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DESCRIPTION

[0001] This invention was made with United States government support awarded by the following agencies: NIH GM081629, RR000167. The United States government has certain rights in this invention.

BACKGROUND

[0002] Embryonic stem (ES) cells hold great promise in science and medicine due to their pluripotent nature, i.e. the ability to replicate indefinitely and differentiate into cells of all three germ layers (Thomson et al., Science 282:1145-1147 (1998)). The application of human ES cells in therapy and regenerative medicine is complicated by the possibility of rejection by the recipient's immune system. Human pluripotent cells that are substantially genetically identical to a particular recipient are, thus, highly desirable. Also, genetic identity may be important for the use of ES cells in designing patient-specific treatment strategies.

[0003] First attempts to generate pluripotent cells from a post-natal primate individual employed somatic nuclear transfer (*see, e.g.,* Byrne, JA et al., Nature 450:497-502 (2007)) and cell fusion (see, e.g., Yu, J et al., Stem Cells 24:168-176 (2006)). However, clinical use of somatic nuclear transfer is impractical due to its low efficiency, while cell fusion results in near tetraploid cells. In 2007, two groups of scientists reprogrammed somatic cells from a post-natal primate individual into pluripotent stem cells (Yu et al., Science 318:1917-1920 (2007) and Takahashi et al., Cell 131:861-872 (2007)). Both groups delivered into, and expressed in, human somatic cells cDNA of four transcription factors using a viral vector system for expressing potency-determining transgenes. The transcription factors of Takahashi *et al.* were *OCT4, SOX2, c-Myc,* and *KLF4,* while Yu *et al.* employed *OCT4, SOX2, NANOG,* and *LIN28.* The expression of these sets of transcription factors induced human somatic cells to acquire ES cell-specific characteristics, including morphology, proliferation, and gene- and surface marker expression. Somatic cells reprogrammed in this manner are referred to as induced pluripotent (iPS) cells. The existence of iPS cells circumvents the need for blastocysts and reduces concerns associated with immune rejection.

[0004] Shortly thereafter, Lowry *et al.* generated patient-specific iPS cell lines through ectopic expression of *OCT4, SOX2, c-Myc,* and *KLF4* (Lowry et al., PNAS 105:2883-2888 (2008)) transgenes. More recently, iPS cells have been generated from a number of different human and murine somatic cell types, such as epithelial, fibroblast, liver, stomach, neural, and pancreatic cells. Further, iPS cells have been successfully differentiated into cells of various lineages (*e.g.*, Dimos et al., Science 321:1218-1221 (2008)). WO 2009149233 refers to methods for the production of IPS cells using non-viral approach.

[0005] Current methods for generating iPS cells employ retroviral vectors such as those derived from lentivirus. These vectors stably integrate into, and permanently change, a target

cell's DNA at virtually any chromosomal locus. This untargeted interaction between reprogramming vector and genome is associated with a risk of aberrant cellular gene expression as well as neoplastic growth caused by viral gene reactivation (Okita et al. Nature 448:313-317 (2007)).

[0006] Moreover, continued presence and expression of the transgenes can interfere with the recipient cell's physiology. Further, ectopic expression of transcription factors used to reprogram somatic cells, such as *c-Myc,* can induce programmed cell death (apoptosis) (Askew et al., Oncogene 6:1915-1922 (1991), Evan et al., Cell 69:119-128 (1992)). Furthermore, continued expression of factors such as *OCT4* can interfere with subsequent differentiation of iPS cells.

[0007] It is desirable to reprogram somatic cells to a state of higher potency without altering the cells' genetic makeup beyond the reprogramming-associated alterations. Recently, Stadtfeld *et al.* generated murine iPS cells using a nonintegrating adenovirus that transiently expressed *OCT4, SOX2, KLF4,* and *c-Myc* (Stadtfeld et al., Sciencexpress, Sep. 25, 2008). To date, primate iPS cells generated without using retroviral vectors have not been reported.

BRIEF SUMMARY

[0008] The present invention provides an enriched population of primate induced pluripotent cells which are substantially genetically identical to a post-natal individual, wherein the population is produced according to a method comprising the steps of:
introducing a plurality of non-viral episomal vectors encoding one or more potency-determining factors into the primate somatic cells under conditions sufficient to express the potency-determining factors, thereby reprogramming the somatic cells to produce the primate pluripotent cells;

wherein at least some of the primate induced pluripotent cells in the enriched population comprise one or more copies of the plurality of non-viral episomal vectors;

wherein the plurality of non-viral episomal vectors is selected from the group consisting of:

1. (a) a first vector comprising, in order, a first promoter, *OCT4,* IRES2, *SOX2,* a second promoter, *SV40 T* antigen, IRES2, and *KLF4,* a second vector comprising, in order, a third promoter, *OCT4,* IRES2, *SOX2,* a fourth promoter, *NANOG,* IRES2, and *KLF4,* and a third vector comprising, in order, a fifth promoter, *c-Myc,* IRES 2, and *LN28,* wherein the promoters need not be identical;
2. (b) a first vector comprising, in order, a first promoter, *OCT4,* IRES2, *SOX2,* a second promoter, *KLF4,* IRES2, *c-Myc,* a third promoter, *NANOG,* IRES2, and *LN28,* a second vector comprising, in order, a fourth promoter, *OCT4,* IRES2, *SOX2,* a fifth promoter, *SV40 T* antigen, IRES2, and *KLF4,* wherein the promoters need not be identical; and
3. (c) a first vector comprising, in order, a first promoter, *OCT4,* IRES2, *SOX2,* a second promoter, *NANOG,* IRES2, and *LN28,* a second vector comprising, in order, a third

promoter, *OCT4,* IRES2, *SOX2,* a fourth promoter, *SV40 T* antigen, IRES2, and *KLF4,* and a third vector comprising, in order, a fifth promoter, *OCT4,* IRES2, *SOX2,* a sixth promoter, *c-Myc,* IRES 2, and *KLF4,* wherein the promoters need not be identical.

[0009] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although suitable materials and methods for the practice or testing of the present invention are described below, other materials and methods similar or equivalent to those described herein, which are well known in the art, can be used.

[0010] Other objectives, advantages and features of the present invention will become apparent from the following specification taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

[0011]

FIG. 1A-B illustrate the effect on reprogramming efficiency of different nucleotide sequences that link transgenes on the vector(s) delivered during the reprogramming methods.

FIG. 2A-C illustrate the effect on reprogramming efficiency of *c-Myc, KLF-4,* and SV40 large T antigen gene expression in human newborn foreskin fibroblasts.

FIG. 3A-C illustrate a suitable construct for carrying transgenes into somatic cells in accord with the method, temporal expression of an episomal vector-mediated transgene, and the effect of vector quantity on cell survival after nucleofection.

FIG. 4A-D illustrate reprogramming of human newborn foreskin fibroblasts with episomal vector-mediated transgene expression.

FIG. 5A-B illustrate related constructs harboring an expression cassette useful in the reprogramming methods.

DETAILED DESCRIPTION

[0012] Described herein is a method for producing a primate pluripotent cell which includes the step of delivering into a primate somatic cell a set of transgenes sufficient to reprogram the somatic cell to a pluripotent state, the transgenes being carried on at least one episomal vector that does not encode an infectious virus, and recovering pluripotent cells. References herein to a "non-viral" vector or construct indicate that the vector or construct cannot encode an

infectious virus.

