A Nuclear receptor and mutant thereof and the use of the same in the Reprogramming of cells

Cross-reference to related application

[0001]. This application claims benefit of, and priority from, Singapore patent application Numbers 200906546-7, filed on 30 September 2009 and 201000140-2, filed on 9 January 2010 the contents of both of which are incorporated herein by reference.

Field of the Invention

[0002]. The present invention relates to a nuclear receptor protein and the use of such proteins in methods of reprogramming a differentiated cell to a pluripotent state.

Background Art

[0003]. Stem cell treatments are a type of cell therapy that introduces new cells into damaged tissue in order to treat a disease or injury. The ability of pluripotent cells to self-renew and differentiate into a range of different cell types offers a large potential to culture tissues that can replace diseased and damaged tissues in the body, without the risk of rejection.

[0004]. A number of stem cell treatments exist, although most are still experimental and/or costly, with the notable exception of bone marrow transplantation. Medical researchers anticipate one day being able to use cells derived from adult somatic cells to treat cancer, diabetes, neurological disorders such as Parkinson's disease, Huntington's disease, Alzheimer's, dementia, as well as cardiac failure and muscle damage, along with many others.

[0005]. The reversion of somatic cells to pluripotent cells is commonly referred to as reprogramming. Somatic cell nuclear transfer and cell fusion are examples of techniques employed in the reprogramming of differentiated cells (Lewitzky, M. & Yamanaka, S. (2007) Curr Opin Biotechnol 18, 467-73). Another method of reprogramming was discovered when mouse fibroblasts were reprogrammed with the retroviral introduction of just four transcription factors Oct4, Sox2, Klf4 and c-Myc (Takahashi, K. & Yamanaka, S. (2006) Cell 126, 663-76). Somatic cells can be reprogrammed back to the pluripotent state by the combined introduction of transcription factors such as Oct4, Sox2, Klf4 and c-Myc (OSKM). These converted cells share many characteristics with embryonic stem cells (ESCs) in terms of morphology, genetic expression and epigenetic marks and are known as induced pluripotent stem cells (iPSCs). Since the discovery of iPSCs, cells from different lineages and a diverse range of species have been successfully reprogrammed (Feng, B., et al. (2009) Cell Stem Cell 4, 301-12). There is a need to enhance the efficiency of such methods.

[0006]. Besides the four reprogramming factors discovered by the groundbreaking study of Yamanaka, other factors such as NANOG and LIN28 were also found to participate in reprogramming (Yu, J. et al. (2007) Science 318, 1917-20). In addition, UTF1, an ESC-specific transcription factor, was shown to enhance the reprogramming of human fibroblasts in conjunction with the four Yamanaka factors as well as the knockdown of p53. Some of the four Yamanaka transcription factors have also been shown to replace factors in reprogramming. For instance, Klf4 can be replaced by Klf2 and Klf5, Sox2 can be substituted by Sox1 and Sox5 while N-myc and L-myc could replace c-Myc. Amongst the four defined reprogramming factors, Oct4 has been shown to be the most critical in inducing pluripotency (Nakagawa, M. et al. (2008) Nat Biotechnol 26, 101-6). However, Oct4 remains irreplaceable by other transcription factors including its close family members such as Oct1 and Oct6 (Nakagawa, M. et al. (2008). No transcription factor has been hitherto shown to be able to substitute Oct4 in the reprogramming of somatic cells.

[0007]. Oct-4 (an abbreviation of Octamer-4) is a homeodomain transcription factor protein of the POU family. Oct-4 expression must be closely regulated; too much or too little will actually cause differentiation of the cell. Oct-4 has been implicated in tumorigenesis of adult germ cells. Ectopic expression of the factor in adult mice has been found to cause the formation of dysplastic lesions of the skin and intestine. The intestinal dysplasia resulted from an increase in progenitor cell population and the upregulation of β-catenin transcription through the inhibition of cellular differentiation.

