

Maturation of skeletal muscle cells

Field of the invention

The present invention relates to modified pluripotent cells and to methods of differentiating
5 said cell into skeletal muscle cells.

Background of the invention

According to the most recent United Nations estimations, the current world population is
7.9 billion in July 2022 [<https://www.worldometers.info/es/poblacion-mundial/#ref-1>] and it is
10 expected to reach 10 billion around the year 2056. This increase will be heterogeneously distributed
around the globe, with nine countries covering half the projected growth of the global population in
the next 30 years, including India, Nigeria, Pakistan, Egypt, and the United States of America.
Population and economic growth are major drivers of increased meat consumption. According to
the United Nations Food and Agricultural Organization (FAO, [https://www.oecd-](https://www.oecd-ilibrary.org/agriculture-and-food/oecd-fao-agricultural-outlook-2022-2031_f1b0b29c-en)
15 [ilibrary.org/agriculture-and-food/oecd-fao-agricultural-outlook-2022-2031_f1b0b29c-en](https://www.oecd-ilibrary.org/agriculture-and-food/oecd-fao-agricultural-outlook-2022-2031_f1b0b29c-en)), an
estimated growth of 15% in global meat consumption is projected by 2031. On the other hand, the
correlation between income growth and higher meat consumption is clearly demonstrable at lower
income rates but once consumers reach an adequate standard of living, they become more
sensitive to environmental, ethical, and animal welfare and health concerns.

20 For this reason, there is a growing interest in finding alternative protein sources which
ideally will be sustainable and will contain the nutrients normally provided by meat in the human
diet. Cultured meat arises as another alternative to traditional animal agriculture that aims to
produce the skeletal muscle and adipose tissues that normally comprise animal meats, except using
in vitro tissue and biological engineering techniques. Despite efforts to develop robust protocols for
25 scalable generation of animal cell types from easily accessible and renewable sources, the
differentiation of animal (pluripotent) stem cells into specific cell types often remains cumbersome,
lengthy, and difficult to reproduce and/or has not been established yet.

Skeletal muscle reprogramming of cells derived from livestock was reported for pig
embryonic fibroblasts and porcine induced pluripotent stem cells (Jeong et al, 2021 & Genovese et
30 al, 2017). In both studies skeletal muscle cells were reprogrammed using MyoD1 overexpression.
Lentiviral overexpression of MyoD1 in pig embryonic fibroblasts showed low differentiation
efficiency, long cultivation time and required serum containing media. Induction of 8 days of porcine
induced pluripotent stem cells with MyoD1 resulted in high conversion rate but was dependent on
5-aza-cytidine and CHIR99021 which are not food safe. In conclusion these protocols result in
35 heterogeneous skeletal muscle maturation, lack scalability, are not food safe and have long
cultivation times of up to 14 days.

Accordingly there remains a need in the art for the production and culturing of mature
skeletal muscle cells that are suitable for human consumption and that can be produced in a
scalable and cost effective manner.

Summary of the invention

The invention concerns the expression of the transcriptional regulator proteins, MYOD and MYOG, in a pluripotent stem cell. In a first aspect therefore, the invention relates to a pluripotent stem cell comprising:

- 5 i) an expression construct for expression of a transcriptional regulator protein inserted into a first genetic safe harbour site;
- ii) an expression construct for expression of a MYOD protein, wherein the coding sequence for the MYOD protein is operably linked to an inducible promoter; and,
- iii) an expression construct for expression of a MYOG protein, wherein the coding sequence
10 for the MYOG protein is operably linked to an inducible promoter;
- wherein the expression constructs of ii) and iii) are inserted into at least one further genetic safe harbour site that is not the first genetic safe harbour site,
- and wherein the inducible promotor is regulated by the transcriptional regulator protein.

In certain embodiments of the invention, the expression constructs of ii) and iii) are both
15 inserted into a second genetic safe harbour site that is different from the first genetic safe harbour site. Preferably, said first and further genomic safe harbour sites are selected from any two of the hROSA26 locus, the AAVS1 locus, the CLYBL gene or the CCR5 gene, preferably wherein the genetic safe harbour site are hROSA26 locus and the AAVS1 locus.

In certain embodiments of the invention, the cell is selected from the group consisting of
20 embryonic stem cells, induced pluripotent stem cells, embryonic cell lines, and somatic cell lines.

In certain embodiments of the invention, the pluripotent stem cells are of a livestock or poultry species. Preferably, the livestock species is porcine or bovine, preferably porcine.

In certain embodiments of the invention, the expression construct that is inserted into the second genetic safe harbour site encodes a MYOD protein a linker and a MYOG protein, preferably
25 wherein the linker is a P2A linker, more preferably wherein the linker comprises the sequence of SEQ ID NO: 6. Preferably, the construct comprises the sequence of SEQ ID NO: 8.

In certain embodiments of the invention, the activity of the transcriptional regulator protein is controlled by an exogenously supplied substance derivative. Preferably, the transcriptional regulator protein is selected from the group consisting of: tetracycline responsive transcriptional
30 activator protein (rtTa), Tetracycline repressor (TetR), VgEcR synthetic receptor or a hybrid transcriptional regulator protein comprising a DNA binding domain from the yeast GAL4 protein, a truncated ligand binding domain from the human progesterone receptor and an activation domain from the human NF-kB, preferably the transcriptional regulator protein is rtTA.

In certain embodiments, the inducible promoter includes a Tet Responsive Element (TRE).

35 In certain embodiments, the inducible promotor is a tetON promotor.

In a second aspect the invention provides for a method for the production of skeletal muscle cells, for example type 1 or type 2 muscle, comprising

 a) culturing the pluripotent stem cell according to any one of the preceding claims in a proliferation medium: followed by

b) inducing skeletal muscle cell differentiation by adding the exogenous substance as described herein. In certain embodiments, the differentiation stage of the method as described herein is at most 10 days, at most 9 days, 8 days, 7 days, 6 days, 5 days, 4 days, 3 days or 2 days.

The medium used for proliferation and the medium used for differentiation may be the same
5 medium or different media.

In certain embodiments, the produced skeletal muscle cells are for human and non-human dietary consumption.

In a further aspect, the invention provides for a use of a pluripotent stem cell as described herein or use of the method for producing a skeletal muscle cells as described herein for tissue
10 engineering, optionally for the production of cultured meat.

In yet a further aspect, the invention provides for a food product comprising the pluripotent stem cell as described herein or the skeletal muscle cells obtained by the method as described herein. In certain embodiments, the food product is cultured meat.

15 **Description of the invention**

Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. One skilled in the art will recognize many methods and materials similar or equivalent to those described herein, which could be used in the practice of the present invention. Indeed, the present invention
20 is in no way limited to the method.

In this document and in its claims, the verb "to comprise" and its conjugations is used in its non-limiting sense to mean that items following the word are included, but items not specifically mentioned are not excluded. In addition, reference to an element by the indefinite article "a" or "an" does not exclude the possibility that more than one of the elements is present, unless the context
25 clearly requires that there be one and only one of the elements. The indefinite article "a" or "an" thus usually means "at least one".

As used herein, the term "and/or" indicates that one or more of the stated cases may occur, alone or in combination with at least one of the stated cases, up to with all of the stated cases.

