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(54) **Title:** METHOD ANK KIT FOR REPROGRAMMING SOMATIC CELLS

(57) **Abstract:** The present invention relates to a method for reprogramming a human somatic cell into a human induced pluripotent stem cell (iPS) comprising a step of culturing said somatic cell into the presence of at least a combination of a DNA methyltransferase (DNMT) inhibitor, a Sirtuin inhibitor and a poly(ADP-ribose)polymerase (PARP) inhibitor.



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METHOD ANK KIT FOR REPROGRAMMING SOMATIC CELLS

FIELD OF THE INVENTION:

5 The invention relates to method and kit for reprogramming somatic cells. In particular, the invention relates to a method for reprogramming somatic cells into pluripotent cells by using a combination of small-molecule compounds and kits comprising such compounds.

BACKGROUND OF THE INVENTION:

10 In August, 2006, Yamanaka's lab reported that mouse embryonic fibroblast cells could be reprogrammed in induced pluripotent stem cells also called iPS cells, through retro-viral introduction of four transcriptional factors, Oct4, Sox2, Klf4 and c-Myc (Takahashi and Yamanaka 2006). After that, Oct4, Sox2, Klf4 and c-Myc were so-called reprogramming factors. In November 2007, Yamanaka's lab and Thomson's lab both reported the generation
15 of human iPS cells from adult human fibroblasts by the combination "Oct4, Sox2, Klf4 and c-Myc" or "Oct4, Sox2, Nanog, Lin-28", respectively (Takahashi *et al.* 2007; Yu *et al.* 2007). IPS cells were very similar to embryonic stem cells in gene profiling, differentiation potential and epigenetic modifications. They were able to self-renew and differentiate into all mature cell types, including neurons, hematopoietic cells, muscle cells and islet cells.

20 iPS cells can be created by over-expression of one or more genes, for example one or more of the following four genes: Oct4, Sox2, c-Myc and Klf4 through retroviral infection, but with low efficiencies. All of these four genes are known to be or considered to be DNA binding proteins, transcription factors. Notably, the oncogene c-Myc used in this approach
25 causes tumor formation in cells derived from the iPS cells. Although iPS cells can be generated with only Oct4, Sox2 and Klf4, the efficiency is even lower; fewer than iPS colonies from out of 100,000 cells. These issues pose significant barriers for creation of iPS cells for therapeutic applications. One obvious concerns is the use of retroviruses, which integrate into chromosomal DNA and can cause ancillary problems (mutations).

30 Thus, chemical-induced reprogramming offers a novel approach to generating iPS cells without any viral vector-based genetic modification. Previous reports showed that several small molecules could improve the efficiency of iPS cell generation (Lukaszewicz *et al.*, 2010) and/or replace some of the reprogramming factors although at least one

transcription factor, in particular Oct4, is still required to generate iPS cells from mouse embryonic fibroblasts (Li *et al.*, 2011). Alternatively, European patent application N° EP2537920 discloses a method for producing an iPS cell, which comprises the steps of: introducing c-Myc into a somatic cell; and culturing said somatic cell in the presence of cell
5 in the presence of a sirtuin inhibitor and/or a poly-ADP ribose polymerase (PARP) inhibitor.

Thus, by using only small molecules, these four exogenous “master genes” would be dispensable for human cell fate reprogramming. Importantly, it has been recently shown that a multi-chemical combination of drugs could reprogram mouse somatic cells into iPS (Hou *et al.* 2013); no similar protocol exists for human cells. Thus developing such protocols could be
10 of paramount significance as this would provide a major advantage for regenerative medicine.

However, until now, human iPS obtained by chemical-induced reprogramming have never been disclosed.

SUMMARY OF THE INVENTION:

In a first aspect, the invention relates to a method for reprogramming a human somatic cell into a human induced pluripotent stem cell (iPS) comprising a step of culturing said somatic cell into the presence of at least a combination of a DNA methyltransferase (DNMT)
20 inhibitor, a Sirtuin inhibitor and a poly(ADP-ribose)polymerase (PARP) inhibitor.

In a second aspect, the invention relates to an human iPS obtained by the method of the invention.

In a third aspect, the invention relates to a kit for reprogramming a somatic cell comprising at least a DNMT inhibitor, a Sirtuin inhibitor and a PARP inhibitor.

DETAILED DESCRIPTION OF THE INVENTION:

The invention is based on the discovery that a specific chemical combination enables the reprogramming of human somatic cells into a human induced pluripotent stem cell (iPS).

Definitions:

Throughout the specification, several terms are employed and are defined in the following paragraphs.

As used herein, the term "**pluripotent**" refers to cells with the ability to give rise to progeny that can undergo differentiation, under appropriate conditions, into cell types that collectively exhibit characteristics associated with cell lineages from the three germ layers (endoderm, mesoderm, and ectoderm). Pluripotent stem cells can contribute to tissues of a prenatal, postnatal or adult organism. A standard art-accepted test, such as the ability to form a teratoma in 8-12 week old SCID mice, can be used to establish the pluripotency of a cell population. However, identification of various pluripotent stem cell characteristics can also be used to identify pluripotent cells. More specifically, human pluripotent stem cells may express at least some, and optionally all, of the markers from the following non-limiting list: SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, TRA-2-49/6E, Alkaline phosphatase (ALP), Sox2, E-cadherin, UTF-I, Oct4, Lin28, Rex1, and Nanog.

As used herein, the term "**induced pluripotent stem cell**" refers to a pluripotent stem cell artificially derived from a non-pluripotent cell. A non-pluripotent cell can be a cell of lesser potency to self-renew and differentiate than a pluripotent stem cell. Cells of lesser potency can be, but are not limited to, somatic stem cells, tissue specific progenitor cells, primary or secondary cells.

As used herein, the term "**reprogramming**" refers to the process of changing the fate of a target cell into that of a different cell type, caused by the expression of a small set of factors (or reprogramming factors) in the target cells. For example, methods for reprogramming fibroblast cells to induced pluripotent stem cells by expressing ectopically Oct3/4, Sox2, c-Myc and Klf4 have been described by Takahashi and Yamanaka, 2006. A "reprogramming factor" may be a transcription factor, which can be used to reprogram a target cell but further includes any analogue molecule that mimics the function of the factor with respect to reprogramming capacity.

As used herein, the term "**somatic cell**" refers to is any cell of the body except germline cells (sperm and egg).

In one embodiment of the invention, the somatic cell is an epithelial cell (such as a mammary gland epithelial cell), a fibroblast, a muscle cell, a cumulus cell, a neural cell, a liver cell, a GI tract cell, a mammary cell, a kidney cell, a blood cell, a vascular cell, a skin cell, an immune system cell, a lung cell, a bone cell, keratinocyte, or a pancreatic islet cell.

As used herein, the term "**DNMT inhibitor**" refers to an inhibitor or antagonist of DNA methyltransferases (DNMT1, DNMT3A and/or DNMT3B) activity. A DNMT inhibitor or antagonist is a compound that selectively inhibits the activity of DNMT. A drug also able to decrease DNMTs expression is also considered as DNMT inhibitor.

DNMT inhibitors are well known from the skilled person. Examples of DNMT inhibitors are described in Fahy *et al.*, Expert Opin. Ther. Patents (2012) 22(12):1427-1442. A person skilled in the art can easily determine whether a compound is capable of inhibiting DNMT activity. Assays for evaluating DNMT activity are for example, described in Kim B Y *et al.*, (Anal Biochem. 2004 Mar. 1; 326(1): 21-4) or in Yan L *et al.*, (Cancer Biol Ther. 2003 September-October; 2(5):552-6). DNMT inhibition may be determined using conventional methods, including for example assays that measure the methylation state of cellular DNA by incubation with 4 U of SssI CpG methylase in the presence of 1.5 μ M S-adenosyl-L-[methyl-3H]methionine, as described in Fang J *et al.*, J Virol, 75(20): 9753-9761, 2001.

Non-limiting examples of DNMT inhibitors include 5-azacytidine (5-aza-CR; vidaza), 5-aza-2'-deoxycytidine (5-aza-dC; decitabine), 1-[beta]-D-arabinofuranosyl-5-azacytosine, dihydro-5-azacytidine, zebularine, sinefungin, 5-fluoro-2'-deoxycytidine (FdCyd).

In a particular embodiment of the invention, the DNMT inhibitor is 5-aza-2'-deoxycytidine (5-aza-dC; decitabine).