[0008]. Oct4, expressed in the inner cell mass (ICM) of the blastocysts, is critical in maintaining pluripotency of cells in the ICM as well as ESCs. Although neural progenitor cells (NPCs) express a high level of endogenous Sox2, ectopic expression of Oct4 alone was still required for their reprogramming. This observation suggests that Oct4 is pivotal in imparting pluripotency in somatic cells. In addition, only a few transcription factors such as Oct4 and the aforementioned transcriptional factors have been reported to contribute to iPSC generation.

[0009]. Nuclear receptors have the ability to directly bind to DNA and regulate the expression of adjacent genes. Nuclear receptors are modular in structure and contain specific domains such as DNA binding domain (DBD) and Ligand binding domain (LBD). They are generally classified into two broad classes according to their mechanism of action and subcellular distribution in the absence of ligand. The 48 known human nuclear receptors have been further categorized into subfamilies based on the sequence homology of the proteins. Subfamily 5 includes two nuclear receptors, Nr5a1, also known as steroidogenic factor 1 (Sf1), and Nr5a2. Similar to other nuclear receptors, Nr5a2 possesses a ligand binding domain (LBD) and a DNA binding domain (DBD). However, being an orphan nuclear receptor, the endogenous ligands of Nr5a2 remains unknown. Unlike most nuclear receptors which function as dimers, Nr5a2 is able to bind DNA in its monomeric state (Galarneau, L. et al. (1996) Mol Cell Biol 16, 3853-65).

Summary of the Invention

[00010]. The present invention seeks to provide alternative transcription factors and the use of such factors in methods of reprogramming a differentiated cell to a pluripotent state.

[00011]. We show that nuclear receptors and sumoylation mutants of nuclear receptors are able to initiate pluripotent stem cells *in vitro*. Further nuclear receptors may be able to replace Oct4 in the derivation of pluripotent stem cells *in vitro*.

[00012]. Accordingly one aspect of the present invention provides a method for inducing pluripotent stem cells *in vitro* comprising the steps of: culturing cells *in vitro*; introducing a polynucleotide that encodes a transcription factor into the cell in the culture, wherein the polynucleotide encoding a transcription factor comprises a nuclear receptor and one or more transcription factor selected from a Sox gene, Krüppel-like factor gene or an myc family of genes to induce the cell to be a pluripotent cell.

[00013]. Another aspect of the invention provides an expression vector comprising a polynucleotide of a nuclear receptor from subfamily 5 selected from: (a) polynucleotides comprising the nucleotide sequence set out in SEQ ID NO. 1, SEQ ID NO. 3, SEQ ID NO. 5 or SEQ ID NO. 8 or a fragment expressing polypeptide SEQ ID NO. 10; (b) polynucleotides comprising a nucleotide sequence capable of hybridising selectively to the nucleotide sequence set out in SEQ ID NO. 1, SEQ ID NO. 3, SEQ ID NO. 5 or SEQ ID NO. 8 or a fragment expressing polypeptide SEQ ID NO. 10; (c) polynucleotides encoding a nuclear receptor polypeptide which comprises the sequence set out in SEQ ID NO. 2, SEQ ID NO. 4, SEQ ID NO. 6 SEQ ID NO. 7, .SEQ ID NO. 9, SEQ ID NO. 11or a homologue, variant, derivative or fragment thereof containing SEQ ID NO. 10; and one or more transcription factor selected from a Sox gene, Krüppel-like factor gene or a gene from the myc family operably linked to a regulatory sequence capable of directing expression of said polynucleotide in a host cell.

[00014]. Another aspect of the invention provides a method for inducing pluripotent stem cells in vitro in the manufacture of a medicament for treating a patient in need of a pluripotent stem cell treatment comprising the steps of: isolating cells from an individual donor; culturing the cells in vitro; introducing a polynucleotide that encodes a transcription factor into the cell in the culture, wherein the polynucleotide encodes a transcription factor comprises a nuclear receptor and one or more transcription factor selected from from a Sox gene, Krüppel-like factor gene or an myc family of genes to induce the cell to be a pluripotent cell; introducing the pluripotent cell to the patient in need of a pluripotent stem cell treatment.