As used herein, with "At least" a particular value means that particular value or more. For
30 example, "at least 2" is understood to be the same as "2 or more" i.e., 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, ... ,etc.

The word "about" or "approximately" when used in association with a numerical value (e.g. about 10) preferably means that the value may be the given value (of 10) more or less 0.1% of the value.

35 The term "heterologous" when used with respect to a nucleic acid (DNA or RNA) or protein refers to a nucleic acid or protein that does not occur naturally as part of the organism, cell, genome or DNA or RNA sequence in which it is present, or that is found in a cell or location or locations in the genome or DNA or RNA sequence that differ from that in which it is found in nature. Heterologous nucleic acids or proteins are not endogenous to the cell into which it is introduced,
40 but has been obtained from another cell or synthetically or recombinantly produced. Generally,

though not necessarily, such nucleic acids encode proteins that are not normally produced by the cell in which the DNA is transcribed or expressed. Similarly exogenous RNA encodes for proteins not normally expressed in the cell in which the exogenous RNA is present. Heterologous nucleic acids and proteins may also be referred to as foreign nucleic acids or proteins. Any nucleic acid or protein that one of skill in the art would recognize as heterologous or foreign to the cell in which it is expressed is herein encompassed by the term heterologous nucleic acid or protein. The term heterologous also applies to non-natural combinations of nucleic acid or amino acid sequences, i.e. combinations where at least two of the combined sequences are foreign with respect to each other.

The terms "expression vector" or "expression construct" refer to nucleotide sequences that are capable of effecting expression of a gene in host cells or host organisms compatible with such sequences. These expression vectors typically include at least suitable transcription regulatory sequences and optionally, 3' transcription termination signals. Additional factors necessary or helpful in effecting expression may also be present, such as expression enhancer elements

As used herein, the term "operably linked" refers to a linkage of polynucleotide elements in a functional relationship. A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a transcription regulatory sequence is operably linked to a coding sequence if it affects the transcription of the coding sequence. Operably linked means that the DNA sequences being linked are typically contiguous and, where necessary to join two protein encoding regions, contiguous and in reading frame. inducible promoter

As used herein, the term "promoter" refers to a nucleic acid fragment that functions to control the transcription of one or more coding sequences, and is located upstream with respect to the direction of transcription of the transcription initiation site of the coding sequence, and is structurally identified by the presence of a binding site for DNA-dependent RNA polymerase, transcription initiation sites and any other DNA sequences, including, but not limited to transcription factor binding sites, repressor and activator protein binding sites, and any other sequences of nucleotides known to one of skill in the art to act directly or indirectly to regulate the amount of transcription from the promoter. A "constitutive" promoter is a promoter that is active in most tissues under most physiological and developmental conditions. An "inducible" promoter is a promoter that is physiologically or developmentally regulated, e.g. by the application of a chemical inducer. In the case of the present invention, the control is effected by the transcriptional regulator protein.

Any reference to nucleotide or amino acid sequences accessible in public sequence databases herein refers to the version of the sequence entry as available on the filing date of this document.

All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

The inventors have surprisingly found that the time period required for differentiation of pluripotent cells into mature skeletal muscle cells can dramatically be reduced by using a modified pluripotent stem cells that comprise an expression construct for expression of a MYOD protein and an expression construct for expression of a MYOG protein or, alternatively, an expression construct which expresses both MYOD and MYOG. As shown in the examples described herein, by using

these modified pluripotent cell lines, full differentiation into mature skeletal muscle cells can be achieved in 6 days or less, for example less than 5 days, for example 4 days or 3 days or more quickly. Apart from significantly reducing the culturing time and the associated cost involved, the use of the herein described pluripotent cells also provides for a more scalable production of mature skeletal muscle cells with more mature skeletal muscle cells being produced as compared to what has been previously described.

The differentiated cells may develop spindle-like elongated morphology, undergo extensive cell fusion and exhibited strong and homogenous myogenic marker expression based on mRNA and protein levels (MHC isoforms/maturity, immunostainings MYOD/MYOG/MYH/TTN/TNNT/PAX3/7), along with high fusion index, length and thickness. Furthermore, spontaneous muscle fiber contractions may be observed, demonstrating the functionality of MYOD/PAX7.

Induction efficiency of the exogeneous compound did not decrease over extended culturing periods, showing robustness and reproducibility of the system (percentage MHC positive cells at different passages).

Accordingly, in a first aspect the invention relates to a pluripotent stem cell comprising:

- i) an expression construct for expression of a transcriptional regulator protein inserted into a first genetic safe harbour site;
- ii) an expression construct for expression of a MYOD protein, wherein the coding sequence for the MYOD protein operably linked to an inducible promoter; and,
- iii) an expression construct for expression of a MYOG protein, wherein the coding sequence for the MYOG protein operably linked to an inducible promoter;

wherein the expression constructs of ii) and iii) are inserted into at least one further genetic safe harbour site that is not the first genetic safe harbour site,

and wherein the inducible promotor is regulated by the transcriptional regulator protein.

A pluripotent stem cell of the invention may optionally comprise no expression constructs other than those set out in i) ii) and iii) above. Alternatively, a pluripotent stem cell of the invention may optionally comprise one or more expression constructs in addition to those set out in i), ii) and iii) above, for example into at least one further genetic safe harbour site that is not the first genetic safe harbour site. Such additional expression constructs may provide for the expression a protein which regulate is a regulator or transcription, for example a transcription factor other than MYOD or MYOG.

In the event that a pluripotent stem cell of the invention is generated which comprises one or more antibiotic resistance markers, one or more of such resistance markers may be removed.

A pluripotent stem cell of the invention may optionally comprise no antibiotic resistance markers.

MYOD, also known as myoblast determination protein 1, is a protein in animals that plays a major role in regulating muscle differentiation. MYOD belongs to a family of proteins known as myogenic regulatory factors. MYOD is one of the earliest markers of myogenic commitment. MYOD is expressed at extremely low and essentially undetectable levels in quiescent satellite cells, but expression of MYOD is activated in response to exercise or muscle tissue damage. The effect of

MYOD on satellite cells is dose-dependent: high MYOD expression represses cell renewal, promotes terminal differentiation and can induce apoptosis. Although MYOD marks myoblast commitment, muscle development is not dramatically ablated in mouse mutants lacking the MYOD gene. This is likely due to functional redundancy from Myf5 and/or Mrf4. Nevertheless, the combination of MYOD and Myf5 is vital to the success of myogenesis. The function of MYOD in development is to commit mesoderm cells to a skeletal myoblast lineage, and then to regulate that continued state. MYOD may also regulate muscle repair. MYOD mRNA levels are also reported to be elevated in aging skeletal muscle. One of the main actions of MYOD is to remove cells from the cell cycle (halt proliferation for terminal cell cycle arrest in differentiated myocytes) by enhancing the transcription of p21 and MYOD. MYOD is inhibited by cyclin dependent kinases (CDKs). CDKs are in turn inhibited by p21. Thus MYOD enhances its own activity in the cell in a feedforward manner. Sustained MYOD expression is necessary for retaining the expression of muscle-related genes. MYOD is also an important effector for the fast-twitch muscle fiber (types IIA, IIX, and IIB) phenotype.

In certain embodiments, the coding sequence for MYOD has the sequence of SEQ ID NO: 1 and the amino acid sequence of SEQ ID NO: 2.