[0013] Also described herein is an enriched population of replenishable reprogrammed pluripotent cells of a primate, including a human primate, wherein, in contrast to existing iPS cells, the at least one vector, including any element thereof having a viral source or derivation is substantially absent from the pluripotent cells. As used herein, this means that the reprogrammed cells contain fewer than one copy of the episomal vector per cell, and preferably no residual episomal vector in the cells. Because asymmetric partitioning during cell division dilutes the vector, one can readily obtain reprogrammed cells from which the vector has been lost. As noted elsewhere herein, on very rare occasions a reprogramming vector can integrate into the genome of the cell, but cells having an integrated vector can be avoided by screening for absence of the vector. Further, in contrast to existing ES cells, the primate pluripotent cells are substantially genetically identical to somatic cells from a fetal or post-natal individual. Fetal cells can be obtained from, e.g., amniotic fluid. The cells of the enriched population are not readily distinguished from existing primate ES and iPS cells morphologically (*i.e.*, round shape, large nucleoli and scant cytoplasm) or by growth properties (*i.e.,* doubling time; ES cells have a doubling time of about seventeen to eighteen hours). Like iPS cells and ES cells, the reprogrammed cells also express pluripotent cell-specific markers (*e.g., OCT-4, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81,* but not *SSEA-1*). Unlike ES cells, the reprogrammed cells are not immediately derived from embryos. As used herein, "not immediately derived from embryos" means that the starting cell type for producing the pluripotent cells is a non-pluripotent cell, such as a multipotent cell or terminally differentiated cell, such as somatic cells obtained from a fetal or post-natal individual. Like iPS cells, the pluripotent cells produced in the method can transiently express one or more copies of selected potency-determining factors during their derivation.

[0014] Described herein are novel methods for reprogramming differentiated primate somatic cells into reprogrammed primate cells that are substantially free of the vectors used in their production by introducing potency-determining factors on a non-viral vector that is present during reprogramming, but is substantially absent from the reprogrammed cells. As used herein, "reprogramming" refers to a genetic process whereby differentiated somatic cells are converted into de-differentiated cells having a higher potency than the cells from which they were derived.

[0015] Advantageously, the higher potency cells produced in the method are euploid pluripotent cells. As used herein, "pluripotent cells" refer to a population of cells that express pluripotent cell-specific markers, have a cell morphology characteristic of undifferentiated cells (*i.e.*, compact colony, high nucleus to cytoplasm ratio and prominent nucleolus) and can differentiate into all three germ layers (*e.g.*, endoderm, mesoderm and ectoderm). When introduced into an immunocompromised animal, such as a SCID mouse, the pluripotent cells form teratomas that typically contain cells or tissues characteristic of all three germ layers. One of ordinary skill in the art can assess these characteristics by using techniques commonly used in the art. *See, e.g.,* Thomson *et al., supra.* Pluripotent cells are capable of both proliferation in cell culture and differentiation towards a variety of lineage-restricted cell populations that

exhibit multipotent properties. Pluripotent cells have a higher potency than somatic multipotent cells, which by comparison are more differentiated, but which are not terminally differentiated. The pluripotent products of primate somatic cell reprogramming methods are referred to herein as "reprogrammed primate pluripotent cells" or as induced pluripotent (iPS) cells. Such cells are suitable for use in research and therapeutic applications currently envisioned for human ES cells or existing iPS cells.

[0016] Differentiated somatic cells, including cells from a fetal, newborn, juvenile or adult primate, including human, individual, are suitable starting cells in the methods. Suitable somatic cells include, but are not limited to, bone marrow cells, epithelial cells, endothelial cells, fibroblast cells, hematopoietic cells, keratinocytes, hepatic cells, intestinal cells, mesenchymal cells, myeloid precursor cells and spleen cells. Another suitable somatic cell is a $CD29^+$ $CD44^+$ $CD166^+$ $CD105^+$ $CD73^+$ and $CD31^-$ mesenchymal cell that attaches to a substrate. Alternatively, the somatic cells can be cells that can themselves proliferate and differentiate into other types of cells, including blood stem cells, muscle/bone stem cells, brain stem cells and liver stem cells. Suitable somatic cells are receptive, or can be made receptive using methods generally known in the scientific literature, to uptake of potency-determining factors including genetic material encoding the factors. Uptake-enhancing methods can vary depending on the cell type and expression system. Exemplary conditions used to prepare receptive somatic cells having suitable transduction efficiency are well-known by those of ordinary skill in the art. The starting somatic cells can have a doubling time of about twenty-four hours.

[0017] The vectors described herein can be constructed and engineered using methods generally known in the scientific literature to increase their safety for use in therapy, to include selection and enrichment markers, if desired, and to optimize expression of nucleotide sequences contained thereon. The vectors should include structural components that permit the vector to self-replicate in the somatic starting cells. For example, the known Epstein Barr oriP/Nuclear Antigen-1 (EBNA-1) combination (see, e.g., Lindner, S.E. and B. Sugden, The plasmid replicon of Epstein-Barr virus: mechanistic insights into efficient, licensed, extrachromosomal replication in human cells, Plasmid 58:1 (2007)) is sufficient to support vector self-replication and other combinations known to function in mammalian, particularly primate, cells can also be employed. Standard techniques for the construction of expression vectors suitable for use in the present invention are well-known to one of ordinary skill in the art and can be found in publications such as Sambrook J, et al., "Molecular cloning: a laboratory manual," (3rd ed. Cold Spring harbor Press, Cold Spring Harbor, N.Y. 2001).

[0018] In the methods, genetic material encoding a set of potency-determining factors is delivered into the somatic cells via one or more reprogramming vectors. Suitable potency-determining factors can include, but are not limited to OCT-4, SOX2, LIN28, NANOG, c-Myc, KLF4, and combinations thereof. Each potency-determining factor can be introduced into the somatic cells as a polynucleotide transgene that encodes the potency-determining factor operably linked to a heterologous promoter that can drive expression of the polynucleotide in the somatic cell. Although SV40 T Antigen is not a potency-determining factor *per se,* it advantageously introduced into somatic cells as it provides the cells with a condition sufficient

to promote cell survival during reprogramming while the potency-determining factors are expressed. Other conditions sufficient for expression of the factors include cell culture conditions described in the examples.

[0019] Suitable reprogramming vectors are episomal vectors, such as plasmids, that do not encode all or part of a viral genome sufficient to give rise to an infectious or replication-competent virus, although the vectors can contain structural elements obtained from one or more virus. One or a plurality of reprogramming vectors can be introduced into a single somatic cell. One or more transgenes can be provided on a single reprogramming vector. One strong, constitutive transcriptional promoter can provide transcriptional control for a plurality of transgenes, which can be provided as an expression cassette. Separate expression cassettes on a vector can be under the transcriptional control of separate strong, constitutive promoters, which can be copies of the same promoter or can be distinct promoters. Various heterologous promoters are known in the art and can be used depending on factors such as the desired expression level of the potency-determining factor. It can be advantageous, as exemplified below, to control transcription of separate expression cassettes using distinct promoters having distinct strengths in the target somatic cells. Another consideration in selection of the transcriptional promoter(s) is the rate at which the promoter(s) is silenced in the target somatic cells. The skilled artisan will appreciate that it can be advantageous to reduce expression of one or more transgenes or transgene expression cassettes after the product of the gene(s) has completed or substantially completed its role in the reprogramming method. Exemplary promoters are the human EF1α elongation factor promoter, CMV cytomegalovirus immediate early promoter and CAG chicken albumin promoter, and corresponding homologous promoters from other species. In human somatic cells, both EF1α and CMV are strong promoters, but the CMV promoter is silenced more efficiently than the EF1α promoter such that expression of transgenes under control of the former is turned off sooner than that of transgenes under control of the latter.