According to some embodiments, NU1025 is provided at a concentration range of between about 0.1 to about 20 μ M, e.g., between about 0.2-20 μ M, e.g., between about 0.5-15 μ M, e.g., between about 1-10 μ M, e.g., between about 2-8 μ M, e.g., between about 3-6 μ M, e.g., about 5 μ M.

As used herein, the term "**Sirtuin inhibitor**" refers to an inhibitor or antagonist of sirtuin family of deacetylase enzymes (SIRT1-7) activity. A Sirtuin inhibitor or antagonist is a compound that selectively inhibits the activity of Sirtuins. A drug also able to decrease Sirtuin expression is also considered as DNMT inhibitor.

Sirtuins inhibitors are well known from the skilled person. Examples of Sirtuins inhibitors are described in Porcu *et al.*, 2005 TRENDS in Pharmacological Sciences Vol.26

No.2 February 2005 and in Alcain *et al.*, Expert Opinion on Therapeutic Patents, March 2009, Vol. 19, No. 3 : Pages 283-294.

A person skilled in the art can easily determine whether a compound is capable of inhibiting Sirtuin activity. Assays for evaluating Sirtuin activity are for example, described in Smith et al., 2209 Anal Biochem. 2009 Nov 1; 394(1):101-109.

Non-limiting examples of compounds which are known as Sirtuins inhibitors and which may be used include compounds and derivatives thereof from the class of Dihydrocoumarin derivatives, Naphthopyranone derivatives, NAD derivatives and 2-Hydroxy-naphthaldehyde derivatives.

In one embodiment of the invention, the Sirtuin inhibitor is selected from the group consisting of Sirtuin inhibitors include Nicotinamide, Carbamido-NAD, NADH, Dihydrocoumarin, A3, Splitomicin, 2-OH-naphthaldehyde, Tenovin 1, Tenovin 6, AK-1, AK-6, Selistat, Sirtinol and M15.

In a particular embodiment of the invention, the Sirtuin inhibitor is Nicotinamide.

According to some embodiments, Nicotinamide is provided at a concentration range of between about 0.05 to about 50 μ M, e.g., from about 0.1 to about 40 μ M, e.g., between about 0.5-30 μ M, e.g., between about 1-25 μ M, e.g., between about 5-20 μ M, e.g., about 15 μ M.

As used herein, the term "**PARP inhibitor**" refers to an inhibitor or antagonist of Poly(ADP-ribose) polymerases (PARP 1 and/or PARP2) activity. A PARP inhibitor or antagonist is a compound that selectively inhibits the activity of PARP. A drug also able to decrease PARPs expression is also considered as PARP inhibitor.

PARP inhibitors are well known from the skilled person. Examples of PARP inhibitors are described in Penning, Current Opinion In Drug Discovery & Development 2010 13 (5): 577-586. A person skilled in the art can easily determine whether a compound is capable of inhibiting PARP activity. Assays for evaluating PARP activity are for example, described in Poly(ADP-ribose) (PAR) polymer is a death signal (Andrabi SA *et al.*, 2006). PARP inhibition may be determined using conventional methods, including for example dot blots (Affar EB *et al.*, Anal Biochem. 1998; 259(2):280-3), and BER assays that measure the direct activity of PARP to form poly ADP-ribose chains for example by using radioactive

assays with tritiated substrate NAD or specific antibodies to the polymer chains formed by PARP activity (K.J. Dillon *et al*, Journal of Biomolecular Screening, 8(3): 347-352 (2003).

Non-limiting examples of compounds which are known as PARP inhibitors and which may be used include compounds and derivatives thereof from the class of Nicotinamides, Benzamides, Isoquinolinones, Dihydroisoquinolinones, Benzimidazoles, indoles, Phthalazin-1 (2H)-ones, quinazolinones, Isoindolinones, Phenanthridines, phenanthhdinones, Benzopyrones, Unsaturated hydroximic acid derivatives and Pyridazines.

In one embodiment of the invention, the PARP inhibitor is selected from the group consisting of NU1025 (8-hydroxy-2-methylquinazolinone), AZD-2281 (olaparib), AG014699 (rucaparib), ABT-888 (veliparib), BSI-201 (iniparib), CEP-9722, MK-4827, BMN-673, AG14361, Nicotinamide and 3-AB (3-aminobenzamide).

In a particular embodiment of the invention, the PARP inhibitor is NU1025 (8-hydroxy-2-methylquinazolinone).

According to some embodiments, NU1025 is provided at a concentration range of between about 0.05 to about 30 μ M, e.g., from about 0.1 to about 30 μ M, e.g., between about 0.2-30 μ M, e.g., between about 0.2-25 μ M, e.g., between about 0.5-20 μ M, e.g., between about 0.8-15 μ M, e.g., between about 1-10 μ M, e.g., between about 2-5 μ M, e.g., about 3 μ M.

In certain embodiments, a compound which is both a sirtuin inhibitor and a PARP inhibitor may be used (such as for instance Nicotinamide).

Accordingly, in a particular embodiment of the invention, the method for reprogramming a human somatic cell into a human iPS comprising a step of culturing said somatic cell into the presence of at least a combination of a DNMT inhibitor as above-described (such as decitabine) and Nicotinamide as a sirtuin inhibitor and a PARP inhibitor.

In some embodiment, the somatic cell is further cultured in the presence of at least one compound selected from the group consisting of a c-Jun N-terminal kinase (JNK) inhibitor, a p38 mitogen-activated protein (MAP) kinase inhibitor and a Sonic Hedgehog (SHH) signaling pathway agonist.

As used herein, the term "**JNK inhibitor**" refers to an inhibitor or antagonist of c-Jun N-terminal kinase (JNK1, JNK2 and/or JNK3) activity. A JNK inhibitor or antagonist is a

compound that selectively inhibits the activity of JNK. A drug also able to decrease JNKs expression is also considered as JNK inhibitor.

JNK inhibitors are well known from the skilled person. Examples of JNK inhibitors are described in Zhang *et al.*, Chem Biol. 2012 Jan 27; 19(1):140-154. A person skilled in the art can easily determine whether a compound is capable of inhibiting JNK activity. Assays for evaluating JNK activity are for example, described in Guenat *et al.*, J Biomol Screen, 2006; 11: pages 1015-1026). JNK inhibition may be determined using conventional methods, including for example kinase assays (e.g. Alpha screen test) that measure the inhibition of human JNK mediated phosphorylation of a c-Jun substrate such as c-Jun, ATF2 and/or Elk-1.

Non-limiting examples of compounds which are known as JNK inhibitors and which may be used include compounds and derivatives thereof from the class of Aryl-oxindole derivatives, Pyrazoloanthrones derivatives, Tetrahydro-pyrimidine derivatives, Benzazoles derivatives, Sulfonyl amino acid derivatives and Sulfonyl hydrazide derivatives.

Non-limiting examples of JNK inhibitors include SP600125, AEG3482 and AS602801 (bentamapimod).

In a particular embodiment of the invention, the JNK inhibitor is AS602801 (bentamapimod).

According to some embodiments, AS602801 is provided at a concentration range of between about 0.05 to about 30 μ M, e.g., from about 0.1 to about 30 μ M, e.g., between about 0.2-30 μ M, e.g., between about 0.2-25 μ M, e.g., between about 0.2-20 μ M, e.g., between about 0.2-15 μ M, e.g., between about 0.2-10 μ M, e.g., between about 0.2-8 μ M, e.g., between about 0.2-6 μ M, e.g., between about 0.5-5 μ M, e.g., between about 1-4 μ M, e.g., about 3 μ M.

As used herein, the term "**p38 inhibitor**" refers to an inhibitor or antagonist of p38 mitogen-activated kinase (MAPK) (p38 α , p38 β , p38 γ , and p38 δ) activity. A p38 inhibitor or antagonist is a compound that selectively inhibits the activity of p38. A drug also able to decrease p38 expression is also considered as p38 inhibitor.

p38 inhibitors are well known from the skilled person. Examples of p38 inhibitors are described in Genovese, Arthritis & Rheumatism Vol. 60, No. 2, February 2009 pp 317-320

and in Buhler & Laufer, Expert Opinion on Therapeutic Patents, May 2014, Vol. 24, No. 5: pages 535-554. Assays for evaluating p38 activity are available such as the CycLex® p38 Kinase Assay/Inhibitor Screening kit purchased from MBL Inc.