MYOG, or Myogenin, is a transcriptional activator encoded by the *MYOG* gene. Myogenin is a muscle-specific basic-helix-loop-helix (bHLH) transcription factor involved in the coordination of skeletal muscle development or myogenesis and repair. MYOG is a member of the MYOD family of transcription factors.

In mice, MYOG is essential for the development of functional skeletal muscle. MYOG is required for the proper differentiation of most myogenic precursor cells during the process of myogenesis. When the DNA coding for myogenin was knocked out of the mouse genome, severe skeletal muscle defects were observed. Mice lacking both copies of myogenin (homozygous-null) suffer from perinatal lethality due to the lack of mature secondary skeletal muscle fibers throughout the body. In cell culture, myogenin can induce myogenesis in a variety of non-muscle cell types.

In certain embodiments, the coding sequence for MYOG has the sequence of SEQ ID NO: 3 and the amino acid sequence of SEQ ID NO: 4.

In certain embodiments, the pluripotent stem cell as described herein comprises iv) a further expression construct for expression of a PAX7 protein, wherein the coding sequence for the PAX7 protein operably linked to an inducible promoter, wherein the expression constructs of iv) is inserted into at least one further genetic safe harbour site that is not the first genetic safe harbour site.

PAX7, Paired box protein, is a protein that in humans is encoded by the PAX7 gene. Pax-7 plays a role in neural crest development and gastrulation, and it is an important factor in the expression of neural crest markers such as Slug, Sox9, Sox10 and HNK-1. PAX7 is expressed in the palatal shelf of the maxilla, Meckel's cartilage, mesencephalon, nasal cavity, nasal epithelium, nasal capsule and pons. Pax7 is a transcription factor that plays a role in myogenesis through regulation of muscle precursor cells proliferation. It can bind to DNA as a heterodimer with PAX3. Also interacts with PAXBP1; the interaction links PAX7 to a WDR5-containing histone

methytransferase complex By similarity. Interacts with DAXX too. PAX7 functions as a marker for a rare subset of spermatogonial stem cells, specifically a sub set of Asingle spermatogonia. These PAX7+ spermatogonia are rare in adult testis but are much more prevalent in newborns, making up 28% of germ cells in neonate testis. Unlike PAX7+ muscle satellite cells, PAX7+ spermatogonia rapidly proliferate and are not quiescent. PAX7+ spermatogonia are able to give rise to all stages of spermatogenesis and produce motile sperm. However, PAX7 is not required for spermatogenesis, as mice without PAX7+ spermatogonia show no deficits in fertility. PAX7 may also function in the recovery in spermatogenesis. Unlike other spermatogonia, PAX7+ spermatogonia are resistant to radiation and chemotherapy. The surviving PAX7+ spermatogonia are able to increase in number following these therapies and differentiate into the other forms of spermatogonia that did not survive. Additionally, mice lacking PAX7 had delayed recovery of spermatogenesis following exposure to busulfan when compared to control mice.

Accordingly in certain embodiments, the pluripotent stem cell can comprise expression constructs that provide for the expression of proteins MYOD, MYOG and PAX7.

In certain embodiments, the nucleic acid molecules encoding the proteins according to the invention may be codon-optimized for expression in mammalian cells. Methods of codon-optimization are known and have been described previously (e.g. WO 96/09378 for mammalian cells). A sequence is considered codon-optimized if at least one non-preferred codon as compared to a wild-type sequence is replaced by a codon that is more preferred. Herein, a non-preferred codon is a codon that is used less frequently in an organism than another codon coding for the same amino acid, and a codon that is more preferred is a codon that is used more frequently in an organism than a non-preferred codon. The frequency of codon usage for a specific organism can be found in codon frequency tables, such as in <http://www.kazusa.or.jp/codon>. Preferably more than one non-preferred codon, preferably most or all non-preferred codons, are replaced by codons that are more preferred. Preferably the most frequently used codons in an organism are used in a codon-optimized sequence. Replacement by preferred codons generally leads to higher expression.

A transcriptional regulator protein is a protein that bind to DNA, preferably sequence-specifically to a DNA site located in or near a promoter, and either facilitating the binding of the transcription machinery to the promoter, and thus transcription of the DNA sequence (a transcriptional activator) or blocks this process (a transcriptional repressor). Such entities are also known as transcription factors.

The DNA sequence that a transcriptional regulator protein binds to is called a transcription factor-binding site or response element, and these are found in or near the promoter of the regulated DNA sequence.

Transcriptional activator proteins bind to a response element and promote gene expression. Such proteins are preferred in the methods of the present invention for controlling inducible cassette expression.

A genetic safe harbour (GSH) site is a locus within the genome wherein a gene or other genetic material may be inserted without any deleterious effects on the cell or on the inserted

genetic material. Most beneficial is a GSH site in which expression of the inserted gene sequence is not perturbed by any read-through expression from neighboring genes and expression of the inducible cassette minimizes interference with the endogenous transcription program. More formal criteria have been proposed that assist in the determination of whether a particular locus is a GSH site in future (Papapetrou et al, 2011, Nature Biotechnology, 29(1), 73-8. doi: 10.1038/nbt.1717.) These criteria include a site that is (i) 50 kb or more from the 5' end of any gene, (ii) 300 kb or more from any gene related to cancer, (iii) 300 kb or more from any microRNA(miRNA), (iv) located outside a transcription unit and (v) located outside ultra-conserved regions (UCR). It may not be necessary to satisfy all of these proposed criteria, since GSH already identified do not fulfil all of the criteria. It is thought that a suitable GSH will satisfy at least 2, 3, 4 or all of these criteria.

In certain embodiments of the invention, the first and further genomic safe harbour sites are selected from any two of the hROSA26 locus, the AAVS1 locus, the CLYBL gene or the CCR5 gene. In certain embodiments the first and further genomic safe harbour sites are located on chr1: 152,360,840-152,360,859, chr1: 175,942,362 -175,942,381, chr1:231,999,396-231,999,415, chr2: 45,708,354 – 45,708,373; chr8: 68,720,172 – 68,720,191 of the human genome.

In certain embodiments of the invention, the first and further genomic safe harbour sites are selected from any two of the safe harbour sites ROSA26, AAVS1, the CLYBL gene or the CCR5 gene.

Preferably, the genetic safe harbour sites are hROSA26 locus and the AAVS1 locus.

In certain embodiments of the invention, the expression construct for expression of MYOD protein as described herein and the expression construct for expression of a MYOG protein as described herein are both inserted into a second genetic safe harbour site that is different from the first genetic safe harbour site. In certain embodiments, the expression construct that is inserted into the second genetic safe harbour site is capable of expressing both the MYOD protein and the MYOG protein simultaneously.

As used herein, the term "pluripotent stem cells" includes embryonic stem cells, embryo-derived stem cells, induced pluripotent stem cells and somatic cells, regardless of the method by which the pluripotent stem cells are derived. Accordingly, in certain embodiments the pluripotent stem cell is selected from the group consisting of embryonic stem cells, induced pluripotent stem cells, embryonic cell lines, and somatic cell lines. In certain embodiments, the pluripotent stem cells are epiblast-derived stem cells (EpiSC). In certain embodiments, pluripotent stem cells express one or more markers selected from the group consisting of: OCT-4, Sox2, Klf4, c-MYC, Nanog, Lin28, alkaline phosphatase, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81. Exemplary pluripotent stem cells can be generated using, methods known in the art. "Induced pluripotent stem cells" (iPS cells or iPSC) can be produced by protein transduction of reprogramming factors in a somatic cell.