[0020] The potency-determining factors can be expressed in the somatic cells in a relative ratio that can be varied to modulate reprogramming efficiency. For example, somatic cell reprogramming efficiency is fourfold higher when *OCT-4* and *SOX2* are encoded in a single transcript on a single vector in a 1:1 ratio than when the two factors are provided on separate vectors, such that the uptake ratio of the factors into single cells is uncontrolled. Preferably, where a plurality of transgenes is encoded on a single transcript, an internal ribosome entry site is provided upstream of transgene(s) distal from the transcriptional promoter. Although the relative ratio of factors can vary depending upon the factors delivered, one of ordinary skill in possession of this disclosure can determine an optimal ratio of factors.

[0021] The skilled artisan will appreciate that the advantageous efficiency of introducing all factors via a single vector rather than via a plurality of vectors, but that as total vector size increases, it becomes increasingly difficult to introduce the vector. The skilled artisan will also appreciate that position of a factor on a vector can affect its temporal expression, and the resulting reprogramming efficiency. As such, Applicants employed various combinations of factors on combinations of vectors. Several such combinations are here shown to support

reprogramming.

[0022] After introduction of the reprogramming vector(s) and while the somatic cells are being reprogrammed, the vectors can persist in target cells while the introduced transgenes are transcribed and translated. Transgene expression can be advantageously downregulated or turned off in cells that have been reprogrammed to a pluripotent state. The reprogramming vector(s) can remain extra-chromosomal. At extremely low efficiency, the vector(s) can integrate into the cells' genome. The reprogramming vector(s) replicate coordinately with the recipient cell's genome and, as such, are reasonably stable for about two weeks, longer than episomal vectors that cannot replicate their DNA. Nevertheless, because the vectors are not partitioned evenly at cell division, in the absence of selective pressure, cells lose the episomal vector(s) so one can readily recover vector-free pluripotent cells in the method. For example, it usually takes two-to-three weeks for oriP/EBNA-1-based episomal plasmids to be stably maintained in somatic cells. During the initial two-to-three weeks, cells quickly lose episomal plasmids. Once the cells are stabilized, the cells continue to lose episomal vector at ~5% per generation.

[0023] Pluripotent cells produced in the method can be cultured in any medium that supports pluripotent cell growth, including but not limited to a defined medium, such as TeSR™ (StemCell Technologies, Inc.; Vancouver, Canada), mTeSR™ (StemCell Technologies, Inc.) and StemLine® serum-free medium (Sigma; St. Louis, Mo.), or a conditioned medium such as mouse embryonic fibroblast (MEF)-conditioned medium. As used herein, a "defined medium" refers to a biochemically defined formulation comprised solely of biochemically-defined constituents which can include constituents of known chemical composition or constituents derived from known sources. As used herein, "conditioned medium" refers to a growth medium that is further supplemented with soluble factors from cells cultured in the medium. Alternatively, cells can be maintained on MEFs in culture medium.

[0024] The invention will be more fully understood upon consideration of the following non-limiting Examples.

EXAMPLES

Example 1

Design and construction of expression cassettes

[0025] Suitable expression cassettes structures were created using conventional methods by direct polymerase chain reaction (PCR) amplification of open reading frames (ORFs) from

some or all of the transgenes, using the first and last 20-22 bases of the coding region as primers, and from the Internal Ribosome Entry Sites listed in Table 1. The sources of SV40 T Antigen and human telomerase reverse transcriptase, plasmids pBABE-puro SV40 LT and pBABE-hygro-hTERT, are commercially available from Addgene, Inc, Cambridge, MA, as plasmids 13970 and 1773, respectively. The sources of IRES1 and IRES2, plasmids pIRESpuro3 and pIRES2EGFP, are commercially available from Clontech Laboratories, Inc., Mountain View, CA. Foot-and-mouth disease virus segment 2, was chemically synthesized. In-frame expression cassettes are described using the codes set forth below in Table 1. For example, "E-02S" refers to an expression cassette having an EF1α promoter upstream of the OCT4 and SOX2 coding regions, with IRES2 therebetween. Likewise, "C-M2K" refers to an expression cassette having a CMV promoter upstream of the c-Myc and Klf4 coding regions, with IRES2 therebetween. In several constructs, none of which was used in subsequent reprogramming, a variant O2S expression cassette ("O2S(2)") was employed that differed from O2S in that it contained a TK promoter - Hyg - TK polyA cassette (compare FIG. 5A and 5B). Cassettes having the indicated structures were selected for subsequent use in reprogramming methods by empirical determination of expression levels of various factors. The promoter designated as EF2 (SEQ ID NO:12) was a slight variant from the known EF1α promoter (SEQ ID NO:11) that did not differ from EF1α in activity and which was not used in subsequent episomal vector reprogramming trials, *infra.* The F2A is a peptide linker that facilitates co-translation of distinct coding regions expressed from a single transcript. F2A was tested but was not used in subsequent reprogramming trials using episomal vectors. IRES1 was tested but was not used in subsequent reprogramming trials using episomal vectors.

[0026] The relative effects of various promoters, IRES sequences, and transgene arrangements on the expression of the upstream and downstream ORFs were evaluated by separately cloning various transgene expression cassettes into pSin4, a modified lentivirus-based vector, to test their ability to reprogram human somatic cells after transfection, as previously described (Yu *et al., supra*). 293FT cells were transfected with lentiviral plasmid vectors expressing *OCT4* and *SOX2* linked by IRES1 or IRES2 using SuperFect (Qiagen, Valencia, CA), as depicted below. Cells were collected two days post-transfection. FIG. 1A shows a Western blot analysis of OCT-4 and SOX2 in 293FT cells. Lane 1, *pSIN4-EF2-OCT4-IRES1-SOX2;* lane 2, *pSIN4-EF2-OCT4-IRES2-SOX2;* lane *3, pSIN4-EF2-OCT4 F2A-SOX2;* lane 4, *pSIN4-EF2-OCT4-IRES1-PURO;* lane 5, *pSIN4-EF2-SOX2-IRES1-PURO;* lane 6, no plasmid (control). Mouse anti-human OCT4 monoclonal antibody (1:500, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, sc-5279) and goat anti-human SOX2 polyclonal antibody (1:500, R&D Systems, Minneapolis, MN AF2018) were used to detect the relative expression of OCT4 and SOX2 respectively.

