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(54) Title: PARTIAL REPROGRAMMING OF SOMATIC CELLS TO INDUCED TISSUE STEM (ITS) CELLS

(57) Abstract: The present invention relates to a method for producing and expanding Induced Tissue Stem (i TS) cells from differentiated cells of neuroectodermic, endodermic and mesodermic lineages.

Partial reprogramming of somatic cells to Induced Tissue Stem (ITS) cells

FIELD OF THE INVENTION

The present invention provides a method for producing and expanding Induced Tissue Stem (ITS) cells from differentiated cells of neuroectodermic, endodermic and mesodermic lineages.

BACKGROUND OF THE INVENTION

10 The body is made up of about 200 different kinds of specialised cells such as muscle cells, nerve cells, fat cells, live cells etc. All specialised cells originate from stem cells.

A stem cell is a cell that is not yet specialised and which has the capacity to self-renew and to give birth to a variety of different kinds of cells through a process called differentiation.

15 A stem cell that can become every type of cell in the organism is called pluripotent, whilst a stem cell that can become only certain types of cells is called multipotent.

Stem cells are found in the early embryo, the foetus, the placenta, umbilical cord, and in many different tissues of the adult body.

Stem cells are often divided into two groups: tissue specific stem cells (often referred to as adult stem cells) and pluripotent stem cells (including embryonic stem cells and induced pluripotent stem cells).

20 Tissue specific stem cells are undifferentiated cells found in foetal or adult tissues or organs. They are capable of self-renewal. Their differentiation is mainly restricted to forming the cell types of that tissue or organ. The chief role of tissue specific stem cells is to maintain and repair the tissue in which they are found.

25 It now appears that all tissues probably contain adult stem cells. The first adult stem cells were isolated from bone marrow and umbilical cord blood which contain relatively high numbers of stem cells. Adult stem cells were also isolated successfully from the brain, blood, muscle, skin, lung, pancreas and liver, although most tissues contain only a very small numbers of stem cells.

30

In each tissue, adult stem cells are used to produce new mature, differentiated cells in replacement of the cells that die in the natural process of ageing. They may also be activated by disease or injury in order to repair the injured tissue.

Hence, it is desirable to produce large quantities of tissue stem cells for therapeutic application such as tissue reconstruction.

However, due to their small numbers, isolation of tissue specific stem cells, or multipotent stem cells, from the adult body, is difficult. Indeed, the isolation methods of the prior art are highly invasive methods and cannot be used safely on humans for reconstructive surgery.

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Thus, there is still a need in the art for a fast, safe and efficient method for obtaining multipotent stem cells.

SUMMARY OF THE INVENTION

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The inventors have developed a new generation of non-pluripotent stem cells called induced tissue stem cells.

20

Thus, the invention relates to a method for producing an induced tissue stem cell from a somatic cell comprising the steps of:

- a) contacting a somatic cell with at least one reprogramming factor,
- b) cultivating said cell in the presence of feeder cells in order to obtain a partially reprogrammed cell,
- c) isolating said partially reprogrammed cell,
- d) cultivating said partially reprogrammed cell in the absence of feeder cells.

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The invention also relates to a population of induced tissue stem cells obtainable by the method as described above.

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The invention also relates to the use of a population of induced tissue stem cells as defined above for non-therapeutic purposes.

The invention also relates to a population of induced tissue stem cells as defined above for use in a method of treatment.

5 DETAILED DESCRIPTION OF THE INVENTION

In one aspect, the invention relates to a method for producing an induced tissue stem cell from a somatic cell comprising the steps of:

- a) contacting a somatic cell with at least one reprogramming factor,
- 10 b) cultivating said cell in the presence of feeder cells in order to obtain a partially reprogrammed cell,
- c) isolating said partially reprogrammed cell,
- d) cultivating said partially reprogrammed cell in the absence of feeder cells, and optionally in the presence of Leukemia Inhibitory Factor (LIF).

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In one aspect, the invention relates to a method for producing an induced tissue stem cell from a somatic cell comprising the steps of:

- a) contacting a somatic cell with at least one reprogramming factor,
- b) cultivating said cell in the presence of feeder cells for a period of time sufficient for
20 obtaining a multipotent cell,,
- c) isolating said multipotent cell,
- d) cultivating said multipotent cell in the absence of feeder cells, and optionally in the presence of Leukemia Inhibitory Factor (LIF).

- 25 As used herein, the expression “induced tissue stem cell”, or “induced multipotent stem cell”, or “partially reprogrammed cell”, refers to a cell which has been reprogrammed, starting from a differentiated somatic cell, into a multipotent cell. Said induced tissue stem (iTS) cell or induced multipotent (iMS) stem cell has the capacity, under different conditions, to differentiate into more than one differentiated cell type of the germ cell
30 layer from which it originates.

Thus, three main types of iTS or iMS can be produced: endodermic iTS, neuroectodermic iTS and mesodermic iTS.

Typically, an iTS obtained by partial reprogramming of a hepatocyte can be differentiated into hepatocytes, bile duct cells and pancreatic cells, all of which belong to
5 the endodermic lineage.

As used herein, the term “somatic cell” has its general meaning in the art. It refers to a differentiated primary cell.

The somatic cell according to the invention can be from any mammalian species, with
10 non-limiting examples including murine, bovine, simian, porcine, equine, ovine, or human cells.

In a preferred embodiment, said somatic cell is a mouse cell.

In another embodiment, said somatic cell is a human cell.

15

Somatic cells can be isolated from any tissue, including, but not limited to, liver, heart, lung, skin, gut, intestine, muscle and spleen.

In one embodiment of the invention, said somatic cell is selected from the group consisting of, epithelial, endothelial, neuronal, adipose, cardiac, skeletal muscle,
20 immune cells, hepatic, splenic, lung, circulating blood cells, gastrointestinal, renal, and pancreatic cells.

In a preferred embodiment, said somatic cell is a hepatocyte.

As used herein, the expression “feeder cell” refers to cells that serve as a basal layer for
25 pluripotent stem cells and provide secreted factors, extracellular matrix, and cellular contacts for the maintenance of stem cells in the undifferentiated state without losing pluripotency. Feeder cells can be inactivated by gamma irradiation or mitomycin. According to an embodiment of the invention, the feeder fibroblasts may be from the group of fibroblasts, more particularly of human fibroblasts and more particularly of
30 dermis fibroblasts, including dermis fibroblast cell lines. Examples of dermis fibroblast

cell lines include but are not limited to CCD-112SK (Hovatta O, et al. 2003) and 3T3-J2 (Rheinwald JG et al. 1975). In a particular embodiment, dermis fibroblasts are previously treated to stop their proliferation before to be coated in the culture surface. Therefore, dermis fibroblasts may be irradiated or treated with a cell cycle blocking agent such as mitomycin.