The pluripotent stem cell according to the invention can be from any species. Embryonic stem cells have been successfully derived in, for example, mice, multiple species of non-human primates, and humans, and embryonic stem-like cells have been generated from numerous additional species. Thus, one of skill in the art can generate embryonic stem cells and embryo-

derived stem cells from any species, including but not limited to, human, non-human primates, rodents (mice, rats), ungulates (cows, sheep, etc.), dogs (domestic and wild dogs), cats (domestic and wild cats such as lions, tigers, cheetahs), rabbits, hamsters, gerbils, squirrel, guinea pig, goats, elephants, panda (including giant panda), pigs, raccoon, horse, zebra, marine mammals (dolphin, whales, etc.) and the like.

Similarly, iPS cells can be from any species.

In certain embodiments, the pluripotent stem cell according to the invention, or for use in the invention is an animal cell. In certain embodiments the pluripotent stem cell according to the invention, or for use in the invention if from an edible animal species.

Preferably, the pluripotent stem cell according to the invention, or for use in the invention is from a livestock or poultry animal or a seafood animal. Livestock species include but are not limited to domestic cattle, pigs, sheep, goats, lamb, camels, water buffalo and rabbits.

Preferably, the pluripotent stem cell according to the invention, or for use in the invention is a porcine or a bovine pluripotent stem cell. Most preferably, a porcine pluripotent stem cell. In certain embodiments, the stem cell according to the invention is a porcine epiblast stem cell (pEpiSCs).

Poultry species include but are not limited to domestic chicken, turkeys, ducks, geese and pigeons. In certain embodiments, the cells originate from common game species such as wild deer, gallinaceous fowl, waterfowl and hare. Preferably a pluripotent stem cell according to the invention, or for use in the invention, is not a human cell.

Seafood species include, but are not limited to, fish and shellfish.

Transcriptional repressor proteins bind to a response element and prevent gene expression.

Transcriptional regulator proteins may be activated or deactivated by a number of mechanisms including binding of a substance, interaction with other transcription factors (e.g., homo- or hetero-dimerization) or coregulatory proteins, phosphorylation, and/or methylation. The transcriptional regulator may be controlled by activation or deactivation.

If the transcriptional regulator protein is a transcriptional activator protein, it is preferred that the transcriptional activator protein requires activation. This activation may be through any suitable means, but it is preferred that the transcriptional regulator protein is activated through the addition to the cell of an exogenous substance. The supply of an exogenous substance to the cell can be controlled, and thus the activation of the transcriptional regulator protein can be controlled. Alternatively, an exogenous substance can be supplied in order to deactivate a transcriptional regulator protein, and then supply withdrawn in order to activate the transcriptional regulator protein.

If the transcriptional regulator protein is a transcriptional repressor protein, it is preferred that the transcriptional repressor protein requires deactivation. Thus, a substance is supplied to prevent the transcriptional repressor protein repressing transcription, and thus transcription is permitted.

Any suitable transcriptional regulator protein may be used, preferably one that is activatable or deactivatable. It is preferred that an exogenous substance may be supplied to

control the transcriptional regulator protein. Such transcriptional regulator proteins are also called inducible transcriptional regulator proteins.

Accordingly, in certain embodiments, the pluripotent stem cell according to the invention is controlled by an exogenously supplied substance.

5 In certain embodiments, the exogenously supplied substance is selected from the group consisting of peptides (such as described by Klotzsche, et al; Journal of Biological Chemistry 280.26 (2005): 24591-24599 or Schlicht et al.; Applied and environmental microbiology 72.8 (2006): 5637-5642) or the inducers described in Goeke, et al. Journal of molecular biology 416.1 (2012): 33-45; incorporated herein by reference), an aptamer (such as the RNA aptamer described in Hunsicker
10 et al. "Chemistry & biology 16.2 (2009): 173-180; incorporated herein by reference), tetracycline, and anhydroteracyclin or a derivative thereof. Preferably, the exogenously supplied substance is doxycycline.

In certain embodiments, the transcriptional regulator protein as described herein is selected from the group consisting of tetracycline responsive transcriptional activator protein (rtTa),
15 Tetracycline repressor (TetR), VgEcR synthetic receptor or a hybrid transcriptional regulator protein comprising a DNA binding domain from the yeast GAL4 protein, a truncated ligand binding domain from the human progesterone receptor or an activation domain from the human NF-kB.

Tetracycline-Controlled Transcriptional Activation is a method of inducible gene expression well known in the art where transcription is reversibly turned on or off in the presence of the antibiotic
20 tetracycline or one of its derivatives (e.g. doxycycline which is more stable). In this system, the transcriptional activator protein is tetracycline - responsive transcriptional activator protein (rtTa) or a derivative thereof. The rtTA protein is able to bind to DNA at specific TetO operator sequences. Several repeats of such TetO sequences are placed upstream of a minimal promoter (such as the CMV promoter), which together form a tetracycline response element (TRE). There are two forms
25 of this system, depending on whether the addition of tetracycline or a derivative activates (Tet-On) or deactivates (Tet-Off) the rTA protein.

In a Tet-Off system, tetracycline or a derivative thereof binds rTA and deactivates the rTA, rendering it incapable of binding to TRE sequences, thereby preventing transcription of TRE-controlled genes. The Tet-On system is composed of two components; (1) the constitutively
30 expressed tetracycline – responsive transcriptional activator protein (rtTa) and the rtTa sensitive inducible promoter (Tet Responsive Element, TRE). This may be bound by tetracycline or its more stable derivatives, including doxycycline (dox), resulting in activation of rtTa, allowing it to bind to TRE sequences and inducing expression of TRE-controlled genes. In preferred embodiments of the invention the transcriptional regulator protein is rtTA.

35 If the transcriptional regulator protein is rtTA, then the inducible promoter inserted into the at least one further GSH that is not the first GSH site includes the tetracycline response element (TRE). Thus, in certain embodiments the inducible promoter includes a Tet Responsive Element (TRE).

In some embodiments, where the transcriptional regulator protein is rtTA and includes TRE the exogenously supplied substance is the antibiotic tetracycline or one of its derivatives.

In certain embodiments of the invention, the expression construct that is inserted into the second genetic safe harbour site is a fusion protein that encodes both the MYOD protein and the MYOG protein as described herein. In certain embodiments, expression construct that is inserted into the second genetic safe harbour site encodes a MYOD protein a linker and a MYOG protein, in preferred embodiments, the construct comprises or consists of SEQ ID NO: 8. The MYOD and MYOG may be arranged in either order, i.e. MYOD positioned 3' to MYOG or vice versa.