5 Non-limiting examples of such p38 MAPK inhibitors include BIRB796 (Doramapimod), SB203580, VX702, SB202190, LY2228820, VX745, Vinorelbine (Navelbine), PH797804, pamapimod, CMPD-1, EO1428, JX401, ML3403, RWJ67657, SB239063, SCIO469 hydrochloride, SKF86002 dihydrochloride, SX011, and TAK715.

10 In a particular embodiment of the invention, the p38 inhibitor is BIRB 796 (doramapimod).

According to some embodiments, BIRB 796 is provided at a concentration range of between about 0.1 to about 10 μ M, e.g., between about 0.5 μ M, e.g., between about 5 μ M, e.g., between about 2 μ M, e.g., between about 6 μ M, e.g., about 1 μ M.

15 As used herein, the term "**SHH signaling pathway agonist**" refers to a compound (natural or synthetic) which potentiates or recapitulates the bioactivity of Hedgehog (Hh), such as to activate transcription of target genes. Preferred hedgehog agonists can be used to overcome a ptc gain-of-function and/or a smoothened loss-of-function, the latter also being referred to as smoothened agonists. Such agonist encompasses any compound that may act by
20 directly activating the normal function of the hedgehog protein, but also to any compound that activates the Hh signalling pathway, and thus inhibits the function of ptc. Hh signaling was first identified in Drosophila as an important regulatory mechanism for embryonic pattern formation. The vertebrate family of Hedgehog genes includes three members that exist in mammals, known as Desert (Dhh), Sonic (Shh) and Indian (Ihh) Hedgehogs.

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SHH signaling pathway agonists are well known from the skilled person. Examples of SHH signaling pathway agonists are described in the international patent applications N° WO0174344 and WO03027234 as well as in Carney & Ingham BMC Biology 2013, 11:37.

30 Non-limiting examples of such SHH signaling pathway agonist include SAG (Hh-Ag1.3) and purmorphamine (9-cyclohexyl-N-(4-morpholinophenyl)-2-(naphthalen-1-yloxy)-9H-purin-6-amine) which activates the Hedgehog pathway by targeting Smoothened.

In a particular embodiment of the invention, the SHH signaling pathway agonist is purmorphamine.

According to some embodiments, purmorphamine is provided at a concentration range of between about 0.1-10 μ M, e.g., between about 0.2-8 μ M, e.g., between about 0.5-5 μ M, e.g., about 1 μ M.

5 As above-mentioned, a compound which is simultaneously a sirtuin inhibitor and to lesser extend a PARP inhibitor may be used such as Nicotinamide.

Accordingly, in one embodiment of the invention, the method for reprogramming a human somatic cell into a human induced pluripotent stem cell (iPS) comprising a step of culturing said somatic cell into the presence of at least a combination of a DNA
10 methyltransferase (DNMT) inhibitor as above described, Nicotinamide and at least a JNK inhibitor and/or a p38 inhibitor and/or a SHH signaling pathway agonist.

In a particular embodiment of the invention, the method for reprogramming a human somatic cell into a human iPS comprising a step of culturing said somatic cell into the presence of at least a combination of a DNMT inhibitor, Nicotinamide, a JNK inhibitor, a p38
15 inhibitor and a SHH signaling pathway agonist (as described in EXAMPLE 4).

In a particular embodiment, the method for reprogramming a human somatic cell into a human iPS comprising a step of culturing said somatic cell into the presence of at least a combination of a DNA methyltransferase (DNMT) inhibitor, a Sirtuin inhibitor and a
20 poly(ADP-ribose)polymerase (PARP) inhibitor and macroH2A inhibitor.

As used herein "macroH2A" refers to H2A histone variants due to the presence of a 30-kDa non-histone domain (macro domain) at their C-termini. macroH2A variants are generally considered transcriptionally repressive in nature. The level of macroH2A is
25 regulated significantly at transcriptional level, several signalling pathways could be involved, typically, Protein kinase A-dependent signalling or Epithelial Mesenchymal Transition could play an important role. The chemical modulation of either of these pathways could result in reduction of macroH2A levels and thus to improve reprogramming, as described in Example 6.

30 As used herein, the term "macroH2A inhibitor" refers to an inhibitor or antagonist of the activity or expression of macroH2A. In some embodiments, the inhibitor of macroH2A expression is a small inhibitory RNAs (siRNAs). macroH2A expression can be reduced by contacting the subject or cell with a small double stranded RNA (dsRNA), or a vector or

construct causing the production of a small double stranded RNA, such that macroH2A expression is specifically inhibited (i.e. RNA interference or RNAi). Methods for selecting an appropriate dsRNA or dsRNA-encoding vector are well known in the art for genes whose sequence is known (e.g. see Tuschl, T. et al. (1999); Elbashir, S. M. et al. (2001); Hannon, GJ. (2002); McManus, MT. et al. (2002); Brummelkamp, TR. et al. (2002); U.S. Pat. Nos. 6,573,099 and 6,506,559; and International Patent Publication Nos. WO 01/36646, WO 99/32619, and WO 01/68836).

In some embodiments, the macroH2A inhibitor expression is an endonuclease. The mechanism behind endonuclease-based genome inactivating generally requires a first step of DNA single or double strand break, which can then trigger two distinct cellular mechanisms for DNA repair, which can be exploited for DNA inactivating: the errorprone nonhomologous end-joining (NHEJ) and the high-fidelity homology-directed repair (HDR). Endonucleases for gene inactivation have come in various forms, which includes CRISPR/CRISPR associated (Cas) systems, mega nucleases (MN), zinc finger nucleases (ZFN), and transcription activator-like effector nucleases (TALEN). Endonucleases for use in the present invention are disclosed in WO 2010/079430, WO2011072246, WO2013045480, Mussolino C, et al (Curr Opin Biotechnol. 2012 Oct;23(5):644-50) and Papaioannou I. et al (Expert Opinion on Biological Therapy, March 2012, Vol. 12, No. 3 : 329-342).

In a particular embodiment, the endonuclease is CRISPR-cas. As used herein, the term “CRISPR-cas” has its general meaning in the art and refers to clustered regularly interspaced short palindromic repeats associated which are the segments of prokaryotic DNA containing short repetitions of base sequences.

In some embodiment, the endonuclease is CRISPR-cas9 which is from *Streptococcus pyogenes*. The CRISPR/Cas9 system has been described in US 8697359 B1 and US 2014/0068797. Originally an adaptive immune system in prokaryotes (Barrangou and Marraffini, 2014), CRISPR has been recently engineered into a new powerful tool for genome editing. It has already been successfully used to target important genes in many cell lines and organisms, including human (Mali et al., 2013, Science, Vol. 339 : 823–826), bacteria (Fabre et al., 2014, PLoS Negl. Trop. Dis., Vol. 8:e2671.), zebrafish (Hwang et al., 2013, PLoS One, Vol. 8:e68708.), *C. elegans* (Hai et al., 2014 Cell Res. doi: 10.1038/cr.2014.11.), bacteria (Fabre et al., 2014, PLoS Negl. Trop. Dis., Vol. 8:e2671.), plants (Mali et al., 2013, Science, Vol. 339 : 823–826), *Xenopus tropicalis* (Guo et al., 2014, Development, Vol. 141 : 707–714.), yeast (DiCarlo et al., 2013, Nucleic Acids Res., Vol. 41 : 4336–4343.), *Drosophila* (Gratz et al., 2014 Genetics, doi:10.1534/genetics.113.160713), monkeys (Niu et al., 2014,

Cell, Vol. 156 : 836–843.), rabbits (Yang et al., 2014, J. Mol. Cell Biol., Vol. 6 : 97-99.), pigs (Hai et al., 2014, Cell Res. doi: 10.1038/cr.2014.11.), rats (Ma et al., 2014, Cell Res., Vol. 24 : 122–125.) and mice (Mashiko et al., 2014, Dev. Growth Differ. Vol. 56 : 122–129.). Several groups have now taken advantage of this method to introduce single point mutations (deletions or insertions) in a particular target gene, via a single gRNA. Using a pair of gRNA-directed Cas9 nucleases instead, it is also possible to induce large deletions or genomic rearrangements, such as inversions or translocations. A recent exciting development is the use of the dCas9 version of the CRISPR/Cas9 system to target protein domains for transcriptional regulation, epigenetic modification, and microscopic visualization of specific genome loci.