[0027] FIG. 1B shows reprogramming using linked potency-determining factors in 0.2×10^6 mesenchymal cells derived (Yu *et al., supra*) from *OCT4* knock-in human ES cells (US Patent Application No. 2006/0128018 and Zwaka and Thomson, Nature Biotechnology 21:319-321 (2003)). This line was maintained under neomycin selection (geneticin: 100 µg/ml, Invitrogen Corp.). Human iPS cell colonies were counted on day 16 post-transduction. The gene combinations *were pSIN4-EF2-OCT4-IRES1-SOX2* (O1S); *pSIN4-EF2-OCT4-IRES2-SOX2*

(O2S); *pSIN4-EF2-OCT4-F2A-SOX2* (OF2AS); *pSIN4-EF2-NANOG-IRES1-LIN28* (N1L); *pSIN4 EF2 NANOG IRES2 LIN28* (N2L); *pSIN4-EF2-OCT4-IRES1-PURO* (O); *pSIN4-EF2-SOX2-IRES1-PURO* (S); *pSIN4-EF2-NANOG-IRES1-PURO* (N); *pSIN4-EF2-LIN28-IRES1-PURO* (L). The abbreviation used for each lentiviral plasmid vector is shown in parentheses after the vector name.

Example 2

Reprogramming human newborn foreskin fibroblasts using lentiviral constructs

[0028] Preliminary reprogramming experiments were conducted by introducing lentiviral vectors into human neonatal foreskin fibroblasts. FIG. 2A shows that *NANOG* has a profound positive effect on reprogramming efficiency when *OCT4, SOX2, LIN28,* and c-*MYC* are also introduced, and that in combination with *OCT4, SOX2,* and *LIN28, NANOG* can support reprogramming, even in the absence of *c-MYC* or *KLF4.* Lentiviral constructs used were *pSIN4-EF2-OCT4-IRES2-SOX2* (02S); *pSIN4-EF2-NANOG-IRES2LIN28* (N2L); *pSIN4-EF2-LIN28-IRES1-PURO* (L); *pSIN4-CMV-c-Myc-IRES1-PURO* (M); *pSIN4-EF2-KLF4-IRES1-PURO* (K). Twenty-one days after transduction, alkaline phosphatase-positive human iPS cell colonies were counted. The number of iPS cell colonies were derived from an input of 2.5×10^4 human newborn foreskin fibroblasts (passage 9). The light gray bars represent the total number of reprogrammed colonies formed having typical human ES cell morphology; dark gray bars indicate the number of large colonies with minimal differentiation.

[0029] FIG. 2B evidences reprogramming using linked potency-determining factors. Lentiviral constructs used were *pSIN4-EF2-c-Myc-IRES2-KLF4* (EF2-M2K); *pSIN4-CMV-c-Myc-IRES2-KLF4* (CMV-M2K); *pSIN4-EF2-KLF4-IRES2-c-Myc* (EF2-K2M); *pSIN4-CMV-KLF 4-IRES2-c-Myc* (CMV-K2M); *pSIN4-CMV-c-Myc-IRES2LIN28* (M2L); *pSIN4-EF2-NANOG-IRES2-KLF4* (N2K). Fourteen days after transduction, alkaline phosphatase-positive human iPS cell colonies were counted. The number of iPS cell colonies were derived from an input of approximately 7.0×10^4 foreskin fibroblasts (passage 12). The asterisk indicates that most of the alkaline phosphatase-positive colonies appeared morphologically loose.

[0030] FIG. 2C shows the effect of SV40 large T antigen gene expression on reprogramming efficiency. SV40 large T antigen prevents c-Myc-induced in murine fibroblasts (Hermeking et al., PNAS 91:10412-10416 (1994)) and enhances reprogramming efficiency (Hanna et al., Cell 133:250-264 (2008); Mali et al., Stem Cells doi: 10.1634/stemcells.2008-0346 (2008)). Abbreviations of gene combinations are the same as in FIG. 2B, with the addition of SV40 large T antigen (T). c-Myc also promotes cell proliferation. Twelve days after transduction, alkaline phosphatase-positive human iPS cell colonies were counted. The number of iPS cell colonies were derived from an input of approximately ~3.5×10^4 foreskin fibroblasts (passage 17). Fig. 2C demonstrates that if present at levels achieved during lentiviral-based

reprogramming, T antigen inhibits final stages of iPS cell derivation. In contrast, see *infra,* wherein T antigen does not have this effect when present for the temporal expression time and/or level achieved during reprogramming using episomal vectors. In addition, T antigen prevents c-Myc-induced apoptosis but does not adversely affect c-Myc-induced cell proliferation.

Example 3

Reprogramming of human newborn foreskin fibroblasts using non-viral episomal constructs

[0031] Human newborn foreskin fibroblasts (Cat# CRL-2097™, ATCC) were maintained in foreskin fibroblast culture medium (DMEM (Cat# 11965, Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS, HyClone Laboratories, Logan, UT), 2 mM Glutamax, 0.1 mM non-essential amino acids, and 0.1 mM β-mercaptoethanol).

[0032] Various combinations of potency-determining factors provided as transgene expression cassettes constructed as in Example 1 and as detailed below in Table 3 were introduced into somatic cells using an episomal construct pCEP4-EGFP (as shown in Fig. 3A) resulting in reprogramming with varying efficiency. pCEP4-EGFP was created from commercially available mammalian episomal expression vector pCEP4 (Invitrogen Corp., Carlsbad, CA) by inserting the EGFP coding region between the pCEP4 BamHI and NheI sites. The episomal vectors of Table 2 were created by inserting the designated expression cassettes into pCEP4-EGFP or into a related backbone lacking P_{CMV} (designated pEP4). See Fig. 3A and Table 2 footnotes for cloning sites into which expression cassettes were inserted.

[0033] Vectors were introduced into the fibroblasts via a single nucleofection event, using Human Dermal Fibroblasts Nucleofector Kit (Normal Human Dermal Fibroblasts, Amaxa, Inc. Cat. No. VPD-1001), in accord with the manufacturer's instructions. After nucleofection, the transfected fibroblasts (~ 0.8 to 1.0 × 10^6 cells each) were immediately plated onto three 10 cm dishes seeded with irradiated mouse embryonic fibroblasts (MEF). Foreskin fibroblast culture medium was replaced every other day. After four days, the foreskin fibroblast culture medium was replaced with human ES cell culture medium (DMEM/F12 culture medium supplemented with 20% KnockOut serum replacer, 0.1 mM non-essential amino acids (all from Invitrogen Corp.), 1 mM Glutamax, 0.1 mM β-mercaptoethanol and 100 ng/ml zebrafish basic fibroblast growth factor (zbFGF) as previously described (Amit et al., Developmental Biology 227:271-278 (2006); Ludwig et al., Nature Methods 3:637-646 (2006)). When the seeded MEF could no longer sustain the reprogramming culture, about 8 to 10 days after plating, human ES cell culture medium conditioned with irradiated MEF was used instead. When appropriate (about 2-3 weeks after transfection), the cultures were stained for alkaline phosphatase as an indication of human iPS colony development.

[0034] To determine suitable parameters for introducing transgene constructs, temporal expression was initially evaluated by measuring EGFP level over time after introduction of EGFP from pEGFP-N2 (control) and pCEP4-EGFP episomal vector into 293FT cells was evaluated (Fig. 3B).