In certain embodiments, the linker sequence may be a cleavable linker. That is, the linker sequence may comprise a sequence of amino acids which is capable of being cleaved. For example, the linker sequence may comprise a sequence capable of acting as a substrate for an enzyme capable of cleaving peptide bonds--i.e. a cleavage site. Many such cleavage sites are known to and can be employed by the person skilled in the art of molecular biology. In some embodiments, the cleavable linker may comprise an autocleavage site. Autocleavage sites are automatically cleaved without the need for treatment with enzymes. For example the family of 2A self-cleaving peptides, or 2A peptides have been described, which includes 2A peptides P2A, E2A, F2A, and T2A. F2A is derived from foot-and-mouth disease virus; E2A is derived from equine rhinitis A virus; P2A is derived from porcine teschovirus-1 2A; T2A is derived from thosea asigna virus 2A. In certain embodiments, the cleavable linker is thus selected from the group consisting of P2A, E2A, F2A, and T2A.

In some preferred embodiments the expression construct comprises a Picornavirus 2A (P2A) linker. Preferably, the expression construct comprises a linker comprising or consisting of the sequence of SEQ ID NO: 6.

In certain embodiments, the expression construct that is inserted into the second genetic safe harbour site encoding a MYOD protein a linker and a MYOG protein as described herein comprises or consists of the sequence of SEQ ID NO: 8.

In certain embodiments, the inducible promotor that is operably linked to the MYOD protein is different than the inducible promotor that is linked to the MYOG protein. In certain embodiments, the inducible promotor that is operably linked to the MYOD protein is the same as the inducible promotor that is linked to the MYOG protein. Inducible promotors are well-known in the art, examples include but are not limited to CMV, CAG, CBh, PGK, SV40, Ferritin heavy or light chains, etc.

In certain embodiments, the inducible promotor used in the present invention a tetOn promotor. Preferably a 3rd generation TetOn promotor.

Culturing methods

The inventors of present application have surprisingly found that the differentiation time needed to obtain skeletal muscle cells can dramatically be reduced by using the pluripotent cells as described herein. Accordingly, in a further aspect the invention relates to a method for the production of skeletal muscle cells, comprising

a) culturing the pluripotent stem cell as described herein in a proliferation medium: followed by

b) inducing skeletal muscle cell differentiation by adding an exogenous substance as described herein.

In certain embodiments, the method of the invention is an *ex vivo* method.

In certain embodiments, the method is for the production of skeletal muscle cells. Mature
5 skeletal muscle cells are herein defined as skeletal muscle cells which show one or more of multinucleation, formation of proteins that are involved in sarcomere formation (eg. Titin and/or one or more myosin heavy chain isoforms) and ultimately contractile functionality.

In certain embodiments, the method of the invention relates to a method for production of type 1 or type 2 muscle.

10 The proliferation medium and the differentiation medium may have the same composition. The same medium may be used for proliferation and differentiation. That is to say, the same culture system can be used for both proliferation and differentiation phases, for example via a medium change or by only adding an inducer of the opti-ox system within the bioreactor. The addition of an inducer for differentiation to the bioreactor is very attractive, as it minimizes capital investment in
15 equipment, processing times and cell manipulation.

Optionally, proliferation and/or differentiation may be carried out in the absence of insulin and/or retinoic acid.

Optionally, proliferation and/or differentiation may be carried out as a single cell suspension. Alternatively, proliferation and/or differentiation may comprise aggregates.

20 The inventors have surprisingly found that use of the pluripotent cell as described herein obviates the need to culture the cells with a commitment induction step. Normally, when skeletal muscle cells are cultured several culturing phases can be distinguished. The commitment or determination phase involves the formation of myoblasts, which have lost the potential to differentiate into other cell types. Differentiation of myoblasts to skeletal muscle cells is promoted
25 by a highly regulated network of transcription factors chronologically expressed to promote skeletal muscle morphologic and biochemical features such as the fusion of single myoblasts into multinucleated skeletal muscle cells and the formation of the sarcomeres that require expression of muscle specific proteins that are highly abundant and can count up to 55% of protein content of a skeletal muscle cell. The differentiation phase is also divided into four stages: growth arrest, mitotic
30 clonal expansion, early differentiation, and terminal differentiation. Use of the pluripotent stem cells as described herein in the method allows the differentiation of the pluripotent stem cells to mature skeletal muscle cells with a very short (2-4 days) myoblast like cell state and can efficiently differentiate into mature skeletal muscle. This short state is particularly advantageous as it reduces the amount of compounds and small molecules that are normally required to be present in the
35 myoblast proliferation medium. The cells as described herein are may be able to differentiate without the presence of fetal bovine serum (FBS), dexamethasone and EGF or FGF2 which are normally required for satisfactory differentiation. The ability to leave out these compounds from the differentiation medium reduces the medium costs and eases the way to regulatory acceptance.

The method as described herein reduces the differentiation time of the pluripotent cells as
40 described herein to mature skeletal muscle cells dramatically. In certain embodiments, the time to

produce skeletal muscle cells using the method as claimed is at most 10 days, 9 days, 8 days, 7 days, 6 days, 5 days, 4 days, 3 days or 2 days. Using the pluripotent cells as described in the method as described, the inventors have observed a conversion rate of at least 95% by day 4 of culture, meaning that at least 95% of the cells are mature after 4 days of culture. Accordingly, in certain embodiments the time to produce at least 95% mature skeletal muscle cells is at most 4 days.

In a further aspect, the invention provides for skeletal muscle cells, preferably mature skeletal muscle cells, as obtained by the method as described herein.

Culturing the cells as described herein can be performed under so called 2D culturing conditions, which is considered the conventional approach to culturing cells. However, the method as described can also easily be adapted to allow culturing under 3D conditions as shown in the examples below.

3D cell culture is an artificially-created environment which enables cells to grow or interact with their surroundings in three dimensions. In such culture, cells typically form 3D colonies, which may be referred to as "spheroids". The 3D culture approach may more accurately model the cells' in vivo growth and behaviour. The skilled person is readily able to carry out 3D cell culture, for example by taking advantage of any of a number of commercially-available culturing tools. For example, the 3D culture may be carried out using scaffold or scaffold-free techniques. Scaffold-based techniques make use of supports such as solid scaffolds and hydrogels to enable the cells to form a 3D culture. Such scaffolds may aim to mimic the natural extracellular matrix (ECM), which is present in vivo. Scaffold-free techniques dispense with the use of the scaffold on which to grow the cells. Instead, 3D spheroids may be established through the use of, for example, low-adhesion plates, hanging-drop plates, micro-patterned surfaces, rotating bioreactors, magnetic levitation and magnetic 3D bioprinting.

Cells that have been transduced with lentiviral vectors are not considered food safe or not safe for human and non-human dietary consumption. The pluripotent cells as described herein of the method as described herein obviate the need to use lentivirally transduced cells. Accordingly, in certain embodiments, the skeletal muscle cells that are produced according to the method as disclosed herein are suitable for human and non-human dietary consumption. In certain embodiments, the produced skeletal muscle cells can be used in the production of cultured meat for human consumption. Meat according to the invention may be derived from any non-human animal, including any species mentioned herein.

In a further aspect, the invention provides for a use of a pluripotent stem cell as described herein or the skeletal muscle cells obtained by the method as described herein for tissue engineering. In certain aspect, the use is for the production of cultured meat.

In a further aspect, the invention provides for a use of a pluripotent stem cell as described herein or the skeletal muscle cells obtained by the method as described herein for tissue engineering, for example in a method of treatment.