In some embodiment, the endonuclease is CRISPR-Cpf1 which is the more recently characterized CRISPR from *Provotella* and *Francisella* 1 (Cpf1) in Zetsche et al. (“Cpf1 is a Single RNA-guided Endonuclease of a Class 2 CRISPR-Cas System (2015); Cell; 163, 1-13).

In some embodiments, the macroH2A inhibitor is a Protein kinase C activator.

As used herein, the term “Protein kinase C” also known as PKC is a family of protein kinase enzymes that are involved in controlling the function of other proteins through the phosphorylation of hydroxyl groups of serine and threonine amino acid residues on these proteins. Typically, PKC activator is Phorbol 12-myristate 13-acetate (PMA), such as described in Tahara et al 2009.

In some embodiments, the macroH2A inhibitor is an EMT inducer.

As used herein, the term “the epithelial-mesenchymal transition” (EMT) is a process by which epithelial cells lose their cell polarity and cell-cell adhesion, and gain migratory and invasive properties to become mesenchymal stem cells; these are multipotent stromal cells that can differentiate into a variety of cell types. Typically, EMT inducers could be TGF-beta and Wnt 5a.

The step of culturing human somatic cells with the combination of compounds of the invention contained in a culture medium shall be carried out for the necessary time required for the production of iPS. The duration of this culture step may be determined easily by one of skill in the art. For instance, during the culture the person skilled in the art can monitor the cultured cells for the expression of markers specifically expressed by iPS (e.g. pluripotency genes such as OCT4, SOX2, NANOG and KLF4) or activity of said cells such as Alkaline Phosphatase. Monitoring of these markers can be performed using for instance RT-PCR

analysis of RNA extracted from cultured cells with specific primers, immunofluorescence analysis with antibodies specific of the markers, ELISA and FACS or any method to detect the RNA/protein/activity corresponding to the specific marker or activity.

Typically, the step of culturing somatic cells may be carried out for 1 to 20 days, preferably 10 days.

If necessary, the culture medium of the invention can be renewed, partly or totally, at regular intervals. Typically, the culture medium of the invention can be replaced with fresh culture medium of the invention every other day, for 10 days.

As used herein, the term "**culture medium**" refers to any medium capable of supporting the growth and the differentiation of definitive endoderm cells into hepatic progenitor cells. Preferred media formulations that will support the growth and reprogramming of a human somatic cell into a human iPS include chemically defined medium (CDM). As used herein, the term "**chemically defined medium**" (CDM) refers to a nutritive solution for culturing cells which contains only specified components, preferably components of known chemical structure. A chemically defined medium is a serum-free and feeder-free medium. As used herein, "serum-free" refers to a culture medium containing no added serum. As used herein, "feeder-free" refers to culture medium containing no added feeder cells.

The culture medium used by the invention may be a water-based medium that includes a combination of substances such as salts, nutrients, minerals, vitamins, amino acids, nucleic acids, proteins such as cytokines, growth factors (such as LIF, EGF and/or FGF) and hormones, all of which are needed for cell survival. For example, a culture medium according to the invention may be a synthetic tissue culture medium such as the DMEM (Dulbecco's Modified Eagle Medium) such as DMEM/F12, the RPMI (Roswell Park Memorial Institute medium) such as RPMI1640 or the CMRL-1066 (Connaught Medical Research Laboratory) for human use, supplemented with additives as is further described below (Section Examples) such as B27. The B-27 supplement (Invitrogen) contains SOD, catalase and other anti-oxidants, and unique fatty acids, such as linoleic acid and linolenic acid.

In a second aspect, the invention relates to an human iPS obtained by the method of the invention.

In a third aspect, the invention relates to a kit for reprogramming a somatic cell comprising at least a DNMT inhibitor, a Sirtuin inhibitor and a PARP inhibitor.

Typically, the kit according to the invention, wherein the DNMT inhibitor is 5-aza-2'-deoxycytidine (5-aza-dC; decitabine), the Sirtuin inhibitor is nicotinamide and the PARP inhibitor is NU1025 (8-hydroxy-2-methylquinazolinone).

The kit according to the invention, further comprising at least a JNK inhibitor, a p38 inhibitor or a SHH signaling pathway agonist.

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

FIGURES:

Figure 1: Levels of expression of pluripotency genes in human cells treated with one compound (A) or with a combination of three compounds (B).

Figure 2: Levels of expression of different genes relative to the level for non-treated hMep cells.

Figure 3: siRNA knockdown of the expression of mH2A1.1 strongly de-repress pluripotency genes in HCT116 isogenic cell line after 3i treatment. HCT116 wt cells were pre-treated with 5uM 5-AZA, 5uM 5-AZA + PARPi or 3i treatment for 3 days and then transfected with siRNA specific for mH2A.1 or with scramble siRNA. Forty-eight hours post-transfection the cells were starved and RNA isolated for RT-PCR analysis. (A) The siRNA treatment efficiently suppresses the expression of mH2A1. The level of mH2A was checked after siRNA knock-down by RT-PCR and normalized to both scramble and housekeeping gene. (B) Real-time PCR quantification of the relative expression of Oct4 in Hct116 pre-treated with 5uM 5-AZA, 5uM 5-AZA + PARPi or 3i transfected with siRNA specific for mH2A.1 or with scramble siRNA.

Figure 4: ETM induction cooperates with 3i in activation of endogenous Oct4 locus. HCT116 cells with Crispr/Cas integrated Cre recombinase into endogenous Oct4 locus were pre-treated with StemXVivo EMT Inducing Media Supplement (R&D Systems) (left panel) or PMA (right panel) and 3i cocktail was added 3 days later. The analysis of endogenous Oct4 locus was carried out 24h later. Bright green cells represent cells expressing high level of endogenous Oct4.

EXAMPLE 1: Identifying the combination of chemical drugs to improve the re-activation of pluripotent genes.

Material & Methods

Cell culture: The human colon cancer cell lines HCT116 were maintained in DMEM containing 10% heat-inactivated FCS, 100 U/ml penicillin and 1mM glutamine. The human breast cancer cell lines MCF7 were maintained in RPMI1640 containing 10% of heat-inactivated FCS, 100 U/ml penicillin and 1mM glutamine and 1mM AA. Both cell lines were incubated at 37°C, 5% CO₂-95% air using standard tissue culture incubators.

Drugs treatment: Both MCF7 and HCT116 cells were sited at 30% confluence on 6 well plate 1 day before treatment. To assess the effect of drugs treatment on reactivation of pluripotent genes, we used the following drugs/concentrations containing 5-aza-dC 5µM; Nicotinamide 15mM; PARPi NU1025 10µM. The drugs were changed every second day and the samples were analysed after 10 days.

Reverse transcription polymerase chain reaction (RT-PCR) and Real-time quantitative PCR: Total RNA was extracted by using a commercial kit (Trizol) according to the manufacturer's instructions (Gibco BRL). c-DNA was prepared using 1µg of total RNA using First Strand c-DNA Synthesis Kit (Thermo SCIENTIFIC) according to manufacturer's instruction. The mRNA transcription levels of pluripotency genes including *OCT4*, *SOX2*, *NANOG* and *KLF4* were determined using a real-time quantitative PCR system. Relative quantitation was performed using the comparative Ct method with data from the ABI PRISM 7000 Detection System following the manufacturer's protocol. The Ct values were measured, and the average Ct of triplicate samples was calculated. Alteration of mRNA expression was

defined as a 3-fold difference in the expression level after treatment, relative to that before treatment and to Gapdh or 18S.

Results

5

It is important to point out that pluripotent genes are silent in adult tissues with only exception for germ cells. Based on our previous analysis of Wip1 deficient cells, we speculated that chemical treatment with a specific set of inhibitors could result in re-activation of pluripotency genes". To access this further, we analyzed the epigenetic regulation of pluripotent-associated genes *NANOG*, *OCT4*, *c-MYC*, *KLF4*, and *SOX2*, and their correlation with gene expression in different cancer cell lines using a combination of epigenetic and DNA damage modulators with known chemical compounds. Our results show that treatment of MCF7 and HCT116 cells with a combination of Sirtuin inhibitor Nicotinamide, inhibitor of PARP PARPi and the methyltransferase inhibitor 5-aza-dC increased the expression of pluripotency genes (Figure 1B). In contrast, single drug treatment did not effect pluripotent gene expression (Figure 1A) suggesting that combination of drugs is required for reprogramming of cancer cells.

