet and debris. Then 100 microliters of media was used for an overnight precipitation at 4°C with ExoQuick buffer (ratio 0.25X). EV precipitates were recovered by centrifugation for 30 min at 1500g. Two precipitations were performed for each sample, one EV precipitation was resuspended in Buffer XE (QIAGEN) for potential further analysis, and a second one in only 50 ul HTG Lysis buffer in order to concentrate by 10-fold before miRNA profiling with HTG.

For miRNA profiling, samples were used directly in the first step of lysis. Thus, Whole sample was used directly and was lysed with Plasma lysis buffer in a ratio 1:1. Next, proteinase K (1/10) was added and the samples were incubated 3h at 50°C at 600rpm on Thermomixer. EVs were resuspended in Lysis buffer and lysed in the same conditions, with an incubation step at 95°C for 10min added before the lysis incubation. 26 µl of lysate was process with 70 µl of oil on the HTG processor following the HTG EdgeSeq miRNA Whole Transcriptome Assay V2 procedure. For indexing and amplification libraries, samples were tagged with Illumina adaptors and indexes by PCR with OneTaq® Hot Start 2X Master Mix GC Buffer (95°C - 4 min; 16 cycles: 95°C-15 sec, 56°C-45 sec, 68°C- 45 sec; 68°C10 min; Hold at 4°C) and AMPure cleaned (ratio 2.5) on a robotic liquid handler SciClone NGS WorkStation (Perkin Elmer). Pools were obtained with our custom pooling program on Hamilton robot. The samples were pooled based on GX touch Chip HS quantification. The pools were purified manually a second time with AMPure Bead (ratio 1.8) to remove potential remaining traces of primer-dimer and quantified with Qubit to adjust the final concentration to 2 nM. And as a last step, for MiSeq sequencing, pools were loaded on MiSeq at 20pM with a 5% PhiX spike and sequenced for 50 base Single read on MiSeq with 150V3 kit.

Briefly, 974 miRNAs detected in the in the cell supernatant which more than 75 of them are highly expressed miRNAs in the milk samples.

The table 3 below lists the top ten highly expressed miRNAs.

miRNA name	log2 counts	CV
miR-21-5p	9.76	0.01
miR-181a-5p	9.07	0.03
miR-30d-5p	8.63	0.01
miR-30b-5p	8.63	0.01
miR-22-3p	8.49	0.01
miR-146b-3p	8.40	0.01

miR-30c-5p	8.12	0.04
miR-30a-5p	7.63	0.02
miR-30e-5p	7.26	0.01
miR-148b-3p	6.77	0.04

Our findings provide a novel iPSC-based 3D-organotypic model for studying the regulation and development of normal mammary cell fate and function
5 as well as breast milk bioactives production.

It should be understood that various changes and modifications to the presently preferred embodiments described herein will be apparent to those skilled in the art. Such changes and modifications can be made without departing
10 from the spirit and scope of the present invention and without diminishing its attendant advantages. It is therefore intended that such changes and modifications be covered by the appended claims.

Claims

1. A method for producing a mammalian milk like product comprising:
 - A) Generating lactocytes mammary-like gland organoids derived from
5 mammalian induced pluripotent stem cells (miPSC);
 - B) Secreting the mammalian milk like product from such lactocytes from
mammalian induced pluripotent stem cells (miPSC).
2. A method according to claim 1 for producing a non-standard human milk like
10 product in vitro comprising:
 - A) Generating lactocytes mammary-like gland organoids derived from human
induced pluripotent stem cells (hiPSC);B) Secreting the human milk like product
from such lactocytes from human induces pluripotent stem cells;Wherein such non standard human milk like product comprises one or more of
15 the nutrients or bioactives selected from the group consisting of proteins,
peptides, lipids, carbohydrates, Vitamins, minerals, choline, myoinositol, L-
carnitine, growth factors, cytokines, probiotics, extracellular vesicles, bioactives
from exosome and secretory IgA.
- 20 3. A method according to claim 1 or 2 which optionally comprises a step C)
whereby the human milk like product of claim 1 is further treated to generate a
modified human milk like product.
- 25 4. A method according to claim 3 wherein step C) is selected in the group consisting
of: a purification step, an isolation process, a fractionation step, an enrichment
process, an enzymatic treatment, the addition of further components and
combinations thereof.