[00015]. Another aspect of the invention provides a method of making pluripotent stem cell lines comprises: culturing cells in vitro; introducing a polynucleotide that encodes a transcription factor into the cell in the culture, wherein the polynucleotide encodes a transcription factor comprising a nuclear receptor and one or more transcription factor selected from from a Sox gene, Krüppel-like factor gene or an myc family of genes to induce the cell to be a pluripotent cell; passaging the pluripotent cells to maintain the cell line.

[00016]. Other aspects of the invention include those apparent to a person skilled in the art with reference to the description and figures of the preferred embodiments.

Brief Description of the Drawings

Figure 1. Nr5a2 enhances reprogramming efficiency and can reprogram MEFs with Sox2 and Klf4, with or without c-Myc.

(a) Screen of 18 nuclear receptor s for the enhancement of MEF reprogramming with Oct4, Sox2, Klf4 and c-Myc (OSKM). Graph depicts the fold change of number of *Pou5f1*-GFP-positive colonies generated from each nuclear factor in conjunction with OSKM with respect to the OSKM (control). Data represent mean ± s.e.m. of three retrovirus-mediated transduction experiments (n=3).

(b) Reprogramming enhancers, Nr1i2 and Nr5a2 were tested for their ability to replace Sox2, Klf4 and Oct4 in the reprogramming of MEFs. A quantitation of GFP-

positive colonies was performed on 14 dpi. For control experiments, no nuclear receptor factors were introduced but only OKM, OSM or SKM retroviruses were added. Data represent mean ± s.e.m. of three retrovirus-mediated transduction experiments (n=3).

(c) Number of GFP-positive colonies generated from the reprogramming of MEFs with Nr5a2 together with Sox2 and Klf4. For control experiment, only SK retroviruses were introduced to MEFs. Data represent mean ± s.e.m. of three retrovirus-mediated transduction experiments (n=3).

(d) Generation of iPSC colonies after retroviral transduction of *Pou5f1*-GFP MEFs with Nr5a2, Sox2 and Klf4. Phase contrast image is shown.

(e) iPSC colonies in d are *Pou5f1*-GFP-positive when viewed under a fluorescence microscope, indicating the reactivation of endogenous *Pou5f1*.

(f) N_2SK iPSCs expressed alkaline phosphatase.

(g) Expression of Nanog in N_2SK iPSCs.

(h) Nuclei in g were counterstained with Hoechst.

(i) SSEA-1 expression in N_2SK iPSCs.

(j) Cells in i were stained with Hoechst to indicate nuclei. Scale bars represent 200 μm in d-f and 50 μm in g-j.

Figure 2. Global expression profiling of Nr5a2-reprogrammed cells.

(a) Correlation analysis (46,643 probes) was carried out to cluster the transcriptome of ESCs, iPSCs (OSKM, N_2SKM #A5, N_2SK #B3 and N_2SK #B11) and MEFs (*actin*-GFP and *Pou5f1*-GFP). OSKM iPSCs were derived from the retroviral introduction of Oct4, Sox2, Klf4 and c-Myc to MEFs.

(b) Heatmap generated from the biological replicate microarray data in a displays the expression profile of 1,000 ESC-associated and MEF-associated genes. Green represents downregulation of gene expression while red represents upregulation of gene expression with respect to MEFs.

Figure 3. Epigenetic states of Nr5a2-reprogrammed cells.