As used herein, the term “dermis fibroblast” refers to a population of cells that synthesizes and maintains the extracellular matrix of dermis. Specific markers of dermis fibroblasts include vimentin and FAP (fibroblast activation protein).

In a preferred embodiment of the invention, said feeder cells are inactivated fibroblasts. Typically, when the somatic cell is a mouse cell, said feeder cells are mouse embryonic fibroblasts (MEF).

Typically, when the somatic cell is a human cell, said feeder cells are dermis fibroblasts.

As used herein, the expressions “reprogramming factors”, “reprogramming nuclear factor” and “reprogramming transcription factor” are used interchangeably. They refer to nuclear proteins that, when expressed in a given target cell, independently or in combination, can be used to change the cell’s fate, i.e. to re-program the cell.

Surprisingly, the inventors have discovered that reprogramming factors used in the prior art for obtaining induced pluripotent stem cells (iPS) could be useful in the method of the present invention for obtaining induced tissue stem cells.

For example, reprogramming factors have been identified in document WO2007/069666. Reprogramming factors are described in Takahashi et al Cell 2006 126: 663-76; Takahashi et al. Cell 2007 131-861-72 and Yu et al. Science 2007 318 : 1917.

Reprogramming transcription factors can be of any mammalian origin. Typically, they can be of murine origin or of human origin. Preferably, the reprogramming transcription factors belong to the same species as the target cell which is to be reprogrammed.

Examples of reprogramming factors include, but are not limited to:

- 5 - Oct-3/4 (*Pou5f1*): Oct-3/4 is one of the family of octamer ("Oct") transcription factors, and plays a crucial role in maintaining pluripotency. The absence of Oct-3/4 in Oct-3/4+ cells, such as blastomeres and embryonic stem cells, leads to spontaneous trophoblast differentiation, and presence of Oct-3/4 thus gives rise to the pluripotency and differentiation potential of embryonic stem cells. Exemplary Oct3/4 proteins are the proteins encoded by the murine *Oct3/4* gene (Genbank accession number NM_013633) and the human *Oct3/4* gene (Genbank
10 accession number NM_002701).

- 15 - Factors of the Sox family: The Sox family of genes is associated with maintaining pluripotency similar to Oct-3/4, although it is associated with multipotent and unipotent stem cells in contrast with Oct-3/4, which is exclusively expressed in pluripotent stem cells. While Sox2 was the initial gene used for induction (Takahashi et al Cell 2006 126: 663-76; Takahashi et al. Cell 2007 131-861-72; Yu et al. Science 2007 318 :1917), other genes in the Sox family have been found to work as well in the induction process. Sox1 yields iPS cells with a similar efficiency as Sox2, and genes Sox3, Sox15, and Sox18 also
20 generate iPS cells.
Exemplary sox-2 proteins are the proteins encoded by the murine *Sox2* gene (Genbank accession number NM_011443) and the human *Sox2* gene (Genbank accession number NM_003106).

- 25 - Factors of the Klf family: Klf4 of the Klf family of genes was initially identified as a factor for the generation of mouse iPS cells and was also demonstrated to be a factor for generation of human iPS cells.
Exemplary Klf4 proteins are the proteins encoded by the murine *klf4* gene (Genbank accession number NM_010637) and the human *klf4* gene (Genbank
30 accession number NM_004235).

- Factors of the Myc family: The Myc family of genes contains proto-oncogenes implicated in cancer. *c-myc* was shown to be a factor implicated in the generation of mouse iPS cells and of human iPS cells.
5 Exemplary *c-myc* proteins are the proteins encoded by the murine *c-myc* gene (Genbank accession number NM_010849) and the human *c-myc* gene (Genbank accession number NM_002467).
- The Nanog family: In embryonic stem cells, Nanog, along with Oct-3/4 and
10 Sox2, is necessary in promoting pluripotency.
- LIN28: LIN28 is an mRNA binding protein expressed in embryonic stem cells and embryonic carcinoma cells associated with differentiation and proliferation. Yu et al. demonstrated it is a factor in iPS generation, although it is not
15 mandatory (Yu et al. Science 2007, vol318:1917-20).

In a preferred embodiment said reprogramming factor is selected from the group consisting of Oct-4, KLF4, sox2, and *c-myc*.

- 20 According to the method of the invention, the reprogramming factor can be delivered to the somatic cell by any suitable technique known in the art.

In a preferred embodiment of the invention, the step of contacting a somatic cell with at least one reprogramming factor is performed by a non-integrating viral vector.

25

As used herein, the term “non-integrating viral vector” refers to a viral vector that does not integrate into the host genome. The expression of the gene delivered by the viral vector is temporary.

Non-integrating viral vectors suitable in the method of the invention include adenoviruses, adenovirus associated viruses, herpesviruses, baculoviruses, vaccinia viruses and Sendai virus.

Contrary to integrating viral vectors, which raises the issue of definitive genetic alteration of the iPS cells and the possible aberrant transcription of the reprogramming factor during differentiation., non-integrating vectors are non-teratogenic.

A relatively high incidence of chromosomal aberrations was identified in iPS cells produced by integrative vectors that may affect the differentiation capacity and increase the tumorigenicity of iPS cells. The genetic abnormalities can arise at different stages of iPS cells generation. Moreover, aberrant DNA methylation of CG dinucleotides was observed in iPS cells that were linked to tumorigenicity. These epigenetics changes were shown to contribute to neoplastic potential of iPS cells since cancer-related epigenetic abnormalities have been recently identified during reprogramming in iPS cells. Thus, several aspects of the programming process remain poorly understood, especially in terms of recurrent genetic and epigenetic changes, suggesting that basic research efforts need to be performed in this field concomitant to permanent and rapid transfer to the biomedical applications.

In a preferred embodiment, iPS cells are produced without using integrative viral vectors and therefore do not have tumoral potential development, as demonstrated using the teratoma assay (see Example 2 below).

In a preferred embodiment, adenoviruses are used. These vectors have the advantage that they are easy to produce at high titers and have the capacity to efficiently transduce a wide range of post-mitotic cells.

Most adenoviral vectors are derived from Ad serotype 5, however, Ad vectors have also been generated from other serotypes, including human Ad2, Ad7, and Ad4 and non-human viruses.

In all adenoviral vectors, the E1 and E3 genes were replaced with the transgene and are thus non-replicative and are propagated in cell lines that provide the E1 gene products in

trans, such as the human 293 cell line. Combining the E1 and E3 deletions provides a total cloning capacity of 8.3 kb in one mutant virus.

In order to reduce the number of viruses necessary to reprogram somatic cells the reprogramming factors can be delivered in a single virus using 2A “self-cleaving”
5 peptides, which support efficient polycistronic expression from a single promoter or an internal ribosome entry site (IRES) sequence between two consecutive open reading frames.