[0035] The effect of the amount of transgene construct introduced on human newborn foreskin fibroblast cell survival was also evaluated in preliminary experiments. FIG. 3C shows the effect of amount of pCEP4-EGFP episomal vector used on nucleofection efficiency and survival of human newborn foreskin fibroblasts, estimated from cell confluence on the day after nucleofection. Approximately 1×10^6 nucleofected foreskin fibroblasts were plated into each well of a 6-well plate. Gray lines represent non-transfected control fibroblasts; black lines represent transfected fibroblasts.

[0036] Fig. 4A depicts schematic transgene expression constructs from Table 3 containing various expression cassettes that when introduced in certain combinations into human newborn foreskin fibroblasts result in reprogramming of the fibroblasts to pluripotent cells. Three combinations of introduced episomal reprogramming vectors have yielded reprogrammed pluripotent cells: (1) pEP4-E-O2S-E-T2K, pEP4-E-O2S-E-N2K and pCEP4-C-M2L; (2) pEP4-E-O2S-C-K2M-E-N2L and pEP4-E-O2S-E-T2K; and (3) pEP4-E-O2S-EN2L, pEP4-E-O2S-E-T2K and pEP4-E-O2S-E-M2K. Table 3 indicates the amount of each vector used in each successful combination. One vector in each successful reprogramming combination encoded T antigen under control of the EF1α promoter.

[0037] FIG. 4B shows a bright-field microscopy image of a typical colony with morphological changes observed 18 days after episomal vector transfection. FIG. 4C shows a bright-field microscopy image of an alkaline phosphatase-positive colony 18 days after episomal vector transfection.

[0038] Twenty-five to thirty days after transfection, the reprogramming cultures were passaged once to fresh 10 cm MEF dishes (1:3 ratio), due to the presence of many non-iPS cell colonies with morphologies similar to human iPS cell colonies. Colonies were then picked for further analysis. FIG. 4D shows a bright-field microscopy image of a human iPS cell colony 6 days after the first passage of day 28 post-transfection reprogramming culture. The scale bar represents 0.1 mm. Reprogrammed cells were maintained for subsequent analysis in feeder-free culture on Matrigel (BD Biosciences, Bedford, MA) with conditioned medium as previously described (Xu et al., Nat. Biotechnol. 19:971 (2001)).

[0039] Advantageously, the reprogramming efficiency of greater than 1% of the newborn foreskin fibroblast cells reprogrammed was achieved, at significantly lower reprogramming time than was achieved using four gene combinations.

TABLE 1 - Reprogramming genes and translation elements

Gene Symbol	Abbr.	Source	SEQ ID NO	Accession # or sequence
OCT4	O	hESC	1	NM_002701
SOX2	S	hESC	2	NM_003106
NANOG	N	hESC	3	NM_024865
LN28	L	hESC	4	NM_024674
c-Myc	M	hESC	5	NM_002467
KLF4	K	hESC	6	NM_004235
SV40 T	T	pBABE-puro SV40 LT p	7	EF579667
TERT	TERT	pBABE hygro-hTERT	8	NM_198253
IRES1	1	pIRESpuro3	--	
IRES2	2	pIRES2EGFP	--	
F2A	F2A	(synthesized)	9	
CMV	C		10	
EF1α	E		11	
EF2α	-		12	

TABLE 2: Episomal constructs

#	Name	Size (bp)
1	pCEP4-EGFP	10984
2[b]	pEP4-E-O2S(2)	13523
3[b]	pEP4-E-M2K	14293
4[a]	pCEP4-M2K	13643
5[b]	pEP4-E-K2M	14268
6[a]	pCEP4-K2M	13636
7[b]	pEP4-E-N2K	13819
8[b]	pEP4-E-T2K	15071
9[a]	pCEP4-M2L	12852
10[b]	pEP4-E-N2L	13020
11[b]	pEP4-E-T2L	14284
12[c]	pEP4-E-O2S-C-M2K	16038
13[c]	pEP4-E-O2S-E-M2K	16680
14[c]	pEP4-E-O2S-C-K2M	16010
15[c]	pEP4-E-O2S-E-K2M	16652

#	Name	Size (bp)
16[c]	pEP4-E-O2S-E-N2K	16206
17[c]	pEP4-E-O2S-E-T2K	17458
18[c]	pEP4-E-O2S-E-N2L	15415
19[c]	pEP4-E-O2S-E-T2L	16679
20[c]	gEP4-U2S-C-M2I.	15247
21[c]	pEP4-E-O2S-E-K2T	17474
22[c]	pEP4-E-O2S-C-M2L-E-N2K	19956
23[c]	pEP4-E-O2S-C-M2K-E-N2L	19956
24[c]	pEP4-E-O2S-C-K2M-E-N2L	19949
25[c]	pEP4-E-O2S-C-M2L-E-T2K	21220
26[c]	pEP4-E-O2S-C-M2K-E-T2L	21220
27[c]	pEP4-E-O2S-C-K2M-E-T2L	21213
28[c]	pEP4-E-O2S-C-M2L-E-K2T	21224

[a] All linked gene cassettes were cloned into the pCEP4-EGFP between BamHI and Nhel restriction sites.

[b] All linked gene cassettes plus the EF1α promoter were cloned into the pCEP4-EGFP between BamHI and SpeI (19) restriction sites.

[c] All expression cassettes were cloned into the pCEP4-EGFP between BamHI and Nrul restriction sites.

TABLE 3: Combinations of episomal constructs tested for reprogramming activity

Equivalent of pCEP4-EGF(µg)	Test #	Plasmids	µg	Morph. Changes	AP+ colony /plate
EXPERIMENT 1					
6.3	1	pEP4-E-O2S-C-M2K	9.2	+/-	0
6.3	2	pEP4-E-O2S-K2Neo	9.3	+/-	0
6.3		pCEP4-M2L	7.4		
6.3	3	pEP4-E-O2S-E-N2K	9.3	+/-	0
6.3		pCEP4-M2L	7.4		
6.3	4	pEP4-E-O2S-E-T2K	10	+++	0
6.3		pCEP4-M2L	7.4		
6.3	5	pEP4-E-O2S-E-TERT2K	10.8	+/-	0
6.3		pCEP4-M2L	7.4		
6.3	6	pEP4-E-O2S-C-M2L	8.7	+/-	0
6.3		pEP4-E-N2K	7.9		
6.3	7	pEP4-E-O2S-C-M2L	8.7	+	0
6.3		pEP4-E-T2K	8.6		
6.3	8	pEP4-E-O2S-C-M2L	8.7	+/-	0
6.3		pEP4-E-TERT2K	9.4		
EXPERIMENT 2					