In yet a further aspect, the invention provides for a food product (also referred to as "foodstuff") comprising the pluripotent stem cells as described herein or the skeletal muscle cells

produced and/or obtained by the method as described. In certain embodiments, the food product further comprises an edible composition for human or non-human consumption. The edible composition for human or non-human consumption for example comprises at least one of skeletal muscle cells, mature muscle cells, minerals, synthetic substances, flavoring substances (such as 5 for examples herbs and spices), plant based proteins or proteins from microbial origin such as yeast proteins. Plant based proteins and yeast proteins suitable for the use in food products are known to the skilled person in the art. In certain embodiments, the food product is cultured meat.

In yet a further aspect, the invention provides for a method of producing a food product, the method comprising combining the pluripotent stem cells as described herein or the produced and/or 10 obtained skeletal muscle cells with an edible composition for human consumption or non-human consumption as described herein. In certain embodiments, the food product is cultured meat.

Description of the sequences

Table 1: Sequences

15

SEQ ID NO:	Name
1	MYOD coding sequence: ATGGAGCTGCTGTGCGCCACCGCTCCGCGACGTAGATTTGACGGGCCCCGACGGCTCTCTCTGCAACTTTGCAACAGCGGACGACTTCTATGATGACCCGTGTTTCGACTCCCCGACCTGCGCTTCTTCGAGGACCTGGACCCGCGCCTCGTGACGTGGGCGCGCTCCTAAAGCCCGAGGAACACTCGCACTTCCCTGCCGCAGCGCACCCGGCCCCGGGAGCTCGTGAGGACGAGCATGTGCGCGCGCCCAGCGGGCACCACCAGGCGGGCCGCTGTCTACTGTGGGCCTGC AAGGCGTGCAAACGCAAGACCACTAACGCCGACCGCCGCAAGGCCGCCACCATGCGCGA GCGGCGCCGCTTGAGCAAAGTCAACGAGGCCTTCGAGACTCTCAAGCGCTGCACGTCTA GCAACCCGAATCAGCGGCTGCCAAGGTGGAAATCCTGCGCAACGCCATCCGCTATATC GAAGGCCTGCAGGCGTGCTTCGCGACCAAGGACGCCGCGCCCCCTGGCGCTGCAGCGG CCTTTTACGCGCCTGGCCCGCTGCCCCCGGGCCGAGGCGGAGAGCACTACAGCGGTGA CTCAGACGCATCCAGCCCGCGCTCCAAGTGTTCGACGGCATGATGGATTATAGCGGCC CCCCCGAGCGGTGCCCGGCGGCGGAAGTCTACGACGGCACCTATTACAGCGAGGCGCC CAGCGAACCCCGGCCCGGAAGAATGCTGCGGTGTCGAGCCTCGACTGTCTGTCCAGCA TCGTGGAGCGCATCTCCACCGAGAGCCCCGCCGCGCCCGCGCTTCTGCTGGCGGACAC GCCGCGGGAGTCGTCTCCGGGCCCGCAAGAGGCGGCCCGCGGGAGCGAGGTTCGAGCG CGGCACCCCCACCCCTTCCCCGGACGCCGCCCGCAGTGCCCCGCGAGCGCGAACCCC AACCTATCTACCAGGTGCTCTGA
2	MYOD amino acid sequence MELLSPPLRDV DLTGPDGSLCNFATADDFYDDPCFDSPLRFFEDLDPRLVHVGALLKPEEHS HFPAAAHPAPGAREDEHVRAPSGHHQAGRCLLWACKACKRKTTNADRRKAATMRERRRLSK VNEAFETLKRCTSSNPQNQLPKVEILRNAIRYIEGLQALLRDQDAAPPAAAAFYAPGLPPGR

GGEHYSGDSDASSPRSNCS DGMMDYSGPPSGARRRNCYDGTYYSEAPSEPRPGKNAAVSS LDCLSSIVERISTESPAAPALLADTPRESSPGPQEAAAGSEVERGTPTPSPDAAPQCPASANP NPIYQVL*	
3	MYOG coding sequence
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4	MYOG amino acid sequence
MELYETSPYFYQEPHFYDGENYLPVHLQGFEPGYPYERTELSLSPEARVPLEDKGLGTPEHCP GQCLPWACKVKRKS SVSDRRRAATLREKRRLKVN EAFEALKRSTLLNPNQRLPKVEILRSA IQYIECLQALLSSLNQEERDLRYRGGGGPQGPVSECSSHSASCSPEWGSALF GPNPGDHL LTADPTDAHNLHSLTSIVDSITVEDVAVAFPDETMPN*	
5	GSG-P2A_linker coding sequence
GGATCTGGAGCAACAACTTCTCACTACTCAAACAAGCAGGTGACGTGGAGGAGAATCCC GGCCCC	
6	GSG-P2A_linker amino acid sequence
GSGATNFSLLKQAGDVEENPGP	
7	MYOD-P2A-MYOG construct coding sequence
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8	MYOD-P2A-MYOG construct amino acid sequence
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Description of the figures

Figure 1: EpiSCs-MYOD1 (Left) and EpiSCs-MYOD1-MYOG (right) differentiated for 8 days in 2D monolayer culture. Cultures were stained with Titin and nuclei were visualized with DAPI.

Figure 2: Comparison between EpiSCs-MyoD1 (left) and EpiSCs-MyoD1-MyoG (right) after 5 days 3D suspension differentiation. Aggregates were stained for Titin and nuclei were visualized with DAPI.

Figure 3: Comparison between EpiSCs-MyoD1 (left) and EpiSCs-MyoD1-MyoG (right) after 5 days of 3D suspension differentiation. Zoom in of figure 1, highlighting (white arrow) sarcomere formation indicating full differentiation into functional skeletal muscle cells. The striated pattern is only visible in the EpiSCs-MyoD1-MyoG cell line.

Figure 4: Comparison between EpiSCs-MyoD1 (left) and EpiSCs-MyoD1-MyoG (right) after 8 days 3D suspension differentiation. Aggregates were stained for Titin and nuclei were visualized with DAPI.

- 5 Figure 5: 3D suspension differentiation of EpiSCs-MYOD1-MYOG for 3 (left), 4 (middle) and 5 days (right). Aggregates were fixated and labeled with anti-Titin antibody and with DAPI nucleus staining.

- 10 Figure 6: (A) Comparison between EpiSCs-MyoD1 (left) and EpiSCs-MyoD1-MyoG1 (right) after 7 and 2 days of 3D suspension differentiation showing whole mount staining (outside staining) and a cryosection of the middle of a representative aggregate stained for titin and Dapi. (B) qRT-PCR analysis of MYH3 expression in time during 2D differentiation of EpiSCs-MYOD1. (C) MYH3 expression of EpiSCs-MYOD1 aggregates differentiated for 7 days and EpiSCs-MYOD1-MYOG aggregates differentiated for 2 days. Expression data are normalized for RPL27 expression.

- 15 Figure 7: EpiSCs-MyoD1-MyoG Aggregates were proliferated and then differentiated for 2 days in either bioreactor or shaker flask and afterwards fixated and labeled with anti-Titin and anti-Myogenin antibody and with DAPI nucleus staining.

Examples

- 20 The present invention is further illustrated by the following examples which should not be construed as limiting the scope of the invention.