Adenoviral vectors encoding up to two, three or four reprogramming factors can be used to generate iTS cells in both embryonic and adult somatic mouse and human cells.

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The step of contacting the somatic cell with at least one reprogramming factor is typically performed by infection of somatic cells with non-integrating viral vectors encoding stem cell-associated genes into an adult, differentiated cell. The multiplicity of infection (MOI) used for the reprogramming of the host cell is dependant on the cell
15 type. It is therefore important to test a variety of MOI ranges for each cell type under the desired culture conditions in order to optimize the conditions of infection. A non-integrating viral vector encoding the Green Fluorescence protein (GFP) is usually used and the efficiency of the infection is achieved after 96 hours by the quantification of GFP positive cells by flow cytometry.

20

In another embodiment, the step of contacting the somatic cell with at least one reprogramming factor is performed by episomal vector or mRNA transfection. These techniques, like the non-integrating viral vectors, limit the risks of teratogenicity.

25 In one embodiment of the invention, small molecules can be added during step a) in order to enhance reprogramming efficiency such as valproic acid, butyrate acid or ascorbic acid.

According to one embodiment of the invention, the method for producing iTS cells further comprises the step of selecting the partially reprogrammed cell for the absence of a stem cell marker.

Typically, the stem cell marker is SSEA1 for murine cells.

- 5 Typically, the stem cell marker is selected from the group consisting of SSEA3, SSEA4, TRA-1-60 and TRA-1-80 for human cells.

This negative selection step can be performed by any method known in the art. These methods include transcriptomic approaches such as Taqman Low Density Array
10 (TLDA), and proteomic approaches, such as immunofluorescence, Western blot, ELISA or Fluorescence-Associated Cell Sorting (FACS).

In a preferred embodiment, murine cells are selected if they do not express SSEA1.

According to one embodiment of the invention, human cells are selected if they do not express TRA1-60. According to another embodiment, human cells are selected if they
15 do not express TRA1-81. According to a preferred embodiment, human cells are selected if they do not express TRA-1-60 or TRA1-81.

According to one embodiment of the invention, the method can further comprise a step of selecting the partially reprogrammed cell for the presence of one or more foetal
20 progenitor markers committed toward a specific lineage.

As used herein, the expression “foetal progenitor markers committed toward a specific lineage” or “lineage-specific markers” refers to genes that are expressed by multipotent cells of a given lineage. According to the tissue from which the somatic cell was taken, said “lineage-specific markers” can be endodermic markers or neurectodermic markers
25 or mesodermic markers.

Typically, endodermic markers include, but are not limited to, Foxa2, Gata4, Afp, Sox17, CER, CXCR4 and Fn1.

Typically, neurectodermic markers include, but are not limited to, Sox1, Sox2, Sox3.

Typically, mesodermic markers include, but are not limited to, brachyury and flk1.

According to one embodiment of the invention, the method further comprises the step of selecting the partially reprogrammed cell for the absence of markers of differentiated mature cells.

Typically, markers of differentiated mature cells can be markers of the somatic cell from which the partially reprogrammed cell was obtained. Typically, when a hepatocyte was used as a somatic cell, the partially reprogrammed cell can be selected for the absence of alpha-anti-trypsin and/or albumine.

The invention also relates to a substantially pure population of induced tissue stem cells obtainable by the method as described above.

The expression “substantially pure homogenous population”, as used herein, refers to a population of cells wherein the majority (e.g., at least about 80%, preferably at least about 90%, more preferably at least about 95%) of the total number of cells have the specified characteristics of the iTS of interest.

As used herein, the term “isolated” refers to a cell or a population of cells which has been separated from at least some components of its natural environment.

In one embodiment, the somatic cell is patient-specific.

In this embodiment, the method of the invention can be used to generate patient-specific iTS cells.

For instance, iTS cells can be obtained from patients affected with monogenic and multigenic diseases or inherited and acquired diseases such as Fanconi anemia (Raya et al. Nature 2009, 460: 53-59); adenosine desaminase immune deficit (ADA-SCID), Gaucher’s disease, muscular dystrophy, Parkinson’s disease, Huntington’s disease, Alzheimer, Wilson disease, hemochromatosis and iron overload disorders, alpha-one antitrypsin and ornithine carbamoyl transferase deficiencies, type 1 or type 2 diabetes and cancer.

Patient-specific iTS cells represent a valuable model to study diseases.

Moreover they are of great interest in regenerative therapy, since they represent a potentially unlimited source of autogenic cells that can be expanded and further re-differentiated into any given tissue.

5 Advantageously, the method of the present invention leads to the production of large amounts of multipotent cells which can then be differentiated into a number of different cell types.

Similar advantages were obtained in the past using embryonic stem (ES) cell lines and/or induced pluripotent stem (iPS) cells.

10

ES cells are pluripotent stem cells derived from the inner cell mass of embryos at the blastocyst stage. They are a valuable tool for the study of developmental processes and represent a potentially unlimited source of material for tissue regeneration. However, their use raises ethical issues which are still not resolved.

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iPS cells are similar to embryonic stem (ES) cells, and open new ways for the study of developmental processes, the modeling of human diseases and for the design of regenerative medical strategies including drug testing.

iPS cells are functionally and molecularly highly similar to ES cells and share similar
20 cell surface markers. Like ES cells, iPS cells produce and express on their cell surface one or more of the following cell surface antigens: SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, alkaline phosphatase, SCA-1, hTERT and Nanog.

Nevertheless, in contrast to ES cells, the benefits of iPS cells could be jeopardized by safety concerns such as their tumorigenicity for several reasons:

25 In the first place, a major limitation of this technology is the use of integrating viral vectors, which raises the issue of definitive genetic alteration of the iPS cells and the possible aberrant transcription of the reprogramming factor during differentiation.

Secondly, a relatively high incidence of chromosomal aberrations was identified in iPS cells produced by integrative vectors that may affect the differentiation capacity and

increase the tumorigenicity of iPS cells. The genetic abnormalities can arise at different stages of iPS cells generation.

Thirdly, aberrant DNA methylation of CG dinucleotides was observed in iPS cells that were linked to tumorigenicity. These epigenetic changes were shown to contribute to neoplastic potential of iPS cells since cancer-related epigenetic abnormalities have been recently identified during reprogramming in iPS cells and partially reprogrammed cells including cancer-specific gene promoter DNA methylation alterations that can persist in iPS clones.

Based on the above mentioned motives, iPS cells that were produced with integrating vectors cannot be administrated safely to patients

The population of iTS cells according to the invention may be suitable for a variety of both non-therapeutic and therapeutic applications.