EXPERIMENT 2					
3.3	1	pEP4-E-O2S-C-M2K	5.0	+/-	0
3.3	2	pEP4-E-O2S-E-M2K	5.0	+/-	0
3.3	3	pEP4-E-O2S-C-K2M	5.0	+/-	0
3.3	4	pEP4-E-O2S-E-K2M	5.0	+/-	0
2.5	5	pEP4-E-O2S(2)	3.0	+/-	0
2.5		pCEP4-M2K	3.0		
2.5	6	pEP4-E-O2S(2)	3.0	+/-	0
2.3		pEP4-E-M2K	3.0		
2.5	7	pEP4-E-O2S(2)	3.0	+/-	0
2.5		pCEP4-K2M	3.0		
2.5	8	pEP4-E-O2S(2)	3.0	+/-	0
2.3		pEP4-E-K2M	3.0		
1.7	9N	pEP4-E-O2S(2)	2.0	+/-	0
1.5		pEP4-E-N2K	2.0		
1.7		pCEP4-M2L	2.0	+/-	0
1.7	10N	pEP4-E-O2S(2)	2.0	+/-	0
1.7		pEP4-E-N2L	2.0		
1.7		pCEP4-M2K	2.0		
1.7	11N	pEP4-E-O2S(2)	2.0	+/-	0
1.7		pEP4-E-N2L	2.0		
1.5		pEP4-E-M2K	2.0		
1.7	12N	pEP4-E-O2S(2)	2.0	+/-	0
1.7		pEP4-E-N2L	2.0		
1.7		pCEP4-K2M	2.0		
1.7	13N	pEP4-E-O2S(2)	2.0	+/-	0
1.7		pEP4-E-N2L	2.0		
1.5		pEP4-E-K2M	2.0		
2.3	14N	pEP4-E-O2S-E-N2K	3.5	+/-	0
2.1		pCEP4-M2L	2.5		
2.5	15N	pEP4-E-O2S-E-N2L	3.5	+/-	0
2.1		pCEP4-M2K	2.5		
2.5	16N	pEP4-E-O2S-E-N2L	3.5	+/-	0
1.9		pEP4-E-M2K	2.5		
2.5	17N	pEP4-E-O2S-E-N2L	3.5	+/-	0
2.1		pCEP4-K2M	2.5		
2.5	18N	pEP4-E-O2S-E-N2L	3.5	+/-	0
1.9		pEP4-E-K2M	2.5		
EXPERIMENT 3					
1.7	9T	pEP4-E-O2S(2)	2.0	++	0
1.4		pEP4-E-T2K	2.0		
1.7		pCEP4-M2L	2.0		
1.7	10T	pEP4-E-O2S(2)	2.0	+	0
1.5		pEP4-E-T2L	2.0		
1.7		pCEP4-M2K	2.0		
1.7	11T	pEP4-E-O2S(2)	2.0	+	0
1.5		pEP4-E-T2L	2.0		
1.5		pEP4-E-M2K	2.0		
1.7	12T	pEP4-E-O2S(2)	2.0	+/-	0
1.5		pEP4-E-T2L	2.0		
1.7		pCEP4-K2M	2.0		
1.7	13T	pEP4-E-O2S(2)	2.0	+/-	0
1.5		pEP4-E-T2L	2.0		
1.5		pEP4-E-K2M	2.0		
2.2	14T	pEP4-E-O2SET2K	3.5	+++	0

2.1		pCEP4-M2L	2.5		
2.3	15T	pEP4-E-O2S-E-T2L	3.5	+	0
2.1		pCEP4-M2K	2.5		
2.3	16T	pEP4-E-O2S-E-T2L	3.5	+	0
1.9		pEP4-E-M2K	2.5		
2.3	17T	pEP4-E-O2S-E-T2L	3.5	+/-	0
2.1		pCEP4-K2M	2.5		
2.3	18T	pEP4-E-O2S-E-T2L	3.5	+/-	0
1.9		pEP4-E-K2M	2.5		
1.9	19	pEP4-E-O2S-E-T2K	3.0	+++	1
2.0		pEP4-E-O2S-E-N2K	3.0		

1.7		pCEP4-M2L	2.0		
EXPERIMENT 4					
6	1	pEP4-E-O2S-C-M2K-E-N2L	10.9	+/-	0
4	2	pEP4-E-O2S-C-M2K-E-N2L	7.3	+++	0
2		pEP4-E-O2S-E-T2K	3.2		
6	3	pEP4-E-O2S-C-K2M-E-N2L	10.9	+/-	0
4	4	pEP4-E-O2S-C-K2M-E-N2L	7.3	++	2
2		pEP4-E-O2S-E-T2K	3.2		
3	5	pEP4-E-O2S-E-N2L	4.2	+/-	0
3		pEP4-E-O2S-E-M2K	4.6		
3	6	pEP4-E-O2S-E-N2L	4.2	++	1
2		pEP4-E-O2S-E-T2K	3.2		
3		pEP4-E-O2S-E-M2K	4.6		
3	7	pEP4-E-O2S-E-N2L	4.2	+/-	0
3		pEP4-E-O2S-C-M2K	4.4		
3	8	pEP4-E-O2S-E-N2L	4.2	+	0
2		pEP4-E-O2S-E-T2K	3.2		
3		pEP4-E-O2S-C-M2K	4.4		
3	9	pEP4-E-O2S-E-N2L	4.2	+/-	0
3		pEP4-E-O2S-E-K2M	4.5		
3	10	pEP4-E-O2S-E-N2L	4.2	+/-	0
2		pEP4-E-O2S-E-T2K	3.2		
3		pEP4-E-O2S-E-K2M	4.5		
3	11	pEP4-E-O2S-E-N2L	4.2	+/-	0
3		pEP4-E-O2S-C-K2M	4.4		
3	12	pEP4-E-O2S-E-N2L	4.2	+	0
2		pEP4-E-O2S-E-T2K	3.2		
3		pEP4-E-O2S-C-K2M	4.4		
2	13	pEP4-E-O2S-C-M2L-E-T2K	3.9	+	0
4		pEP4-E-O2S-E-N2K	5.9		
6	14	pEP4-E-O2S-C-M2K-E-T2L	11.6	+	0
3	15	pEP4-E-O2S-C-M2K-E-T2L	5.8	+	0
3		pEP4-E-O2S-E-N2K	4.4		
6	16	pEP4-E-O2S-C-K2M-E-T2L	11.6	+/-	0
3	17	pEP4-E-O2S-C-K2M-E-T2L	5.8	+	0

3		pEP4-E-O2S-E-N2K	4.4		

6	18	pEP4-E-O2S-C-M2L-E-K2T	11.6	+/-	0
3	19	pEP4-E-O2S-C-M2L-E-K2T	5.8	+/-	0
3		pEP4-E-O2S-E-N2K	4.4		
3	20	pEP4-E-O2S-E-K2T	4.8	+/-	0
3		pEP4-E-O2S-E-N2K	4.4		
2		pEP4-E-O2S-C-M2L	2.8		

+/-: No or very few colonies with morphological change were observed (~~fig.~~ FIG. 4B).
+, ++ and +++: Different number (from less to more) of colonies with morphological change were observed.