EXAMPLE 2: Induction of pluripotent genes in primary human cells and improvement of the protocol with additional cocktail of the chemical compounds.

Material & Methods

Human primary mammary gland epithelial cells (hMep) were plated on 6-well plates in DMEM/F12 medium containing 20ng/ml human LIF and B27. The drug treatment started on day 2 after plating and continued for 10 days. The drugs were changed every second day. We used the following drug combinations: 1% DMSO as a control; 3i (containing 5-aza-dC 5µM; Nicotinamide 15mM; PARPi NU1025 10µM) and 6i (3i + JNK inhibitor Bentamapimod 10 µM; p38 inhibitor BIRB 796 3 µM and SHH agonist Purmorphamine 1 µM). On day 11, the cells were harvested and mRNA was purified for analysis of pluripotent genes. Total RNA from cells was isolated using RNeasy mini kit (Qiagen) according the standard protocol. 1µg of RNA was reverse transcribed into oligo-dT primed cDNA with PowerScript II Reverse transcriptase (Life Technology) and 40-50 ng of cDNA were used for individual PCR. The quantitative PCR were performed using KAPA SYBR Fast qPCR kit

(KAPA Biosystems) according to manufacturer protocol in Applied Biosystems 7300 Real-Time PCR System. The collected Ct data were normalised to GAPDH Ct and quantified to obtain relative fold changes using Microsoft Excel program.

5 **Results**

10 The results in Figure 2 show the levels of expression of different genes relative to the level for non-treated hMep cells. The results show that while 3i had a significant effect on re-activation of pluripotent genes in primary human epithelial cells, the 6i combination provided even better induction. These data suggest that while 3i could be used as a base combination for reactivation of pluripotent genes, there are further ways of improving this protocol by inclusion of additional chemical compounds.

15

EXAMPLE 3: Acquisition of pluripotent state after 10 days of treatment.

Material & Methods

20 Human primary mammary gland epithelial cells (hMep) were plated on 6-well plates in DMEM/F12 medium containing 10ng/ml EGF, 10 ng/ml FGF and 1% B27. The drug treatment started second day after plating and continued for 10 days. The drugs were changed every day for the first 4 days and subsequently every second day for the rest of the treatment. We used the following drug combinations: 1% DMSO as a control; 3i (containing 5-aza-dC
25 5µM; Nicotinamide 15mM; PARPi NU1025 10µM) and 6i (3i + JNK inhibitor Bentamapimod 10 µM; p38 inhibitor BIRB 796 3 µM and SHH agonist Purmorphamine 1 µM). On day 11 cells were washed with PBS and fixed 15 min at room temperature in Histochoice MB ("Amresco"). The undifferentiated state of chemically-induced pluripotent cells was analyzed by the activity of Alkaline Phosphatase using BCIP/NBT solution
30 ("Amresco"). Alkaline phosphatase activity is considered to be a marker of pluripotency in embryonic stem cells and is detected in iPS after the reprogramming process using a standard protocol with overexpression of 4 factors, Oct4, Klf4, Sox2 and Myc.

Results

A significant number of colonies that were formed after 10 days of treatment with 6i showed very strong alkaline phosphatase activity. At the same time, no positive clones were observed either in DMSO or 3i-treated samples. These data strongly support that 6i protocol is efficient in reprogramming human primary mammary gland epithelial cells into embryonic-like stem cells.

EXAMPLE 4: Acquisition of pluripotent state requires inhibition of PARP.

Material & Methods

Human primary mammary gland epithelial cells (hMep) were plated on 6-well plates in DMEM/F12 medium containing 10ng/ml EGF, 10ng/ml FGF and 1% B27. The drug treatment started second day after plating and continued for 10 days. The drugs were changed every day for the first 4 days and subsequently every second day for the rest of the treatment. We used the following drug combinations: 4i (containing 5-aza-dC 5 μ M; JNK inhibitor Bentamapimod 10 μ M; p38 inhibitor BIRB 796 3 μ M and SHH agonist Purmorphamine 1 μ M) plus Nicotinamide 15 μ M; 4i plus Tenovin-1 10 μ M; 4i plus Tenovin-6 10 μ M; 4i plus AK-1 10 μ M; 4i plus AK-7 10 μ M; 4i plus Selistal 10 μ M. All chemicals were from Axon Medchem. On day 11 cells were washed with PBS and fixed 15 min at room temperature in Histochoice MB ("Amresco"). The undifferentiated state of chemically-induced pluripotent cells was analyzed by the activity of Alkaline Phosphatase using BCIP/NBT solution ("Amresco"). Alkaline phosphatase activity is considered to be a marker of pluripotency in embryonic stem cells and is detected in iPS after the reprogramming process using a standard protocol with overexpression of 4 factors, Oct4, Klf4, Sox2 and Myc.

Results

This set of experiments was to determine whether Parp inhibition is required for reprogramming. This question arises because it was reported that Nicotinamide can inhibit Sirt but also to lesser extend Parp. To address this question, we used 5 additional selective inhibitors of Sirt as stated in Table 1. This table shows that alkaline-positive colonies were

formed after 10 days of treatment only when Nicotinamide was used; no positive colonies appeared when selective inhibitors of Sirt were used.

This data strongly suggest that simultaneous inhibition of Sirt and Parp is required for efficient reprogramming of human somatic cells.

5

**Table 1: Analysis of different Sirt inhibitors
in chemical reprogramming of human somatic cells.**

Sirt inhibitors used in addition to 4i combination	Presence of alkaline phosphatase-positive clones
Nicotinamide	+++
Tenovin 1	---
Tenovin 6	---
AK-1	---
AK-7	---
Selistat	---

10

EXAMPLE 5: OPTIMIZATION OF THE EFFECT 3i ON INDUCTION OF REPROGRAMMING

In order to further optimize the effect of 3i on induction of reprogramming, we identified a transcriptional repressor, macroH2A (presented by 2 isoforms macroH2A.1 and macroH2A.2), as a bottleneck in rapid activation of endogenous Oct4 gene. The incubation with 3i results in DNA demethylation of Oct4 locus, which naturally should results in transcriptional activation. However, this may not happen under conditions when macroH2A is compensatory accumulated. To understand if this is the case, we knocked down the level of macroH2A in HCT116 cells. We found an efficient reduction in the level of macroH2A.1 isoform (the major isoform) throughout different conditions as shown in Figure 3A. Next we analyzed the level of endogenous Oct4. We found that a knockdown of macroH2A.1 efficiently reactivated endogenous Oct4 locus only in combination with 3i treatment (Figure

15

20

3B). No cooperative effect was found when cells were pretreated with either individual drugs or in a combination of any of the 2 drugs. This data strongly suggest that the effect of 3i treatment could be strongly improved by reducing the level of macroH2A.

5 **EXAMPLE 6: EFFECT OF 3i DURG TREATMENT ON REACTIVATION OF ENDOGENOUS OCT4**

As a knockdown of macroH2A significantly improved the effect of 3i drug treatment on reactivation of endogenous Oct4 locus, we started to look for conditions that would allow a
10 downregulation of macroH2A expression. Our rational was that if such conditions exist, cells treatment with 3i under these conditions will result in rapid reactivation of endogenous Oct4 locus. For this type of experiments, we generated a novel cell line where we targeted endogenous Oct4 locus with Cre-ERT2 inducible system using Crispr/Cas technology. We further introduce a reporter system that could produce a GFP when Oct4 is expressed and
15 tamoxifen is present. This novel system is an example of a lineage tracing experiments in human cells HCT116.

So far, we identified 2 conditions that allow efficient induction of endogenous Oct4 gene in the presence of 3i treatment. One is the condition of induction of epithelial-mesenchymal transition (EMT). As shown in Figure 2, left panel, a pre-treatment with
20 StemXVivo EMT Inducing Media Supplement (R&D Systems) for 3 days can significantly improve the effect of 3i on induction of endogenous Oct4 gene.

The second condition is the pre-treatment with Protein kinase C activator, PMA. Similar to EMT induction, this pre-treatment has a strong cooperative effect with 3i treatment
25 on induction of endogenous Oct4 locus (Figure 2, right panel).

As a conclusion, we believe that any condition that can reduce the level of expression of macroH2A would work in cooperation with 3i treatment to induce endogenous Oct4 locus and thus in reprogramming into iPS.