The inventors have found that, unexpectedly, it was possible to obtain partially reprogrammed cells from somatic cells, using the same reprogramming factors as those used for obtaining induced pluripotent stem cells. Further, they have discovered that this partially reprogrammed state corresponds to a dedifferentiated multipotent and foetal state, where the cells are capable of differentiating into several cell types of a given lineage.

Without wishing to be bound by theory, it is believed that this partially reprogrammed state is an intermediate stage in the reprogramming phenomenon which brings the cells back to a multipotent, self renewing state that had never been identified in the prior art.

The step of isolating said partially reprogrammed cells enables to “capture” the cells in a partially reprogrammed state, rather than carrying out the reprogramming all the way back to pluripotent state.

Advantageously, the differentiation of iTS to specific lineages is less time consuming and costly compared to the multiple steps used for the differentiation of iPS cells that are cumbersome and costly. Moreover, the iTS-derived cells are produced with higher purity.

Therefore, the method according to the present invention represents an easy way to produce tissue specific stem cells and their derived lineages for drug and toxicity assays, and for further therapeutic applications.

5 Accordingly, the invention also relates to the use of a substantially pure population of iTS cells as defined above for non-therapeutic methods, such as patient-specific disease modelling, study of molecular mechanisms underlying the pathogenesis, drug screening, predictive toxicity testing. Typically, said non-therapeutic methods can include screening methods for compounds that are involved in differentiation pathways.

10

Tissue specific precursor cell lines have been described in the art. For instance, D2 discloses a mouse pancreatic stem cell line, HN-5, and a human cell line PANC-1. These cell lines are epithelial carcinoma derived from human and mouse pancreas respectively, which are therefore immortalized. They have major genetic perturbations with 63
15 chromosomes. In contrast, iTS cells according to the invention are not immortalized cells but have the property of self-renewal since: 1) they highly express the receptor of leukemia inhibitory factor (LIF). The LIF is preferably added daily to the growth medium allowing iTS cells in vitro to continue proliferating without differentiating. The binding of LIF on its receptor triggers the activation of the latent transcription factor
20 STAT3, a necessary event in vitro for the continued proliferation of mouse iTS. 2) As embryonic stem cells, when cultured in the absence of LIF, iTS cells differentiate spontaneously, forming three-dimensional (3D) aggregates called embryoid bodies. 3) As embryonic stem cells, iTS can proliferate and differentiate spontaneously in vivo when injected intra-muscularly into NOD/SCID mice. However, in contrast to
25 embryonic stem cells, iTS cells according to the present invention are not pluripotent. Their differentiation into somatic cells is faster.

Sandt et al. (PLoS ONE, 2012, vol 7, p1-7) have recently described an infrared microspectroscopy technique which allows to follow the spectral modification of
30 somatic cells during the reprogramming into iPS cells. Using this technique, iTS cells

according to the present invention can be distinguished from somatic cells (fully differentiated cells) on the one hand, and from iPS cells (fully undifferentiated) on the other hand.

- 5 In another aspect, the invention also relates to a pharmaceutical composition comprising a substantially pure population of iTS cells as defined above and optionally a pharmaceutically acceptable carrier or excipient.

As used herein, the term “pharmaceutically acceptable carrier or excipient” refers to a carrier medium which does not interfere with the effectiveness of the biological activity of the progenitor cells, and which is not excessively toxic to the host at the concentrations at which it is administered. Examples of suitable pharmaceutically acceptable carriers or excipients include, but are not limited to, water, salt solution (e.g., Ringer’s solution), oils, gelatines, carbohydrates (e.g., lactose, amylose or starch), fatty acid esters, hydroxymethylcellulose, and polyvinyl pyrrolone. Pharmaceutical compositions may be formulated as liquids, semi-liquids (e.g., gels) or solids (e.g., matrix, lattices, scaffolds, and the like).

Further, the invention also relates to a substantially pure population of iTS cells as defined above or to a pharmaceutical composition as defined above for use in a method of treatment.

, such as; patient-specific disease modelling, study of molecular mechanisms underlying the pathogenesis, drug screening, predictive toxicity testing

The invention also relates to a method for treating a subject comprising the step of administering to said subject an efficient amount of a substantially pure population of iTS cells as defined above.

As used herein, the term “subject” refers to a mammal, preferably a human being, that can suffer from pathology associated with skin damage, but may or may not have the pathology.

In the context of the invention, the term “treating” or “treatment”, as used herein, refers to a method that is aimed at delaying or preventing the onset of a pathology, at reversing, alleviating, inhibiting, slowing down or stopping the progression, aggravation
5 or deterioration of the symptoms of the pathology, at bringing about ameliorations of the symptoms of the pathology, and/or at curing the pathology.

As explained above, the population of partially reprogrammed cells or induced pluripotent cells of the invention can be used in order to produce large amounts of a
10 given cell type, by differentiation under suitable conditions. This differentiation is less time-consuming and leads to iTS-derived somatic cells with a high purity, compared to those obtained by differentiating iPS cells or ES cells.

Accordingly, in one embodiment, the invention relates to a method for amplifying a
15 somatic cell comprising the steps consisting of:

- a) contacting said somatic cell with at least one reprogramming factor,
- b) cultivating said cell in the presence of feeder cells in order to obtain a partially reprogrammed cell,
- c) isolating said partially reprogrammed cell,
- 20 d) cultivating said partially reprogrammed cell in the absence of feeder cells and in the presence of Leukemia Inhibitory Factor (LIF),
- e) differentiating said partially reprogrammed cell in the absence of LIF and/or in the presence of one or several factors, such as HGF, oncostatin M..

25 Therefore, the method of the invention can be used as a method for obtaining a population of substantially pure, fully differentiated cells, by selecting the suitable factors in step e).

In one embodiment, the method can be used for obtaining hepatocytes, which express albumin, liver enzymes systems such as proteins of the cytochrome P450 superfamily.

In another embodiment the method can be used for obtaining insulin-producing pancreatic beta cells.

In the following, the invention will be illustrated by means of the following examples and figures.

FIGURE LEGENDS

Figure 1: Histological (A) and molecular (B) analysis of endodermc-derived iTS cells

10 Figure 2: Molecular signature of endodermic iTS in comparison with murine hepatocytes

Figure 3: Spontaneous differentiation of iTS cells into hepatocytes expressing albumin mRNA for 5 different subclones.