5. A method for producing a human milk like product according to claim 2 to 4 wherein culture conditions according to step A) are adapted to generate lactocytes derived from human induced pluripotent stem cells (hiPSC) capable to secrete a non-standard human milk like product.
6. A method according to anyone of claims 3 to 5 wherein a modified human milk like product is obtained under step C) by adding to the human milk like product of step B) one or more human breast milk components that are not secreted by the lactocytes in step B).
7. A method according to anyone of claims 1 to 6 wherein the lactocytes are part of a mammary gland-like organoid structure generated under step A).
8. A human milk like product which is obtainable according to the method described in anyone of claims 1 to 7.
9. A human milk like product according to claim 8 for use in therapy.
10. Use of a human breast milk like product according to claim 8 as a breast-feeding substitute.
11. A method for producing a human milk like product in vitro comprising:
- A) Generating lactocytes mammary-like gland organoids derived from human induced pluripotent stem cells (hiPSC);
 - B) Secreting the human milk like product from such lactocytes from human induces pluripotent stem cells;

wherein such step A) comprises:

- i) directing hiPSCs to differentiate towards non-neural ectoderm cells by culturing them in an appropriate culture medium, for example MammoCult medium, in an appropriate 3D culture system, for example 3D-suspension condition, for at least 12 days and
- ii) growing the formed mEBs (mammospheres) in an appropriate 3D embedding system, for example a mixed floating gel composed of matrix protein such as Matrigel and/or Collagen I for at least 30 days, for example for 32 days, to generate lactocytes.

12. A method for producing a human milk product according to claim 11 wherein step A)i) is defined as follows:

- i) generation of embryoid bodies (EBs) from hiPSCs by incubation in standard iPSC medium E8 comprising DMEM/F12, L-ascorbic acid-2-phosphate magnesium, sodium selenium, FGF2, insulin, NaHCO₃ and transferrin, TGFβ1 or NODAL or in medium mTeSR™ for two days, and producing mEBs (mammospheres) highly enriched in non-neural ectodermal cells by incubation of EBs in complete MammoCult medium comprising the basal medium, proliferation supplement and supplemented with heparin, and hydrocortisone for 10 days, and wherein
- step A)ii) is distinguished into further substeps and comprises the following steps: ii), iii) and iv):
- ii) incubation of mEBs (mammospheres) in complete EpiCultB medium supplemented with EpiCult proliferation supplement and Parathyroid hormone (pThrP) for 5 days ,
- iii) promotion of branch and alveolar differentiation and mammary cell specification by incubating mEBs (mammospheres) in EpiCultB medium supplemented with EpiCult proliferation supplement, hydrocortisone, insulin, FGF10 and HGF for 20, and
- iv) induction of milk protein expression by incubating mEBs (mammospheres) in EpiCultB medium supplemented with EpiCult proliferation supplement, hydrocortisone, insulin, FBS, prolactin, progesterone and β-estradiol for 7.

13. A method for producing a human milk product according to any of claims 11 or 12 wherein step A)i) is defined as follows:

- 5 i) generation of embryoid bodies (EBs) from hiPSCs by incubation in standard iPSC medium E8 comprising DMEM/F12, L-ascorbic acid-2-phosphate magnesium, sodium selenium, FGF2, insulin, NaHCO₃ and transferrin, TGFβ1 or NODAL for two days, and producing mEBs (mammospheres) highly enriched in non-neural ectodermal cells by incubation of EBs in MammoCultB medium supplemented with MammoCult proliferation supplement, hydrocortisone and heparin for 10 days, and wherein step
- 10 A)ii) is distinguished into further substeps and comprises the following steps ii), iii) and iv):
- ii) embedding the formed mEBs (mammospheres) in a mixture of Matrigel and Collagen I floated in EpiCultB medium supplemented with EpiCult proliferation supplement and Parathyroid hormone (pThrP) for 5 days ,
- 15 iii) promotion of branch and alveolar differentiation and mammary cell specification by incubating embedded mEBs (mammospheres) in EpiCultB medium supplemented with EpiCult proliferation supplement, hydrocortisone, insulin, FGF10 and HGF for 20 days, and
- iv) induction of milk protein expression by incubating mEBs (mammospheres) in
- 20 EpiCultB medium supplemented with EpiCult proliferation supplement, hydrocortisone, insulin, FBS, prolactin, progesterone and β-estradiol for 7 days.

14. A method for producing a human milk like product according to anyone of claims 11 to 13 wherein steps iii) and/or iv) lead to formation of/differentiation into at least

25 breast cells, luminal cells, and basal cells.

15. A method for producing a human milk like product according to claim 14 wherein breast cells express one or more markers selected from the group consisting of: β-

Casein, milk protein, and hormone receptors, luminal cells express one or more all markers selected from the group consisting of: EpCAM, MUC1, CD49F, GATA3, CK8, and CK18, basal cells express one or more markers selected from the group consisting of: CK14, α -smooth muscle actin and P63.