(a) *Pou5f1* and *Nanog* promoter methylation analysis of Nr5a2-reporgrammed cells. Bisulphite genomic sequencing was performed to analyze the methylation status of the promoter region of *Pou5f1* and *Nanog* in ESCs, MEFs and Nr5a2-reprogrammed

cells (N_2SKM #A5, N_2SK #B3 and N_2SK #B11*)*. For each cell line, ten random clones were sequenced and the results are displayed in circles in which open circles represent unmethylated CpG dinucleotides while red circles represent methylated CpG dinucleotides.

(b) Bivalent chromatin marks in Nr5a2-reprogrammed cells. Following ChIP assay, quantitative real-time PCR was performed to analyze the enrichment of trimethylated histone H3K4 and H3K27 chromatin marks in ESCs, MEFs and Nr5a2-reprogrammed cells. Data represents Log_2 enrichment for reported bivalent gene loci (*Zfpm2, Sox21, Pax5, Lbx1h, Evx1* and *Dlx*). Data shown are mean ± s.e.m. of three independent experiments (n=3).

Figure 4. N_2SK iPSCs can generate mouse chimaeras.

(a) Brightfield image of the male gonad dissected from the E13.5 N_2SK #B3 chimaeric embryo.

(b) GFP fluorescence image of a. Positive GFP signals were observed in the gonads, indicating germline incorporation of the Nr5a2-reprogrammed cells.

(c) N_2SK #B11 adult chimaeras. Nr5a2-reprogrammed cells, derived from 129S2/SV *Pou5f1*-GFP MEFs were microinjected into B6(Cg)-Tyr^{c-2J}/J embryos and generated chimaeras with mixed fur coat color.

Figure 5. Expression of viral constructs harboring the screened nuclear receptor genes verified by PCR amplification of cDNA with a virus specific primer and a gene-specific primer.

Figure 6. Nr5a2 reprograms MEFs with Sox2, Klf4 and c-Myc.

(a) Phase contrast image of iPSC colonies derived from the retroviral transduction of *Pou5f1*-GFP MEFs with Nr5a2, Sox2, Klf4 and c-Myc.

(b) Fluorescence image of **a** show the restoration of endogenous *Pou5f1* in Nr5a2-reprogrammed cells.

(c) Alkaline phosphatase expression in N2SKM iPSCs.

(d) Nanog expression in N2SKM iPSCs.

(e) Nuclei in **d** are counterstained with Hoechst.

(f) Expression of SSEA-1 in N2SKM iPSCs.

(g) Cells in **f** are stained with Hoechst to mark nuclei.

(h) A screen of the other nuclear receptor s for their ability to replace Oct4. MEFs were co-transduced with SKM viruses and viruses encoding each of the nuclear receptor . SKM+Nr5a2 were used as a positive control. Control experiment represents transduction of MEFs with only SKM viruses. Number of GFP-positive colonies was counted on 14 dpi. Data represent mean ± s.e.m. of three retrovirus-mediated transduction experiments (n=3).

(i) Adult mouse chimaera generated from the microinjection of N2SKM #A5 iPSCs derived from 129S2/SV *Pou5f1*-GFP MEFs into C57BL/6J embryos. Scale bars represent 200 μm in **a-c** and 50 μm in **d-g**.

Figure 7. Karyotypic and genotypic analysis of Nr5a2-reprogrammed cells.

(a) N2SKM #A5, N2SK #B3 and N2SK #B11 iPSC lines displayed normal male karyotype.

(b) PCR verified the genomic integration of retroviral genes, Nr5a2, Sox2, Klf4 and c-Myc in Nr5a2-reprogrammed cells. PCR was performed on genomic DNA harvested from ESCs, MEFs and iPSCs with a viral-specific primer and a gene specific primer. OSKM iPSCs were derived from the viral transduction of MEFs with Oct4, Sox2, Klf4 and c-Myc. PCR amplification of a region of the *p21* gene was performed on all the samples and is shown in the control panel.

Figure 8. Nr5a2-reprogrammed cells differentiate into lineages of the three major germ layers in the *in vitro* and *in vivo* differentiation assays.