15 EXAMPLES

Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

20

Example 1

The production of iTS cells from mouse hepatocytes was attempted using an adenovirus vector, which allows transient and high level expression of exogenous genes without
25 integrating into the host genome. For this purpose cDNAs of Oct4, Sox2, cMyc and Klf4 were introduced into non-replicative adenoviral vectors deleted of E1 and E3 under the control of the hCMV immediate early promoter. Hepatocytes are highly permissive for adenoviral infection and 20 MOI (multiplicity of infection or number of virus per cell) of adenovirus encoding GFP led to an infection efficiency of 100% of cells expressing
30 GFP. The dose of 20 MOI was thus used to infect adult murine hepatocytes. Adherent

liver cultures were established from 6 to 8 week old mice. Briefly, the liver was isolated and a catheter was inserted into the vena cava and maintained using clamps. The liver was washed with 50 ml of 0.5mM EGTA HEPES buffer and then perfused with the HEPES/CaCl₂/Collagenase buffer. Once the perfusion was completed, the clamps were removed and the liver excised and placed in a 10 cm Petri dish with 20 ml of chilled William's medium containing 10% serum and antibiotic. The liver was then cut in several locations and the dispersed cells were filtered through a 40 µm cell strainer. The cells were then re-suspended in warm William's medium containing 0.5 mg/ml of bovine serum albumin , 1x Insulin-Transferrin-Selenium, and 10⁻⁷ M dexamethasone on collagen I-treated petri dish of 10 cm. Approximately one million hepatocytes were infected with 20 MOI of adenovirus encoding Oct4, Sox2, cMyc and Klf4 and after 5 days of infections, cells were manually pooled and plated on mitomycin C-arrested Mouse Embryonic fibroblasts (MEF) in daily changed ES cell culture medium; DMEM medium supplemented with specific cytokines, in particular Leukemia Inhibitory Factor (LIF). Murine somatic cells were supplemented with 15% SVF, 1% Non-Essential Amino Acids, 1x Penicillin-Streptomycin, 100 µM β-mercaptoethanol and 1000 U/ml of LIF. After 10 to 20 days, colonies appeared and were picked and cultured in MEF-free conditions on collagen I-treated plates in ES cell culture medium. In this manner, more than 50 colonies have been isolated. For two of them (clones 3 and 6), single cells were added in a 96-well cell culture plate for subcloning. After 1 to 2 weeks of culture each subclone (6.1 to 6.8 and 3.1 and 3.14) was scraped and plated onto collagen-coated 12 well microtiter plates and expanded. Each clone and subclone was called an endodermic iTS cell and further characterized by the following techniques.

25 **Flow cytometry**

All clones and subclones were tested by flow cytometry for the expression of pluripotent markers such as SSEA1 and oct-4. As expected, all clones were negative for both molecules, and were considered as iTS cells and not iPS cells which are always positive for both markers.

Murine Stem Cell Pluripotency Low Density Array

Gene expression of different clones and subclones cells was investigated using Human Stem Cell Pluripotency TLDA (Taqman Low Density Array). The micro-fluidic card (Applied Biosystems, Foster City, CA, USA) consisted of 4 identical sets of 96 genes (90 target genes and six endogenous controls). Target genes included genes expressed in undifferentiated cells, genes involved in the maintenance of pluripotency, stemness-related genes and differentiation marker genes. Quantitative PCR was carried out with the ABI PRISM 7900HT Sequence Detection System. Clustering gene expression patterns were determined using hierarchical algorithms of StatMiner software. In the present study, Euclidean distance and complete linkage methods were applied even though clustering results were identical whatever the linkage method and distance measure used.

By TDLA we first noted a very different hierarchical clustering of pluripotent gene expression and differentiation genes in different endodermic iTS cells in comparison to murine ES cells (mES D3) and hepatocytes of origin.

We then quantified the transcripts of ectodermic, endodermic and mesodermic markers in those cells and showed that only endodermic markers are strongly expressed in endodermic iTS cells in comparison to mES D3 cells, including genes involved in the visceral and parietal endoderm development, in liver development and in pancreas development.

Thus we were able to show that:

- Endodermic iTS cells do not express by TLDA, ectodermic and mesodermic markers.
- Endodermic iTS cells express by TLDA: Foxa2, Gata4, Afp and Fn1 mRNAs involved in visceral and parietal endoderm and liver development.
- Endodermic iTS cells express by TLDA, Iapp mRNAs. Iapp (Islet amyloid polypeptide) is a hormonal factor secreted from pancreas beta cells that was shown to be colocalized with insulin and glucagon in fetal and adult pancreatic islet cells.

Cellular characterization of endodermic iTS cells

Different clones and subclones were stained with May-Grünwald-Giemsa (MGG) for cells morphology analysis. Clones 3 and 6 showed polymorphic cells with one or two nucleus, whereas subclones were monomorphic and mononuclear cells with a high nuclear-to-cytoplasmic ratio, which typically characterizes undifferentiated and stem cells as opposed to differentiated cells. Transmission electron microscopy of all clones and subclones analyzed, showed the presence of fusiform cells with membranes showing numerous villousities. The nucleus showed an abundant euchromatin denoting that, in contrast to hepatocytes, iTS cells are transcriptionally active cells. The cytoplasm showed numerous mitochondria undergoing fusion or division as well as very rare lipidic structures, granular and agranular endoplasmic reticulum and golgi apparatus.

Molecular characterization of endodermic iTS cells

All subclones generated from clones 3 and 6 were tested by RT-PCR to further identify their differentiation potential. We thus performed the amplification of mRNA involved in the development of hepatocyte-bile duct cells, of hepatocytes, as well as in the development of pancreatic islet β -cells.

We were able to show that endodermic iTS cells are tripotent progenitor cells since they express specific markers that can be fated to definitive endoderm bile duct cells, hepatocytes and to pancreatic cells.

In particular endodermic iTS cells were shown to express by RT-PCR:

-Markers of definitive embryonic endoderm such as GATA4, Sox17, Foxa2 (Hepatocytes nuclear factor 3 beta), Foxa1 (Hepatocytes nuclear factor 3 alpha) and CXCR4.

-Markers of liver progenitor cells such as Cytokeratin (CK) 8 and CK18, Hepatocytes nuclear factor 4 alpha (HNF4a) and alpha-fetoprotein (AFP).

-Markers of biliary progenitor cells such as: Hepatocytes nuclear factor 6 (HFN6), Hepatocytes nuclear factor 1 alpha (HNF1a), and Hepatocytes nuclear factor 1 beta (HNF1b).

-Markers of pancreatic progenitor cells such as: Pdx1, Pax4, Pax6, Neurogenin 3 (Ngn3), HFN6, and HNF1a.

Production of hepatocyte-like cells from murine endodermic iTS cells producing albumin.

We first showed that endodermic iTS cells negative for albumin at transcriptional levels
5 can be differentiated into hepatocyte-like cells producing high levels of liver-specific
markers. We have optimized the differentiation procedure permitting the elimination of
serum and fibroblast feeder cells. Endodermic iTS cells were maintained in monolayers
cultured on plates coated or not with collagene in mES culture media under standard
conditions (37°C; 5%CO₂/ambient O₂). When the cells reached approximately 80% of
10 confluence, the media was replaced with hepatocyte culture medium consisting of
William's medium containing 1x Insulin-Transferrin-Selenium, 10⁻⁷ M dexamethasone
and 20 ng/ml of Oncostatin M, an interleukin-6 family cytokine that was initially found
to induce maturation of fetal hepatic cells derived from embryonic day 14.5 liver in
vitro. After five days of culture under these conditions, the cells were found to express
15 high levels of albumin. An average of 52% of cells were albumin-positive based on flow
cytometry analysis. After completion of the protocol, the cells were also found to display
several known hepatic functions. Periodic acid-Schiff (PAS) staining indicated glycogen
synthesis by the differentiated cells and red O staining identified the presence of lipid
droplets.