Materials and Methods

- 25 *Porcine Epiblast-derived Stem Cells (pEpiSCs) differentiation to skeletal muscle cells.*

- Undifferentiated pEpiSCs (Opti-Ox MyoD1 and Opti-Ox MyoD1-MyoG) were grown in N2B27 proliferation media (50% DMEM Ham's F-12 (L0093-500, Biowest), 50% Neurobasal media (21103049, Thermofisher), B27 supplement (17504044, Thermofisher), N2 supplement (17502001, Thermofisher), glutamax (35050061, Thermofisher), 10 mM 2-Mercaptoethanol (31350010, Thermofisher), 0,02µg/mL Activin A (QK001, Q-kine), 0,10 µg/mL FGF2 (QK002, Q-kine), 0,625 µg/mL XAV939 (X3004, Sigma Aldrich) on hESC-qualified geltrex (A1413301, Thermo Scientific) - coated plates. For 2D skeletal muscle differentiation, single cells were obtained after gentle cell dissociation reagent (07174, STEMCELL technologies) treatment and cell number and viability were assessed. Single pEpiSCs were seeded in the corresponding cell culture plates at a density of 100000-200000 cells/cm². After an overnight incubation in proliferation media with 10 µm Rock Inhibitor (Y-27632 (HBF2297, HelloBio), cells were cultivated in DMEM high glucose media or DMEM Ham's F-12 (L0093-500, Biowest) supplemented with 1% Knockout Serum Replacement (10828-028, Thermofisher), and 1x Insulin-Transferrin-Selenium (ITS-G) (41400045, Thermofisher), for the first 4 days of differentiation. On day 4, Knockout Serum Replacement concentration was increased to 15% to initiate maturation of the skeletal muscle cells. Media was

refreshed every two days. Doxycycline (1 µg/mL, D9891, Sigma Aldrich) was added to the differentiation media to activate the Opti-OX system in these cells.

For spheroids or aggregates differentiation experiments, undifferentiated single-pEpiSCs (Opti-Ox MyoD1 and Opti-Ox MyoD1-MyoG) were seeded at 3 million cells/mL in 150 mL shaker flasks containing 12.5 mL of proliferation media (containing 50% DMEM Ham's F-12 (L0093-500, Biowest), 50% Neurobasal media (21103049, Thermofisher), B27 supplement (17504044, Thermofisher), N2 supplement (17502001, Thermofisher), glutamax (35050061, Thermofisher), 10 mM 2-Mercaptoethanol (31350010, Thermofisher), 0,02µg/mL Activin A (QK001, Q-kine), 0,10 µg/mL FGF2 (QK002, Q-kine), 0,625 µg/mL XAV939 (X3004, Sigma Aldrich), 2X % Knockout Serum Replacement (10828-028, Thermofisher) KSR and 10ng/mL FGF2). After two days of aggregate formation media was replaced with skeletal muscle differentiation media as described above and was refreshed every two days until the end of the experiment.

Results

Example 1: Development of an Inducible Transgene Overexpression Method by Dual Genomic Safe Harbor (GSH) Targeting in Animal Cells

The Opti-ox approach to generating cells in which transcription may be controlled as described in WO2018096343A1 was employed in these Examples. This approach involved insertion of an inducible cassette so that transcription of the genetic material within that inducible cassette may be controlled by using a dual genomic safe harbour targeted system.

To explore the myogenic reprogramming potential of OPTi-OX for porcine pluripotent stem cells (pPSCs), we generated MYOD OPTi-OX pPSCs. We sequentially targeted the rTA cassette into the porcine ROSA26 GSH under control of a CAG promoter and a MYOD transgene into the porcine AAVS1 GSH under control of a doxycycline-inducible element. We observed robust and homogeneous inducible transgene expression. Further, we observed that MYOD reprogramming of pPSCs directly generates myotubes after 8 days of expression (Figure 1, left). This finding demonstrated that MYOD can directly reprogram pPSCs into myotubes in 2D cultures.

MYOD and MYOG were selected for use in a combined cellular reprogramming strategy. We designed a targeting vector that has MYOD, a P2A "self-cleaving" peptide linker and MYOG, all in one open reading frame to simultaneously express MYOD and MYOG by doxycycline induction. 2A peptide linkers are well-characterized short peptide linkers of 18-22 amino acids that result in two separate gene products expressed from a single open reading frame due to ribosome skipping during translation. Stable knock-ins in the AAVS1 GSH were selected by incorporation of a puromycin-resistance cassette and selected with puromycin addition to the cell culture medium. Following selection, single pEpiSC cells were plated and clonal cell lines were isolated for outgrowth

and analysis. Incorporation of doxycycline-inducible MYOD-P2A-MYOG was subsequently confirmed using PCR genomic analysis, Sanger sequencing and RT-qPCR. Using the dual GSH-targeting approach, we selected clones that were targeted on both alleles and thus carrying two copies of each of the transgenes, and observed that homozygous targeting of both elements
5 allowed inducible overexpression (data not shown). Importantly, the dual GSH-targeting approach did not affect SCself-renewal or differentiation capacity of pEpiSCs as determined by RT-qPCR (data not shown).

Advantages of using MyoD/MyoG line over MyoD alone is the shorter differentiation time from 8 days to 3 days and increase in the amount of multinucleated skeletal muscle cells indicating
10 improved maturation. This all together, suggest that skeletal muscle cells generated from MyoD/MyoG have a protein profile that is more comparable to native meat.

Example 2: EpiSCs-MYOD-MYOG 2D differentiation into multinucleated skeletal muscle cells

15 pEpiSCs Opti-Ox MYOD and pEpiSCs Opti-Ox MYOD-MYOG were expanded in 3D suspension culture, single celled and seeded in coated plastic wells with 200k cells/cm². One day after attachment doxycycline was added to medium to start the differentiation. Cells were differentiated for 8 days . Both lines showed the formation of titin positive skeletal muscle cells, however MyoD1-MyoG resulted in thicker skeletal muscle cells (Figure 1).

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Example 3: 3D culture of EpiSCs-MYOD and EpiSCs-MYOD-MYOG

For the expansion (proliferation) phase of cell culture in the production of cultivated meat, shakers and/or bioreactors are often used to scale up to large volumes and generate the amounts of cell
25 mass needed for a cultivated meat product at a cost competitive price. Commonly, primary cells are used derived from muscle biopsies. However, primary cultures have limited self-renewal, lose their capacity to differentiate during expansion and are variable in quality between biopsies. Other sources such as ESCs and iPSCs have high expansion capacity but are in an undifferentiated state and differentiation is induced either using small molecules to mimic embryonic development (review
30 Chal et al., 2017 Jun 15;144(12):2104-2122. doi: 10.1242/dev.151035) or via ectopic expression of the transcription factors MYOD1 or PAX7. For meat production, the differentiation of cells into myotubes and subsequently into myofibers is an integral part of the process, which usually occurs in a subsequent separate step. Differentiation of skeletal muscle cells requires often distinct conditions in terms of nutrients and physical environment. The necessary nutrients can be provided
35 by switching from a nutrient rich proliferation medium to a differentiation medium consisting of low serum, but providing the physical environment that the cells require in order to differentiate is more challenging. In addition, the substrate requirements for the proliferation and differentiation phases are typically different in terms of surface chemistry and topography. A particular challenge for skeletal muscle cells in suspension is that skeletal muscle cells need attachment sides to poles, a
40 scaffold, or a surface to form elongated stretched multinucleated myotubes.