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16. A method for producing a human milk like product according to anyone of claims 11 to 13 wherein after induction of mEBs (mammospheres) in step ii) and/or iv) mammary like gland organoids are obtained, that express one or more markers selected from the group consisting of: β -Casein, milk protein, and hormone receptors, comprising luminal cells that express one or more markers selected from the group consisting of: EpCAM, MUC1, CD49F, GATA3, CK8, CK18, and basal cells that express one or more markers selected from the group consisting of: CK14, α -smooth muscle actin and P63.

15 17. A method for producing a human milk like product according to anyone of claims 11 to 16 wherein the human milk like product is a standard human milk product which comprises: proteins, peptides, lipids (including linoleic acid and alpha-linolenic acid), carbohydrates, Vitamins (including Vitamin A, Vitamin D3, Vitamin E, Vitamin K, Thiamin, Riboflavin, Niacin, Vitamin B6, Vitamin B12, Pantothenic acid, folic acid, 20 Vitamin C and Biotin), minerals (including iron, calcium, phosphorus, magnesium, sodium, chloride, potassium, manganese, iodine, selenium, copper and zinc), choline, myoinositol and L-carnitine, and optionally also comprises at least one bioactive selected from the group consisting of: growth factors, cytokines, probiotics, extracellular vesicles (e.g. milk fat globules and or exosomes), bioactives from exosome 25 (for example miRNA) and secretory IgA.

18. A method for producing a human milk like product according to anyone of claims 11 to 16 wherein the human milk like product is a non-standard human milk product

which comprises one or more of the nutrients or bioactives selected from the group consisting of proteins, peptides, lipids, carbohydrates, Vitamins, minerals, choline, myoinositol, L-carnitine, growth factors, cytokines, probiotics, extracellular vesicles, bioactives from exosome and secretory IgA.

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19. A method according to anyone of claims 11 to 18 which comprises a step C) whereby the human milk like product is further treated to generate a modified human milk like product.

10 20. A method according to claim 19 wherein step C) is selected in the group consisting of: a purification step, an isolation process, a fractionation step, an enrichment process, an enzymatic treatment, the addition of further components and combinations thereof.

15 21. A method for producing a human milk like product according to anyone of claims 11 to 20 wherein culture conditions according to step A) are adapted to generate lactocytes derived from human induced pluripotent stem cells (hiPSC) capable to secrete a non-standard human milk like product.

20 22. A method according to anyone of claims 11 to 21 wherein a modified human milk like product is obtained under step C) by adding to the human milk like product of step B) one or more human breast milk components that are not secreted by the lactocytes in step B).

25 23. A method according to anyone of claims 11 to 22 wherein the lactocytes are part of a mammary gland-like organoid structure generated under step A).

24. A human milk like product which is obtainable according to the method described in anyone of claims 11 to 23.

25. A human milk like product according to claim 24 which comprises bioactives from the group comprising or consisting of: lactose, 6'SL, C-4:0 fatty acid, C-8:0 fatty acid, C-10:0 fatty acid, C-14:0 fatty acid, C-15:0 fatty acid, C-16:0 fatty acid, C-16:1n7 fatty acid, C-17:0 fatty acid, C-18:0 fatty acid, C-18:1 n9 fatty acid, C-18:1 fatty acid, C-18:2 n6 fatty acid, C-20:0 fatty acid, C-20:1 n9 fatty acid, C-18:3 n3 fatty acid, C-22:0 fatty acid, lactoferrin, albumin, prolactin, Alpha S1-casein, Hemoglobin subunit beta, Hemoglobin subunit alpha, α -lactalbumin, Alpha-2-macroglobulin, β -casein, bile salt-activated lipase, κ -casein, lactadherin, CD14, fatty acid synthase, IgA, plgR, Serum albumin, Xanthine dehydrogenase, exosomes, miR-21-5p, miR-181a-5p, miR-30d-5p, miR-30b-5p, miR-22-3p, miR-146b-3p, miR-30c-5p, miR-30a-5p, miR-30e-5p and miR-148b-3p.

26. A human milk like product according to claim 24 or 25 which is a standard human milk like product which comprises: proteins, peptides, lipids, carbohydrates, Vitamins, minerals, choline, myoinositol and L-carnitine, and optionally also comprises at least one bioactive selected from the group consisting of: growth factors, cytokines, probiotics, extracellular vesicles, bioactives from exosome (for example miRNA) and secretory IgA.

27. A human milk like product according to claim 24 or 25 which is a non -standard human milk like product which comprises one or more of the nutrients or bioactives selected from the group consisting of proteins, peptides, lipids, carbohydrates, Vitamins, minerals, choline, myoinositol, L-carnitine, growth factors, cytokines, probiotics, extracellular vesicles, bioactives from exosome and secretory IgA.

28. A human milk like product according to anyone of claims 24 to 27 for use in therapy.

29. Use of a human breast milk like product according to anyone of claim 24 to 27 as a breast-feeding substitute.