REFERENCES:

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
5 reference into the present disclosure.

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Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson JA. Induced pluripotent stem cell lines derived from human somatic cells. Science 318 (5858), 1917-1920 (2007).

CLAIMS:

1. A method for reprogramming a human somatic cell into a human induced pluripotent stem cell (iPS) comprising a step of culturing said somatic cell into the presence of at least a combination of a DNA methyltransferase (DNMT) inhibitor, a Sirtuin inhibitor and a poly(ADP-ribose)polymerase (PARP) inhibitor.
2. The method according to claim 1, wherein the DNMT inhibitor is selected from the group consisting of 5-azacytidine (5-aza-CR; vidaza), 5-aza-2'-deoxycytidine (5-aza-dC; decitabine), 1-[beta]-D-arabinofuranosyl-5-azacytosine, dihydro-5-azacytidine, zebularine, sinefungin, 5-fluoro-2'-deoxycytidine (FdCyd).
3. The method according to claim 1, wherein the Sirtuin inhibitor is selected from the group consisting of Nicotinamide, Carbamido-NAD, NADH, Dihydrocoumarin, A3, Splitomicin, 2-OH-naphtaldehyde, Sirtinol and M15.
4. The method according to claim 1, wherein the PARP inhibitor is selected from the group consisting of NU1025 (8-hydroxy-2-methylquinazolinone), AZD-2281 (olaparib), AG014699 (rucaparib), ABT-888 (veliparib), BSI-201 (iniparib), CEP-9722, MK-4827, BMN-673, AG14361, Nicotinamide and 3-AB (3-aminobenzamide).
5. The method according to any one claim 1 to 4, wherein the DNMT inhibitor is 5-aza-2'-deoxycytidine (5-aza-dC; decitabine), the Sirtuin inhibitor is Nicotinamide and the PARP inhibitor is NU1025 (8-hydroxy-2-methylquinazolinone).
6. The method according to any one claim 1 to 4, wherein the Sirtuin inhibitor and the PARP inhibitor is Nicotinamide.
7. The method according to any one claim 1 to 6, wherein the somatic said cell is further cultured in the presence of at least one compound selected from the group consisting of a c-Jun N-terminal kinase (JNK) inhibitor, a p38 mitogen-activated protein (MAP) kinase inhibitor and a Sonic Hedgehog (SHH) signaling pathway agonist.
8. The method according to claim 7, wherein the JNK inhibitor is AS602801 (bentamapimod).

9. The method according to claim 7, wherein the p38 inhibitor is BIRB-796 (doramapimod).

10. The method according to claim 7, wherein the SHH signaling pathway agonist is purmorphamine.

5 11. The method according to claims 1 to 6, wherein the somatic said cell is further cultured in the presence of macroH2A inhibitor.

12. The method according to claim 11, wherein the macroH2A inhibitor is siRNA.

10 13. The method according to any one claim 1 to 12, wherein the somatic cell is an epithelial cell, a fibroblast, a muscle cell, a cumulus cell, a neural cell, a liver cell, a GI tract cell, a mammary cell, a kidney cell, a blood cell, a vascular cell, a skin cell, an immune system cell, a lung cell, a bone cell, keratinocyte, or a pancreatic islet cell.

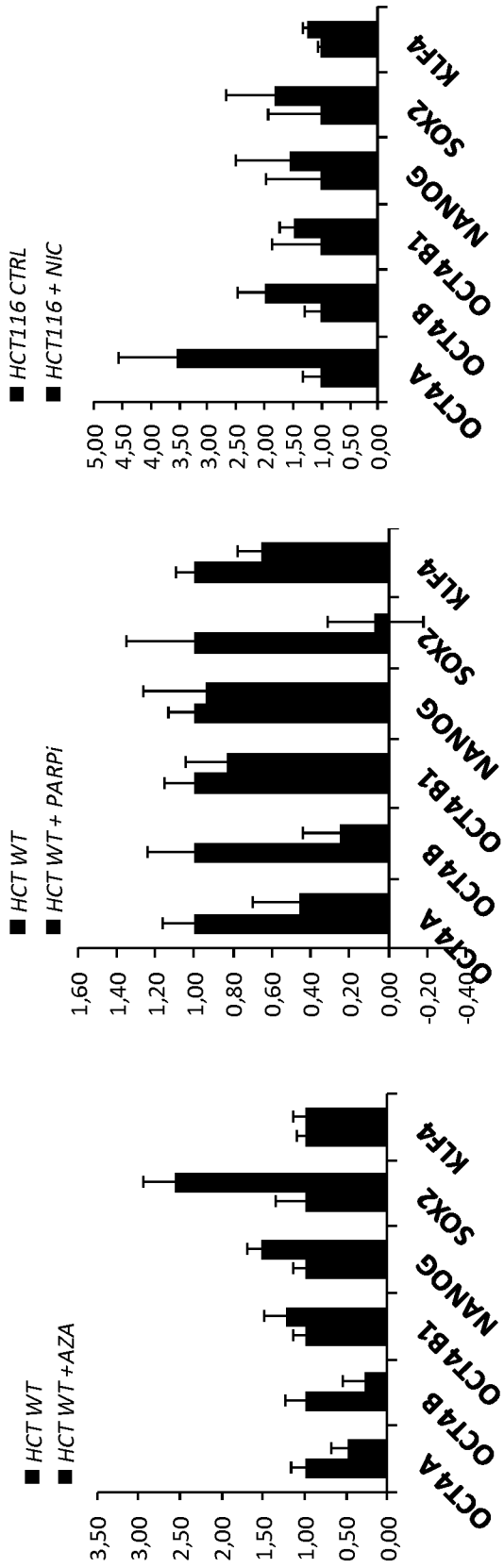
14. A human iPS obtained by the method according to any one of claims 1 to 13.

15. A kit for reprogramming a somatic cell comprising at least a DNMT inhibitor, a Sirtuin inhibitor and a PARP inhibitor.

15 16. The kit according to claim 15, wherein the DNMT inhibitor is 5-aza-2'-deoxycytidine (5-aza-dC; decitabine), the Sirtuin inhibitor is nicotinamide and the PARP inhibitor is NU1025 (8-hydroxy-2-methylquinazolinone).

17. The kit according to claim 15 or 16, further comprising at least a JNK inhibitor, a p38 inhibitor or a SHH signaling pathway agonist.

A.



B.