(a) Embryoid body-mediated *in vitro* differentiation assay showed that Nr5a2-reprogrammed cells could differentiate into cells of the three major embryonic lineages. Cells differentiated from Nr5a2-reprogramemd cells stained positive for Gata-4 (endoderm), Nestin (ectoderm) and α-Smooth Muscle Actin (mesoderm). Differentiation markers were stained red and Hoechst dye counterstained the nuclei blue.

(b) Nr5a2-reprogrammed cells differentiated into tissues of the three primary germ layers in the teratoma assay. Teratomas sectioned and stained with Mallory's tetrachrome revealed ectodermal tissue (neural ectoderm), mesodermal tissue

(muscle and cartilage) and endodermal tissue (gut epithelium and pancreatic cells). Scale bars represent 100 μm in **a** and 50 μm in **b**.

Figure 9. Nr5a1 reprograms MEFs with Sox2, Klf4 and c-Myc.

(a) Nr5a1 enhances the efficiency of reprogramming with Oc4, Sox2, Klf4 and c-Myc. Graph depicts the fold change of number of *Pou5f1*-GFP-positive colonies generated from Nr5a1 in conjunction with OSKM with respect to the OSKM (control). Data represent mean ± s.e.m. of three retrovirus-mediated transduction experiments (n=3).

(b) Nr5a1 replaces Oct4 in the reprogramming of MEFs. Nr5a1 was investigated for its ability to replace Sox2, Klf4 and Oct4 by co-transducing Nr5a1 in conjunction with OKM, OSM and SKM respectively. Control experiments were performed with OKM, OSM or SKM retroviruses in the absence of Nr5a1. Data represent mean ± s.e.m. of three retrovirus-mediated transduction experiments (n=3).

(c) Phase contrast image of iPSC colonies generated from the the retroviral transduction of *Pou5f1*-GFP MEFs with Nr5a1, Sox2, Klf4 and c-Myc.4

(d) Fluorescence image of *Pou5f1*-GFP-positive N1SKM iPSC colonies in **c**.

(e) Nr5a1-reprogrammed cells stained positive for alkaline phosphatase.

(f) Nanog was expressed in Nr5a1-reprogrammed cells

(g) Hoechst staining of **f** indicates nuclei.

(h) Nr5a1-reprogrammed cells stained positive for SSEA-1

(i) Cells in **h** were stained with Hoechst to indicate nuclei.

(j) Normal male karyotype of a Nr5a1-reprogrammed cell line

(k) Embryoid body-mediated *in vitro* differentiation assay performed on Nr5a1-reprogrammed cells show that it can differentiate to cells of the three major embryonic lineages. Differentiated cells stained positive for Gata4 (endoderm), Nestin (ectoderm) and α-Smooth Muscle Actin (mesoderm). Lineage markers were stained red and nuclei were stained blue with Hoechst. Scale bars represent 200 um in **c-e** and 50 um in **f-i**.

Figure 10. Nr5a2 and Nr5a1 together boost reprogramming of MEFs with Sox2, Klf4 and c-Myc.Introduction of both Nr5a2 and Nr5a1 in conjunction with Sox2, Klf4 and c-Myc enhances the number of GFP-positive colonies generated as compared to

when either Nr5a2 or Nr5a1 is transduced with SKM. Control experiment was the transduction of MEFs with only the SKM viruses. Data represent mean ± s.e.m. of three retrovirus-mediated transduction experiments (n=3).

Figure 11. DNA binding domain (DBD) is important for Nr5a2 to reprogram MEFs whereas ligand binding domain (LBD) of Nr5a2 is dispensable for its function as a reprogramming factor.

(a) Western analysis of cell extracts harvested from 293-T cells transfected with either retroviral vectors encoding Nr5a2 WT, Nr5a2 A368M (LBD mutant) and Nr5a2 G190V, P191A (DNA mutant) showed equal expression of Nr5a2 protein. 293-T cells transfected with retroviral vectors harboring the GFP gene was used as a negative control. Western blot of actin was performed as a loading control.