20

Production of pancreatic-like cells from murine endodermic iTS cells producing Insulin.

We were able to show that several subclones express pancreatic progenitors such as
Pdx1, Pax4, Ngn3, HFN6, and HNF1a and we described that endodermic iTS cells
25 generated from differentiated hepatocytes can give rise to pancreatic-like cells capable
of generating insulin. We differentiated endodermic iTS cells into pancreatic-like cells
by replacing the mES medium by a medium containing DMEM/F12 supplemented with
100 ng/ml Activin A and 10 mmol/L nicotinamide and cultured for 5 days. Under these
conditions we were able to detect insulin by ELISA in the cell supernatants.

30

Teratome formation

We performed a teratoma assay in order to document the potency of endodermic iTS cells. ITS cells were harvested by trypsinization and injected intramuscularly into the flanks of NOD/SCID mice, using ~ 5 millions cell per injection. Mice were sacrificed 3
5 months later and teratomas were isolated and processed for histological analysis. Three clones and sub-clones were injected showing two kinds of tissues: a tissue with an architecture of the hepatic parenchyma with cells expressing albumin and a tissue with bile duct structure positive for glycogen, for acid mucosubstances after PAS and Alcian blue staining respectively and for EA1/EA3 cytokeratin.

10

Liver and pancreas implantation of autologous endodermic iTS

In order to document the potential of endodermic iTS cells to colonize tissues of endodermic origin, endodermic iTS cells were injected in the pancreas and in the spleen of partially hepatectomized mice. For these experiments, endodermic iTS cells were
15 infected with a retrovirus encoding the luciferase gene and we used a firefly bioluminescence system for a weekly detection of the graft cells. We showed that all endodermic iTS cells tested have the potential to be grafted into the pancreas over a period of 56 days. Furthermore all endodermic iTS cells have the potential to recolonize the liver of the mice over a period of 35 days.

20

In conclusion, the inventors have shown that the method of the invention enable the production of induced tissue stem cells (iTS cells) of a given lineage. The iTS cells obtained by said method were multipotent stem cells of the endodermic lineage, able to
25 differentiate both *in vitro* and *in vivo* into any cell type of the endodermic lineage.

Similar experiments were performed with somatic cells of the ectodermic/neurodermic lineage and with somatic cells of the mesodermic lineage.

Example 2: Non-oncogenicity of iTS cells

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The in vivo teratoma model described above also provides an accessible system for the evaluation of the oncogenicity of the cells.

The inventors have shown that the injection of endodermal iTS cells as described above, leads to a complete absence of ectodermal and mesodermal tissues and the absence of malignant tissues. Two kinds of tissues were observed by histological analysis: a tissue with an apparent architecture of a hepatic parenchyma with cells expressing albumin, and a tissue with bile duct structure (Figure 1A). RT-PCR analysis of these tissues revealed the presence of albumin and G6P mRNA, that were absent in the original iTS cells (Figure 1B).

Example 3: Gene expression profile of iTS cells

Gene expression of endodermic iTS and murine hepatocytes were investigated using Human Stem Cell Pluripotency TLDA (Taqman Low Density Array). The micro fluidic card (Applied Biosystems, Foster City, CA, USA) consisted of 4 identical 96 gene sets (90 target genes and six endogenous controls). Target genes included genes expressed in undifferentiated cells, genes involved in the maintenance of pluripotency, stemness-related genes and differentiation marker genes.

TLDA analysis showed very different gene-expression patterns between endodermic iTS cells and hepatocytes (see Figure 2A) and molecular signature has been established for endodermic iTS cells in comparison to hepatocytes of origin (see Figure 2B).

Thus, eleven specific markers were found which were overexpressed (DCt>3.3) in endodermic iTS in comparison to murine hepatocytes; KIT, LIFR, TERT, NR5A2, GABRB3, IMP2, LAMA1, SOX17, SERPINA1, PAX4, TAT.

Example 4: Spontaneous differentiation of iTS cells upon removal of LIF

Human iPS and ES cells can adopt a hepatic cell fate in vitro by using a cocktail of different specific factors: Activin A, BMP-4, FGF-2, HGH and oncostatin M. In contrast

to iPS cells, iTS cells are able to differentiate spontaneously to albumin producing hepatocytes cells when LIF are removed from the growth medium. Five subclones have been tested with and without LIF and we were able to show that albumin transcripts and protein are produced without LIF (see Figure 3).

CLAIMS

1. A method for producing an induced tissue stem cell from a somatic cell comprising the steps of:
- 5 a) contacting a somatic cell with at least one reprogramming factor,
b) cultivating said cell in the presence of feeder cells in order to obtain a partially reprogrammed cell,
c) isolating said partially reprogrammed cell,
d) cultivating said partially reprogrammed cell in the absence of feeder cells, and
10 optionally in the presence of Leukemia Inhibitory Factor (LIF).
2. A method according to claim 1, wherein said somatic cell is a mouse cell.
3. A method according to claim 1, wherein said somatic cell is a human cell.
- 15 4. A method according to any of the above claims, wherein said somatic cell is selected from the group consisting of, epithelial, endothelial, neuronal, adipose, cardiac, skeletal muscle, immune cells, hepatic, splenic, lung, circulating blood cells, gastrointestinal, renal, and pancreatic cells.
- 20 5. A method according to any of the above claims, wherein said somatic cell is a hepatocyte.
6. A method according to any of the above claims, wherein said feeder cells are
25 inactivated fibroblasts.
7. A method according to any of the above claims, wherein said reprogramming factor is selected from the group consisting of Oct-4, KLF4, sox2 and c-myc.

8. A method according to any of the above claims, wherein the step of contacting a somatic cell with at least one reprogramming factor is performed by a non-integrating viral vector.
- 5 9. A method according to any of the above claims, further comprising the step of selecting the partially reprogrammed cell for the absence of a stem cell marker.
- 10 10. A method according to any of the above claims, further comprising the step of selecting the partially reprogrammed cell for the expression of one or more foetal progenitor markers committed toward a specific lineage.
- 11 11. A method according to any of the above claims, further comprising the step of selecting the partially reprogrammed cell for the absence of markers of differentiated mature cells.
- 15 12. A substantially pure population of induced tissue stem cells obtainable by the method according to any one of claims 1 to 11.
- 20 13. Use of a substantially pure population of induced tissue stem cells according to claim 12 for non-therapeutic purpose.
- 25 14. A pharmaceutical composition comprising a substantially pure population of induced tissue stem cells according to claim 12 and optionally a pharmaceutically acceptable carrier or excipient.
15. A substantially pure population of induced tissue stem cells according to claim 12 for use in a method of treatment.