REFERENCES CITED IN THE DESCRIPTION

Cited references

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PATENTKRAV

1. Beriget population af primatinducerede pluripotente celler, der i alt væsentligt er genetisk identiske med et post-natalt individ, hvor populationen er frembragt ifølge en fremgangsmåde, der omfatter følgendde trin:

Introduktion af en flerhed af ikke-virale episomale vektorer, der koder for én eller flere potensbestemmende faktorer into de somatiske primatceller under forhold, der er tilstrækkelige til at udtrykke de potensbestemmende faktorer, hvorved de somatiske celler omprogrammeres til at frembringe de pluripotente primatceller;

hvor mindst nogle af de pluripotente primatinducerede celler i den berigede population omfatter én eller flere kopier af flerheden af ikke-virale episomale vektorer;

hvor flerheden af ikke-virale episomale vektorer er valgt fra gruppen bestående af:

(a) en første vektor, der i rækkefølge omfatter en første promotor, *OCT4*, IRES2, *SOX2*, en anden promotor, *SV40-T* antigen, IRES2, og *KLF4*, en anden vektor, der i rækkefølge omfatter en tredje promotor, *OCT4*, IRES2, *SOX2*, en fjerde promotor, *NANOG*, IRES2, og *KLF4*, og en tredje promotor, der i rækkefølge omfatter en femte promotor, *c-Myc*, IRES 2 og *LN28*, hvor promotorerne ikke skal være identiske;

(b) en første vektor, der i rækkefølge omfatter en første promotor, *OCT4*, IRES2, *SOX2*, en anden promotor, *KLF4*, IRES2, *c-Myc*, en tredje promotor, *NANOG*, IRES2 og *LN28*, en anden vektor, der i rækkefølge omfatter en fjerde promotor, *OCT4*, IRES2, *SOX2*, en femte promotor, *SV40-T*-antigen, IRES2 og *KLF4*, hvor promotorerne ikke skal være identiske; og

(c) en første vektor, der i rækkefølge omfatter en første promotor, *OCT4*, IRES2, *SOX2*, en anden promotor, *NANOG*, IRES2 og *LN28*, en anden vektor, der i rækkefølge omfatter en tredje promotor, *OCT4*, IRES2, *SOX2*, en fjerde promotor, *SV40-T* antigen, IRES2 og *KLF4*, og en tredje promotor, der i rækkefølge omfatter en femte promotor, *OCT4*, IRES2, *SOX2*, en sjette promotor, *c-Myc*, IRES 2 og *KLF4*, hvor promotorerne ikke skal være identiske.

2. Population ifølge krav 1, hvor flerheden af ikke-virale episomale vektorer er valgt fra gruppen bestående af: (1) pEP4-E-O2S-E-T2K, pEP4-E-O2S-E-N2K og pCEP4-M2L; (2) pEP4-E-O2S-C-K2M-E-N2L og pEP4-E-O2S-E-T2K og (3) pEP4-E-O2S-E-N2L, pEP4-E-O2S-E-T2K og pEP4-E-O2S-E-M2K, hvor pEP4 og pCEP4 er plasmider, E er en EF1α-promotor; O er et OCT4-kodende område, S er et SOX2-kodende område, T er et SV40-T-antigenkodende område, N er et NANOG-kodende område, K er et KLF4-kodende område, M er et c-Myc-kodende område, C er en CMV-promotor, og L er et LIN28-kodende område.

3. Population ifølge krav 1 eller 2, hvor de somatiske primatceller er opnået fra et post-natalt individ.

4. Population ifølge et hvilket som helst af kravene 1-3, hvor primaten er et menneske.

5. Population ifølge et hvilket som helst af kravene 1-4, hvor cellerne er euploide.

6. Population ifølge krav 1(a), hvor hver af den første, anden, tredje og fjerde promotor er en elongationsfaktor-lα (EFlα) genpromotor.

7. Population ifølge krav 1(b), hvor hver af den første, tredje, fjerde og femte promotor er en EFlα-genpromotor, og hvor den anden promotor er en umiddelbar tidlig cytomegalovirus-gen- (CMV) promotor.