(b) Analysis of Nr5a2 mutants for ability to retain its function as a reprogramming factor. *Pou5f1*-GFP MEFs were transduced with SKM viruses and viruses encoding either Nr5a2 WT, Nr5a2 A368M or Nr5a2 G190V, P191A. Control experiment denotes infection of MEFs with only SKM viruses. Data represent mean ± s.e.m. of three retrovirus-mediated transduction experiments (n=3).

Figure 12. Nr5a2 reprograms MEFs with Sox2, Klf4 and with or without c-Myc

(A) Screen of 19 nuclear receptors for the enhancement of OSKM reprogramming. Graph depicts fold change of number of GFP-positive colonies generated from each nuclear receptor together with OSKM with respect to OSKM (control).

(B) Kinetics of OSKM reprogramming with either Nr5a2 or Nr1i2.

(C) Reprogramming assay of reprogramming enhancers Nr1i2 and Nr5a2 for their ability to replace Sox2, Klf4 and Oct4. For control experiments, the respective combinations of retroviruses were added without Nr5a2 or Nr1i2..

(D) Number of GFP-positive colonies generated from the reprogramming of MEFs with Nr5a2, Sox2 and Klf4. For control experiment, only SK retroviruses were introduced. Data in A to D represent mean ± s.e.m. of three retrovirus-mediated transduction experiments (n=3).

(E) Generation of iPSC colonies after retroviral transduction of *Pou5f1-GFP* MEFs with Nr5a2, Sox2 and Klf4. Phase contrast image is shown.

(F) Colonies in E are GFP-positive when viewed under a fluorescence microscope, indicating the reactivation of endogenous *Pou5f1*.

(G) N_2SK iPSCs expresses alkaline phosphatase.

(H) Expression of Nanog in N_2SK iPSCs.

(I) Nuclei in H were counterstained with Hoechst.

(J) SSEA-1 expression in N_2SK iPSCs.

(K) Cells in J were stained with Hoechst to indicate nuclei. Scale bars represent 200 μm in E-G and 50 μm in H-K.

(L) Brightfield image of the male gonad dissected from the E13.5 N_2SK #B3 chimaeric embryo.

(M) GFP fluorescence image of L. Positive GFP signals were observed in the gonads, indicating germline incorporation of Nr5a2-reprogrammed cells.

(N) N_2SK #B11 adult chimaera generated from Nr5a2-reprogrammed cells derived from F1 (129S2/SV x Pou5f1-*GFP*) MEFs which were microinjected into B6(Cg)-Tyr^{c-2J}/J embryos.

(O) Offsprings generated from the mating of N_2SK #B11 adult chimaera with an albino B6(Cg)-Tyr^{c-2J}/J mouse. Agouti and black offsprings are indicative of germline transmission of the Nr5a2-reprogrammed cells.

Figure 13. Nr5a1-mediated reprogramming and the effect of mutations on reprogramming capability of Nr5a2

(A) Nr5a1 enhances the reprogramming efficiency with OSKM. Graph depicts fold change of number of GFP-positive colonies generated from Nr5a1 in conjunction with OSKM with respect to the control (OSKM).

(B) Nr5a1 replaces Oct4 in the reprogramming of MEFs. Nr5a1 was investigated for its ability to replace Sox2, Klf4 and Oct4 by co-transducing Nr5a1 in conjunction with OKM, OSM or SKM, respectively. Control experiments were performed with OKM, OSM or SKM retroviruses in the absence of Nr5a1.

(C) Phase contrast image of iPSC colonies generated from the retroviral transduction of *Pou5f1-GFP* MEFs with Nr5a1 and SKM.

(D) GFP-positive N_1SKM iPSC colonies in C.

(E) Nr5a1-reprogrammed cells stained positive for alkaline phosphatase.