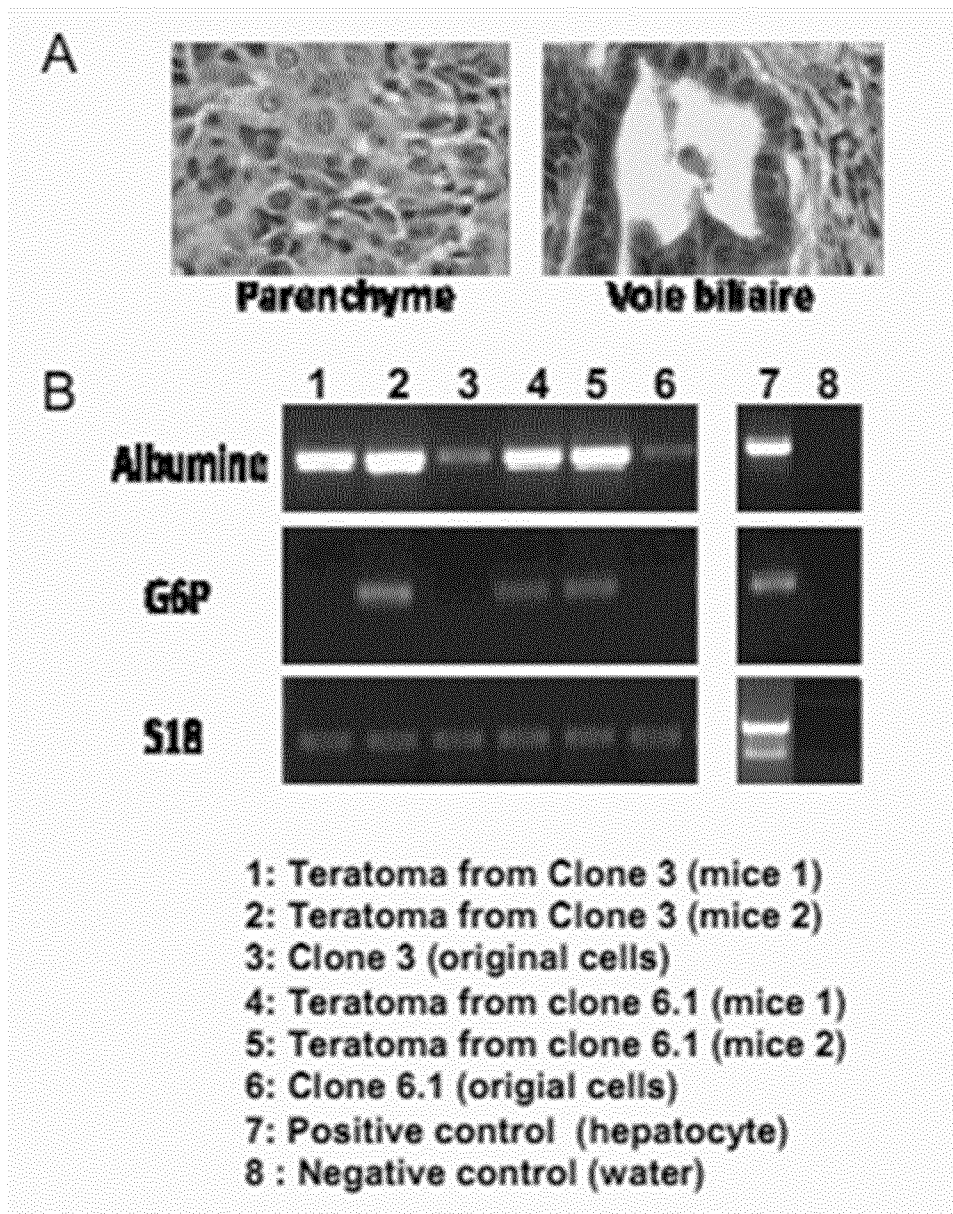


Figure 1

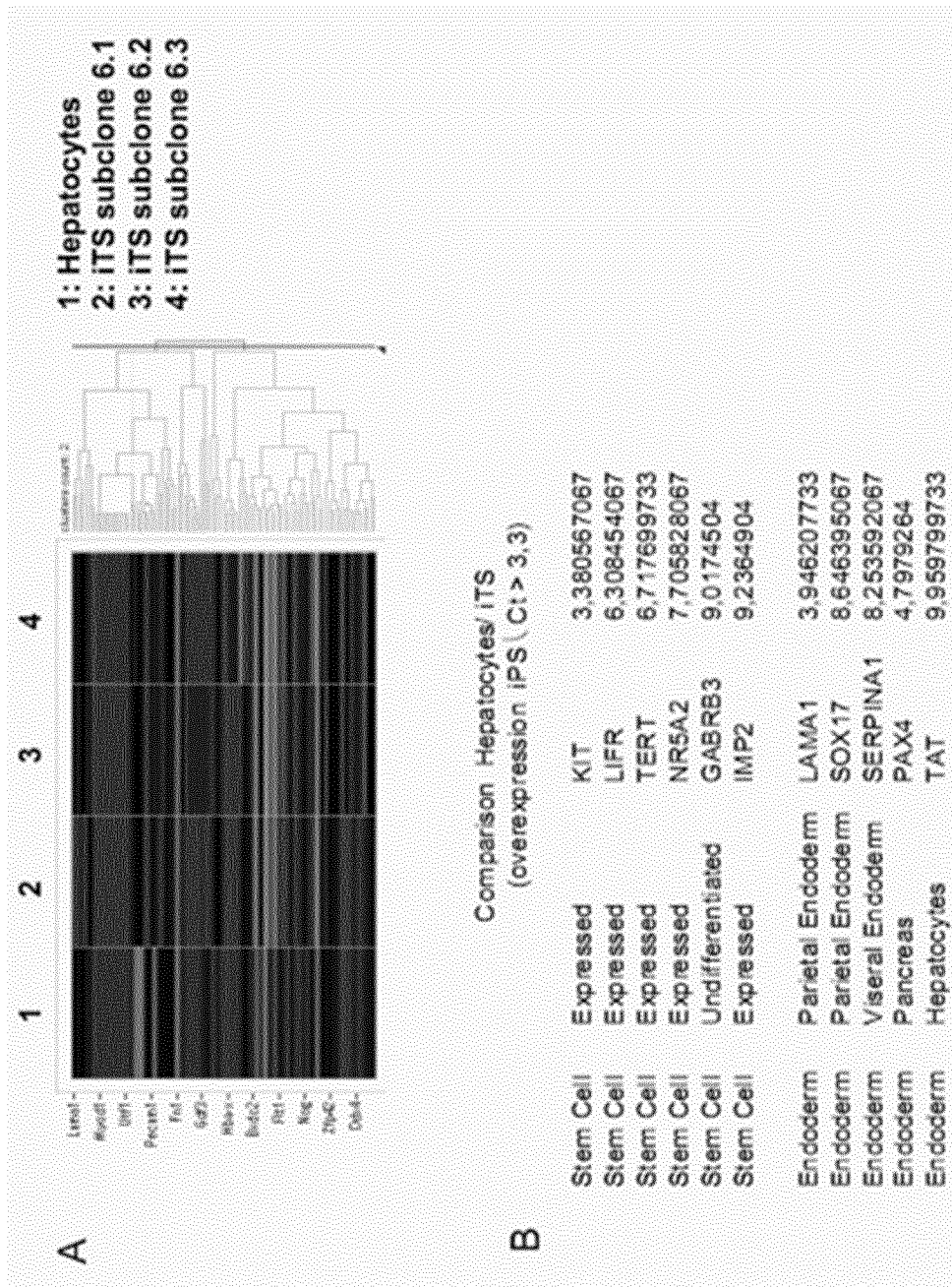


Figure 2

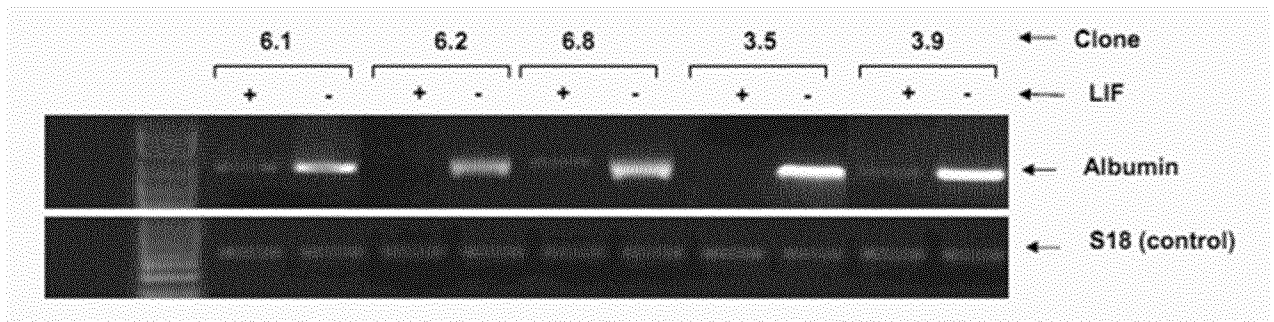


Figure 3

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/060903

A. CLASSIFICATION OF SUBJECT MATTER INV. C12N5/071 C12N5/074 ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) C12N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, BIOSIS, EMBASE, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TATEISHI KEISUKE ET AL: "Generation of insulin-secreting islet-like clusters from human skin fibroblasts", JOURNAL OF BIOLOGICAL CHEMISTRY, THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, INC, US, vol. 283, no. 46, 14 November 2008 (2008-11-14), pages 31601-31607, XP009147429, ISSN: 0021-9258, DOI: 10.1074/JBC.M806597200 [retrieved on 2008-09-09] e.g. abstract; page 31602, left-hand column, paragraph 4; paragraph bridging page 31602 and 31603; page 31604, left-hand column, last paragraph - page 31605; fig. 3; the whole document ----- -/--	1-15
<input checked="" type="checkbox"/>	Further documents are listed in the continuation of Box C.	<input checked="" type="checkbox"/> See patent family annex.
* Special categories of cited documents :		
"A" document defining the general state of the art which is not considered to be of particular relevance	"E" earlier application or patent but published on or after the international filing date	"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means	"P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
		"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
		"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
		"&" document member of the same patent family
Date of the actual completion of the international search	Date of mailing of the international search report	
11 July 2012	20/07/2012	
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Gruber, Andreas	

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/060903