DRAWINGS

Fig. 1A

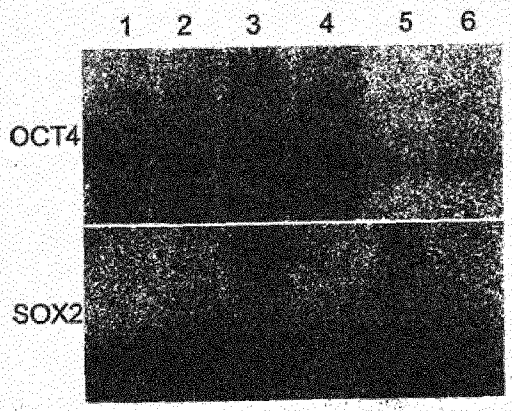

Fig. 1B

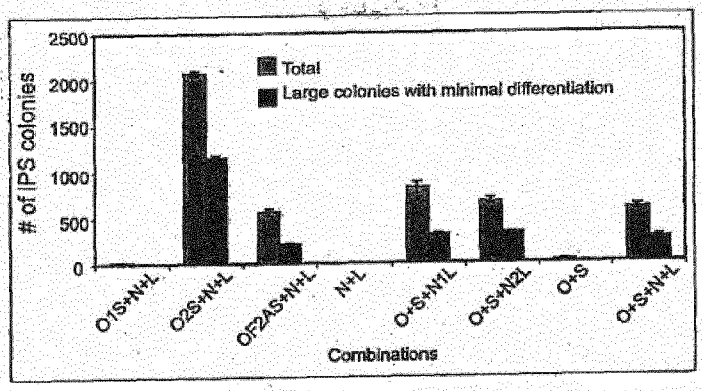

FIGURE 1

Fig. 2A

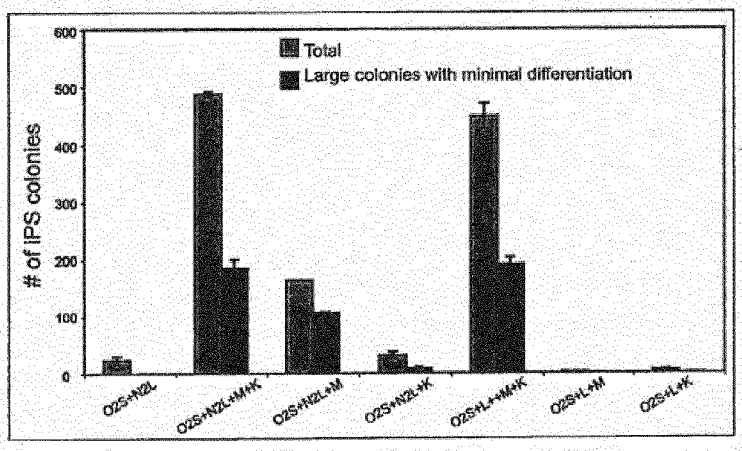

Fig. 2B

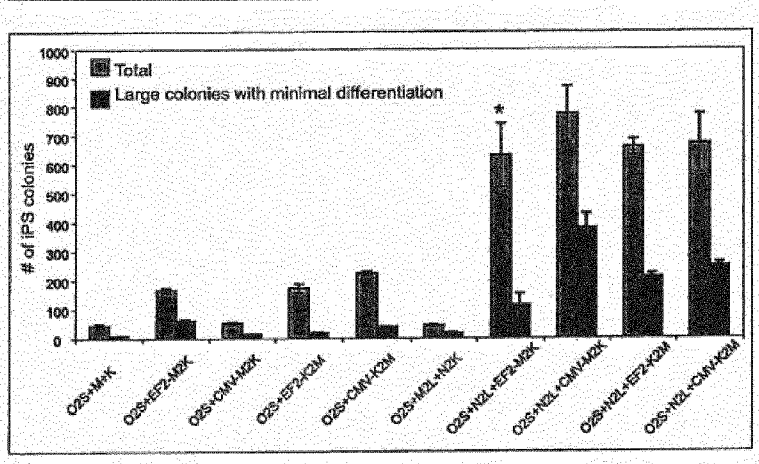

Fig. 2C

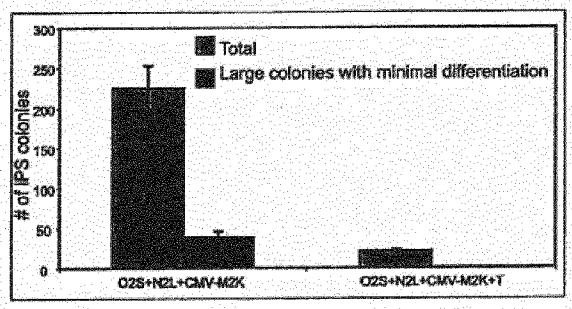

FIGURE 2

Fig. 3A

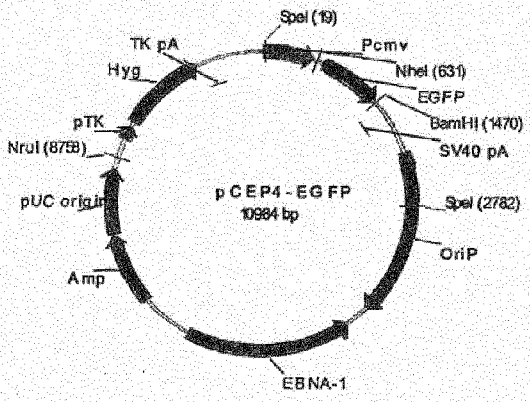

Fig. 3B

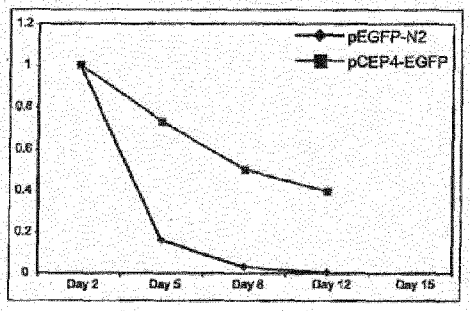

Fig. 3C

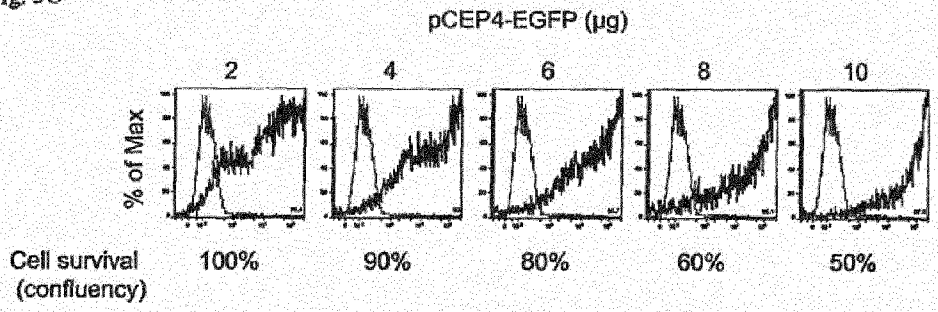

FIGURE 3

Fig. 4A

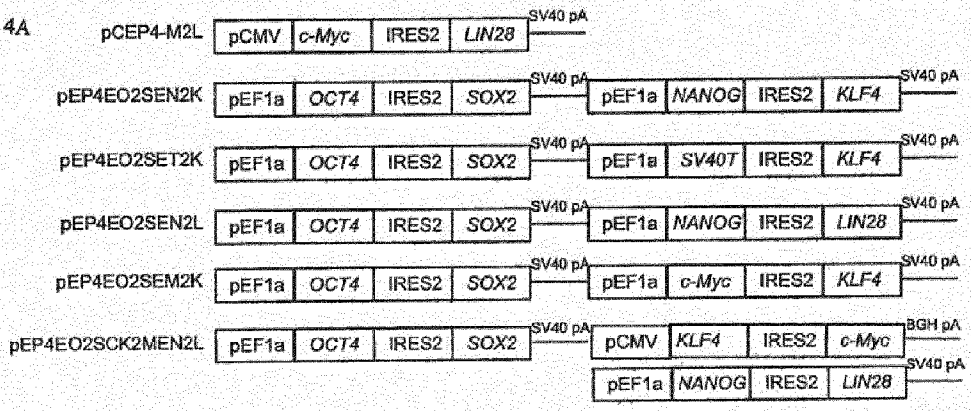

Fig. 4B

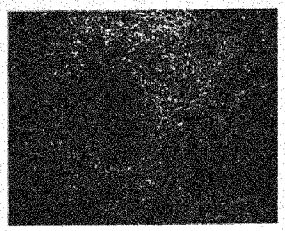

Fig. 4C

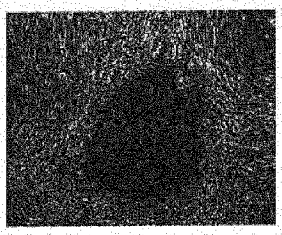

Fig. 4D

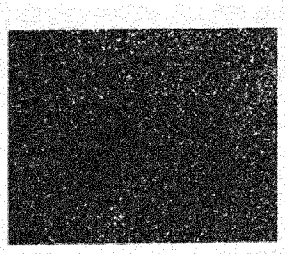

FIGURE 4

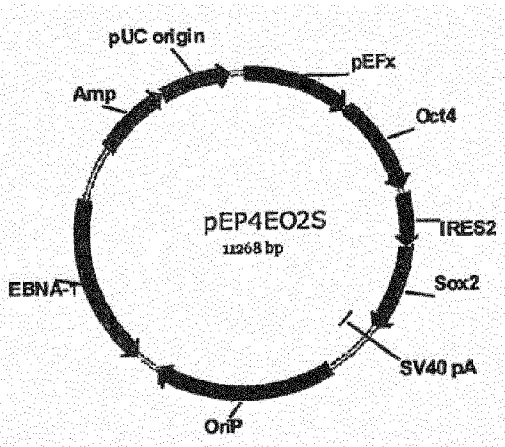

Fig. 5A

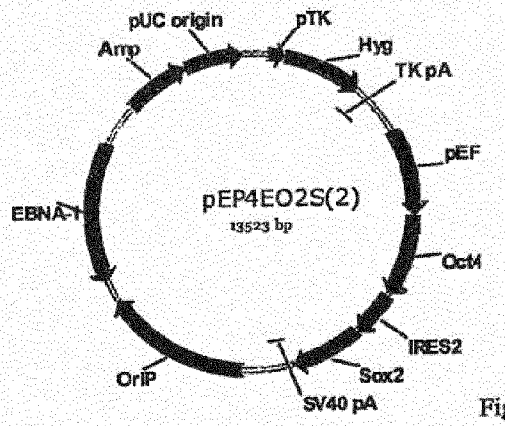

Fig. 5B

FIGURE 5

SEKVENSLISTE

Sekvenslisten er udeladt af skriftet og kan hentes fra det Europæiske Patent Register.

The Sequence Listing was omitted from the document and can be downloaded from the European Patent Register.