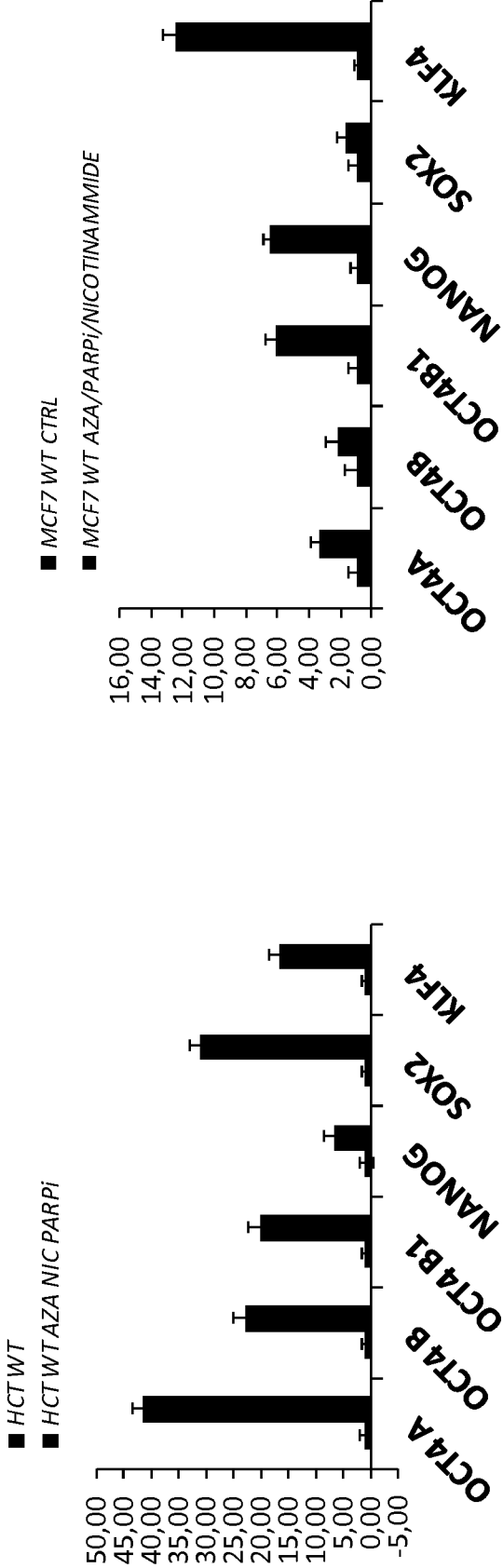


Figure 1

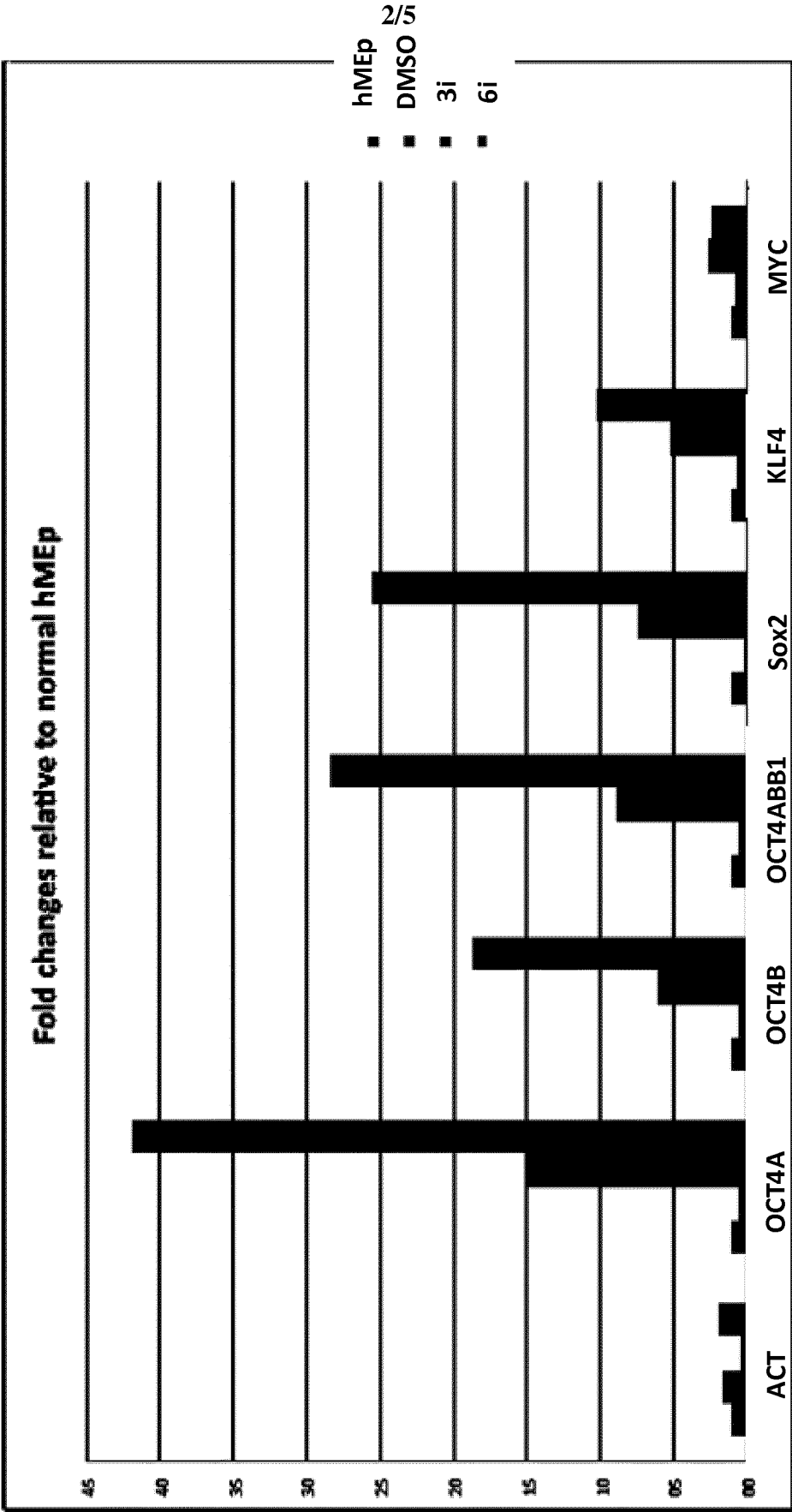


Figure 2

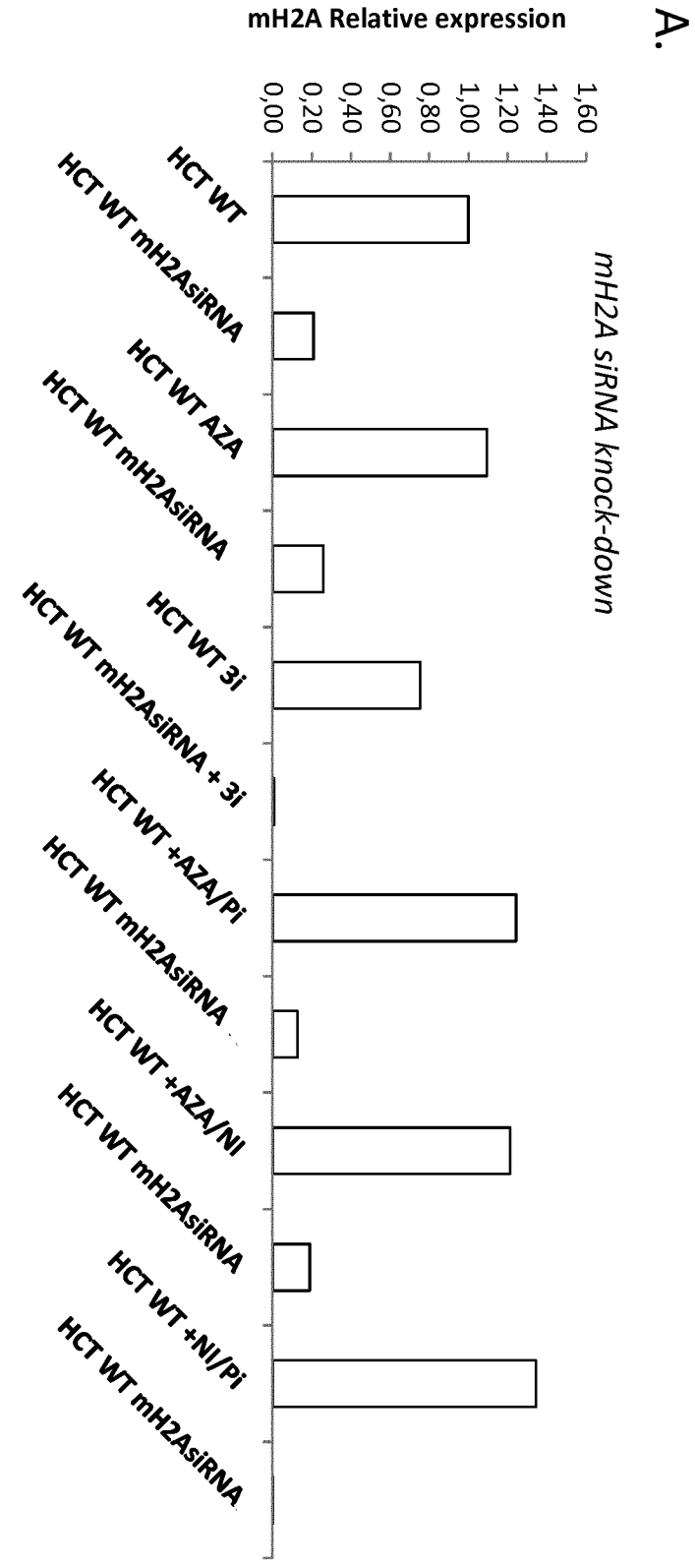


Figure 3A

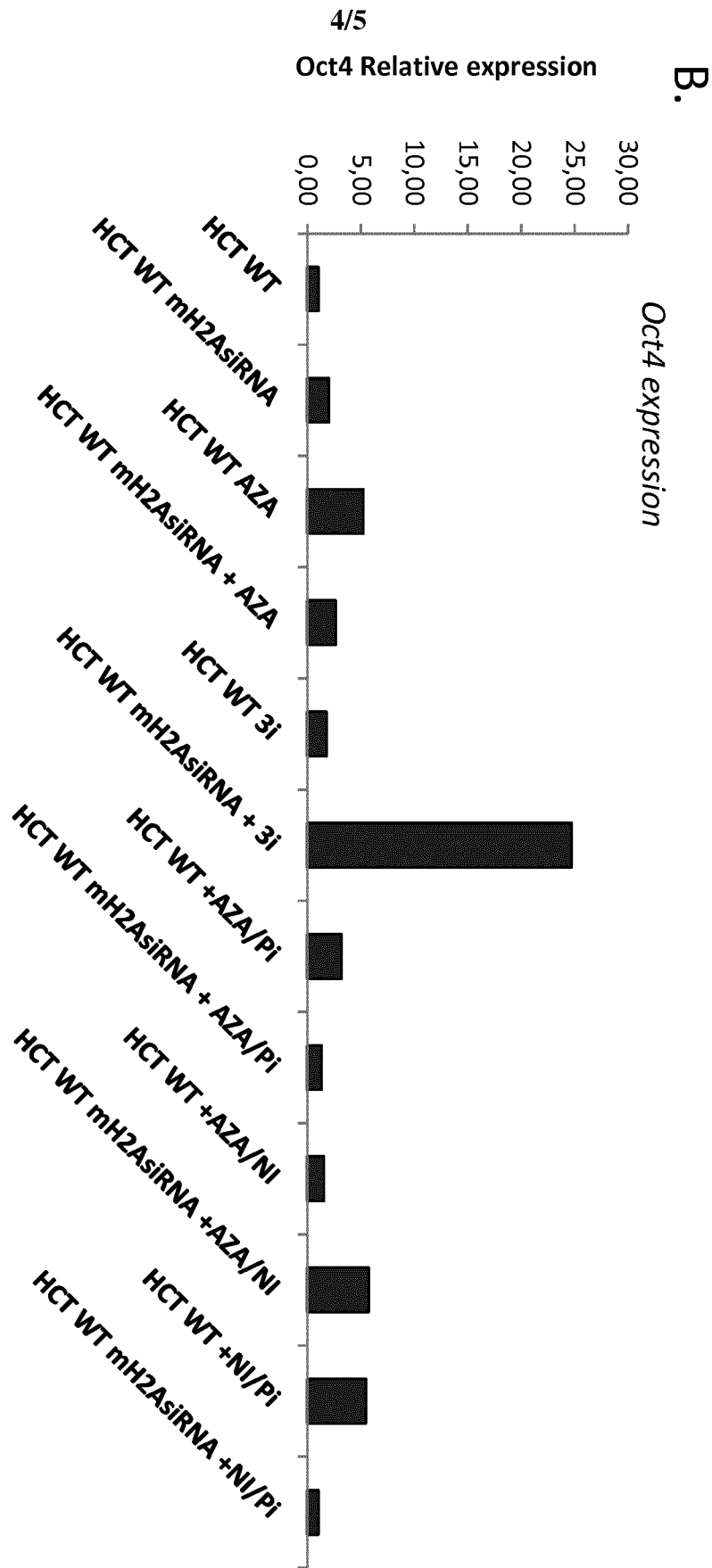


Figure 3B

5/5

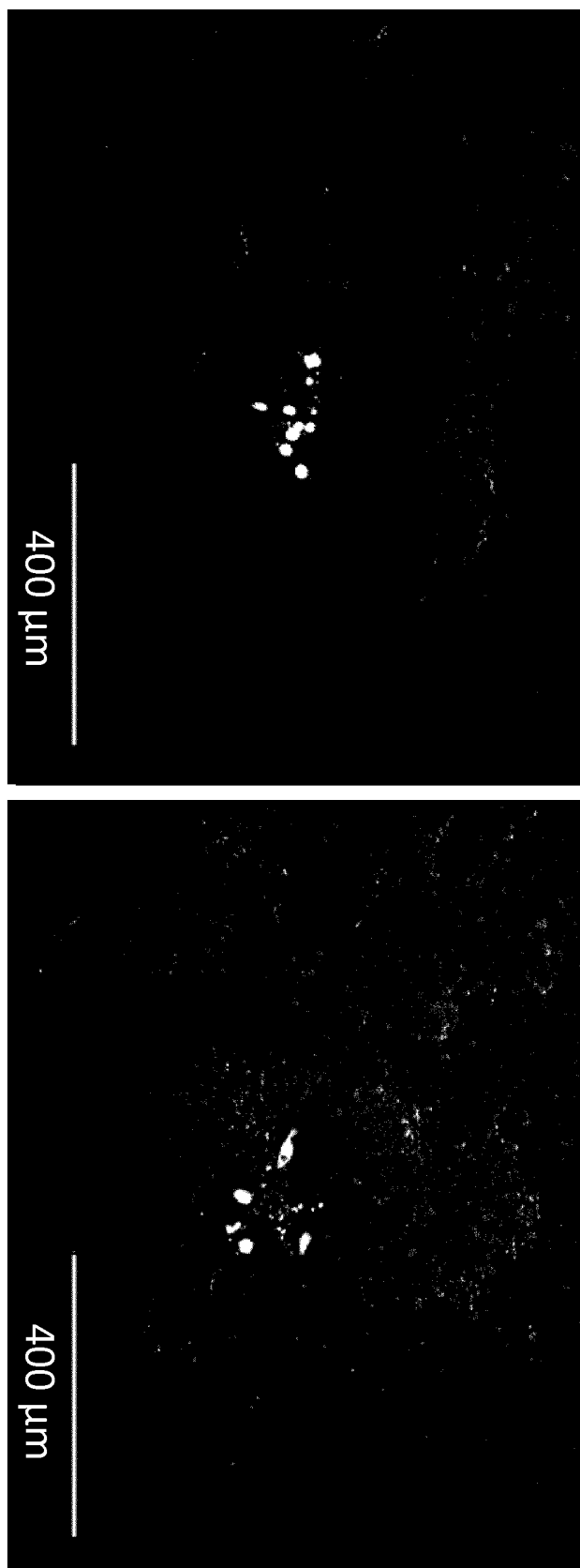


Figure 4

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/055029

A. CLASSIFICATION OF SUBJECT MATTER
INV. 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	EP 2 537 920 A1 (UNIV SAITAMA MEDICAL [JP]) 26 December 2012 (2012-12-26) cited in the application example 7	1-10, 13-17
X	----- WO 2009/102983 A2 (HARVARD COLLEGE [US]; HUANGFU DANWEI [US]; MELTON DOUGLAS A [US]; MAEH) 20 August 2009 (2009-08-20) the whole document	1-10, 13-17
X	----- WO 2009/061442 A1 (CHILDRENS MEDICAL CENTER [US]; PARK IN-HYUN [US]; DALEY GEORGE Q [US];) 14 May 2009 (2009-05-14) the whole document ----- -/-	14



Further documents are listed in the continuation of Box C.



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

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"O" document referring to an oral disclosure, use, exhibition or other means

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"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

7 April 2016

Date of mailing of the international search report

14/04/2016

Name and mailing address of the ISA/

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Authorized officer