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>NOGUCHI HIROFUMI ET AL: "Induction of pancreatic stem/progenitor cells into insulin-producing cells by adenoviral-mediated gene transfer technology", CELL TRANSPLANTATION, US, vol. 15, no. 10, 1 January 2006 (2006-01-01), pages 929-938, XP009122625, ISSN: 0963-6897, DOI: 10.3727/000000006783981431 e.g. abstract; page 931, right-hand column, paragraph 3 - page 932; page 936; the whole document</p> <p style="text-align: center;">-----</p>	12-15
X	<p>BLYSZCZUK P ET AL: "Expression of Pax4 in embryonic stem cells promotes differentiation of nestin-positive progenitor and insulin-producing cells", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE, WASHINGTON, DC; US, vol. 100, no. 3, 4 February 2003 (2003-02-04), pages 998-1003, XP002233762, ISSN: 0027-8424, DOI: 10.1073/PNAS.0237371100 e.g. abstract; page 998, left-hand column, paragraph 1; page 1003, left-hand column, last paragraph and right-hand column, paragraph 3; the whole document</p> <p style="text-align: center;">-----</p>	12-15
X	<p>COLMAN A: "Making new beta cells from stem cells", SEMINARS IN CELL AND DEVELOPMENTAL BIOLOGY, ACADEMIC PRESS, GB, vol. 15, no. 3, 1 June 2004 (2004-06-01), pages 337-345, XP002365752, ISSN: 1084-9521, DOI: 10.1016/J.SEMCDB.2004.02.003 e.g. page 341, right-hand column, paragraph 3; page 342, right-hand column, last paragraph; the whole document</p> <p style="text-align: center;">-----</p>	12-15
X	<p>WO 2010/075575 A1 (BIOPIPS INC [US]; ZHU YONG [US]; WU SHILI [US]; BAO JUN [US]) 1 July 2010 (2010-07-01) e.g. example 2; claim 1-32; the whole document</p> <p style="text-align: center;">-----</p> <p style="text-align: center;">-/--</p>	12-15

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/060903

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2009/126927 A2 (JOSLIN DIABETES CENTER INC [US]; WEIR GORDON [US]; NAGAYA MASAKI [US]) 15 October 2009 (2009-10-15) e.g. claim 1-19; the whole document -----	12-15

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2012/060903

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2010075575	A1	01-07-2010	
		AU 2009329893 A1	30-06-2011
		CA 2748009 A1	01-07-2010
		CN 102272142 A	07-12-2011
		EP 2379577 A1	26-10-2011
		US 2011258713 A1	20-10-2011
		WO 2010075575 A1	01-07-2010

WO 2009126927	A2	15-10-2009	NONE