In this Example, EpiSCs-MYOD and EpiSCs-MYOD-MYOG were adapted to 3D suspension cell growth and grown as aggregates; EpiSCs expanded in an adherent 6-well cell plate were single celled with Accumax (00-4666-56, ThermoFisher Scientific) according to the manufacturer's instructions and transferred to a 150mL shaker flask in 12,5mL media and a RHO/ROCK pathway inhibitor and thereafter expanded for at least 3 cycles. Media during expansion in 3D was refreshed daily. 3D adapted EpiSCs were subsequently used in shaker and bioreactor experiments.

To explore skeletal muscle differentiation in 3D suspension culture, 0.5 million cells/mL were inoculated in a 150 mL shaker flask containing 12,5 mL of media and EpiSCs-MYOD or EpiSCs-MYOD-MYOG were differentiated using a differentiation protocol. Media was changed every second day. Already after 5 days of differentiation of EpiSCs-MYOD-MYOG, multinucleated skeletal muscle cells were formed with elongated morphology, while in the EpiSCs-MYOD only a round morphology was observed (Figure 2). Both lines produced at this timepoint titin protein as observed with the immunofluorescence staining, however only in EpiSCs-MYOD-MYOG a striated pattern of titin was detected indicating already formation of functional sarcomeres and improved maturation (Figure 3 arrow). Extension of differentiation period to 8 days further matured both EpiSCs lines (Figure 4) in skeletal muscle cells, but aggregates containing EpiSCs-MYOD-MYOG showed much bigger and longer skeletal muscle cells that included many nuclei. In the EpiSCs-MYOD aggregates mainly mono nucleated cells were detected. As we observed matured skeletal muscle cells after 5 days of differentiation with the EpiSCs-MYOD-MYOG aggregates we further decreased culture time to 3 and 4 days and compared this to 5 days of differentiation (figure 5). On all time points we observed the formation of titin positive multinucleated skeletal muscle cells showing that 3 days of differentiation is enough to induce proper formation of skeletal muscle cells from EpiSCs with exogenous expression of MYOD-MYOG. MYH3 is a major contractile protein that is a downstream target of MYOD1. We next determined MYH3 expression at different timepoints during 2D differentiation of EpiSCs-MYOD1 cells (Figure 6B). MYH3 expression increased during differentiation and we detected a 7-fold increase in MYH3 expression between day 2 and day 8. Next we compared MYH3 expression between MyoD and MyoD/MyoG lines at day 7 and day 2 of differentiation respectively (Figure 6C). In both lines we detected similar expression, showing faster upregulation of MYH3 for MyoD/MyoG cells and confirming the results shown in figure 6A. Comparison of cells grown in a bioreactor or shaker flask (Figure 7) reveal that in both culture conditions the formation of titin positive multinucleated skeletal muscle cells and expression of MHC and MyoG can be detected.

The simplified bioprocess shown, where the same culture system can be used for both proliferation and differentiation phases via a medium change or by only adding and inducer of the Opti-ox system within the bioreactor/only adding a inducer for differentiation to the bioreactor is very attractive, as it minimizes capital investment in equipment, processing times and cell manipulation.

Claims

1. A pluripotent stem cell comprising:
 - i) an expression construct for expression of a transcriptional regulator protein inserted into
5 a first genetic safe harbour site;
 - ii) an expression construct for expression of a MYOD protein, wherein the coding sequence for the MYOD protein is operably linked to an inducible promoter; and,
 - iii) an expression construct for expression of a MYOG protein, wherein the coding sequence for the MYOG protein is operably linked to an inducible promoter;
- 10 wherein the expression constructs of ii) and iii) are inserted into at least one further genetic safe harbour site that is not the first genetic safe harbour site, and wherein the inducible promotor is regulated by the transcriptional regulator protein.
2. A pluripotent stem cell according to claim 1, wherein the expression constructs of ii) and
15 iii) are both inserted into a second genetic safe harbour site that is different from the first genetic safe harbour site.
3. The pluripotent stem cell according to claim 1 or 2, wherein the cell is selected from the group consisting of embryonic stem cells, induced pluripotent stem cells, embryonic cell lines, and
20 somatic cell lines.
4. The pluripotent stem cell according to any one of claims 1 -3, wherein the pluripotent stem cells are of a livestock or poultry species.
- 25 5. The pluripotent stem cell according to claim 4, wherein the livestock species is porcine or bovine, preferably porcine.
6. The pluripotent stem cell according to any one of claims 2-5, wherein the expression construct that is inserted into the second genetic safe harbour site encodes a MYOD protein a
30 linker and a MYOG protein, preferably wherein the linker is the P2A linker, more preferably wherein the linker comprises the sequence of SEQ ID NO: 6.
7. The pluripotent stem cell according to claim 6, wherein the construct comprises the sequence of SEQ ID NO: 8.
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8. The pluripotent stem cell according to any one of the preceding claims, wherein the activity of the transcriptional regulator protein is controlled by an exogenously supplied substance derivative.

9. The pluripotent stem cell according to any one of the preceding claims, wherein the transcriptional regulator protein is selected from the group consisting of: tetracycline responsive transcriptional activator protein (rtTa), Tetracycline repressor (TetR), VgEcR synthetic receptor or a hybrid transcriptional regulator protein comprising a DNA binding domain from the yeast GAL4 protein, a truncated ligand binding domain from the human progesterone receptor and an activation domain from the human NF-kB, preferably the transcriptional regulator protein is rtTA.
10. The pluripotent stem cell according to any one of the preceding claims, wherein the inducible promoter includes a Tet Responsive Element (TRE).
11. The pluripotent stem cell according to any one of the preceding claims, wherein the inducible promotor is a tetON promotor.
12. The pluripotent stem cell according to any one of the preceding claims, wherein said first and further genomic safe harbour sites are selected from any two of the hROSA26 locus, the AAVS1 locus, the CLYBL gene or the CCR5 gene, preferably wherein the genetic safe harbour site are hROSA26 locus and the AAVS1 locus.
13. A method for the production of skeletal muscle cells comprising
- a) culturing the pluripotent stem cell according to any one of the preceding claims in a proliferation medium: followed by
- b) inducing skeletal muscle cell differentiation by adding the exogenous substance according to claim 8.
14. The method according to claim 13, wherein the proliferation and differentiation medium have the same composition.
15. The method according to claim 13 or 14, wherein the differentiation stage is at most 10 days, at most 9 days, 8 days, 7 days, 6 days, 5 days, 4 days or 3 days.
16. The method according to any one of claims 13 to 15 wherein the produced skeletal muscle cells are for human and non-human dietary consumption.
17. Use of a pluripotent stem cell according to any one of claims 1 to 12 or use of the method for producing a skeletal muscle cells according to any one of claims 13 to 16 for tissue engineering, optionally for the production of cultured meat.
18. A food product comprising the pluripotent stem cell according to any one of claims 1 to 12 or a skeletal muscle cell obtained by the method according to any one of claims 13 to 16.

19. The food product according to claim 18, wherein the food product is cultured meat