Trommsdorff, Marion

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2016/055029

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
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X	HUANGFU DANWEI ET AL: "Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds", NATURE BIOTECHNOLOGY, NATURE PUBLISHING GROUP, US, vol. 26, no. 7, 1 July 2008 (2008-07-01), pages 795-797, XP002502536, ISSN: 1087-0156, DOI: 10.1038/NBT1418 [retrieved on 2008-06-22] last paragraph; figure 1 -----	15-17
A	MIKKELSEN TARJEI S ET AL: "Dissecting direct reprogramming through integrative genomic analysis", NATURE, NATURE PUBLISHING GROUP, UNITED KINGDOM, vol. 454, no. 7200, 3 July 2008 (2008-07-03), pages 49-55, XP002564354, ISSN: 0028-0836, DOI: 10.1038/NATURE07056 [retrieved on 2008-05-28] figure 4 -----	1-17
A	AGNÈS I. LUKASZEWICZ ET AL: "Small Molecules and Stem Cells. Potency and Lineage Commitment: The New Quest for the Fountain of Youth", JOURNAL OF MEDICINAL CHEMISTRY, vol. 53, no. 9, 13 May 2010 (2010-05-13), pages 3439-3453, XP055191381, ISSN: 0022-2623, DOI: 10.1021/jm901361d the whole document -----	1-17
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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2016/055029

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2016/055029

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