(F) Nanog expression in Nr5a1-reprogrammed cells

(G) Hoechst staining of F indicates nuclei.

(H) Nr5a1-reprogrammed cells stained positive for SSEA-1

(I) Hoechst staining of H indicates nuclei.

(J) PCR verification of genomic integration of retroviral gene *Nr5a1* in a N_1SKM line. The control panel shows PCR amplification of a region of the *p21* gene.

(K) Normal karyotype of a Nr5a1-reprogrammed line.

(L) EB-mediated *in vitro* differentiation assay performed on Nr5a1-reprogrammed cells. Differentiated cells stained positive for Gata4 (endoderm), Nestin (ectoderm) and α-Smooth Muscle Actin (mesoderm). Lineage markers were stained red and nuclei were stained blue with Hoechst.

(M) Teratoma assay of Nr5a1-reprogrammed cells. Scale bars represent 200 μm in C-E, 100 μm in L and 50 μm in F-I, M.

(N) PCR verification of viral transcript expression of *Nanog*, *Sall4*, *Stat3*, *Zfx*, *Tcfcp2l1*, *Klf2*, *Klf5*, *N-Myc* and *Esrrb*.

(O) Screen of transcription factors that bind to *Pou5f1* regulatory regions in combination with SKM. Control represents transduction of only SKM viruses into MEFs. Nr5a1 and Nr5a2 with SKM were used as positive controls.

(P) Western analysis of cell extracts harvested from 293-T cells transfected with either retroviral vectors encoding Nr5a2 WT, Nr5a2 A368M and Nr5a2 G190V, P191A. 293-T cells transfected with retroviral vector harboring the *GFP* gene was used as a negative control.

(Q) SKM reprogramming with Nr5a2 ligand and DNA binding mutants. *Pou5f1-GFP* MEFs were transduced with SKM viruses and viruses encoding either Nr5a2 WT, Nr5a2 A368M or Nr5a2 G190V, P191A. Control experiment denotes infection of MEFs with only SKM viruses.

(R) Western analysis of cell extracts harvested from 293-T cells transfected with either retroviral vectors encoding Nr5a2 WT, Nr5a2 2KR and Nr5a2 5KR. 293-T cells transfected with retroviral vector not harboring any gene was used as a negative control.

(S) OSKM reprogramming with Nr5a2 SUMO mutants. Control experiment denotes infection of MEFs with only OSKM viruses. Graph depicts fold change of number of GFP-positive colonies generated from the infection of *Pou5f1-GFP* MEFs with OSKM viruses and viruses encoding either Nr5a2 WT, Nr5a2 2KR or Nr5a2 5KR with respect to that of the control. Data in A-B, O, Q and S represent mean ± s.e.m. of three retrovirus-mediated transduction experiments (n=3).

Figure 14. Genome-wide mapping of Nr5a2 binding sites

(A) Motif of Nr5a2 generated by the *de novo* motif discovery algorithm MEME which scans for overrepresented sequences of Nr5a2-bound sites.

(B) Heat map depicting the co-occurrence of Nr5a2 and other transcription factors. Each square in the heat map denotes the frequency of co-localization between two transcription factors (red represents less frequently co-localized and yellow represents more frequently co-localized). Transcription factors have been clustered along both axes based on the similarity in their co-localization with other factors. Transcription factors demarcated by the blue box tend to co-localize with Nr5a2.

(C) Genes important in various cellular roles such as maintenance of ESC identity and cell proliferation that are bound by Nr5a2, Sox2 and Klf4.

Figure 15. *Nanog* is a downstream target of Nr5a2 in reprogramming

(A) Nr5a2 binds to the *Nanog* enhancer during the reprogramming of MEFs. ChIP assay was performed on MEFs 8 days after being co-transduced with OSKM and HA-Nr5a2 viruses. Quantitative real-time PCR was performed to analyze the enrichment of HA-Nr5a2 on the *Nanog* enhancer using an anti-HA antibody. Data shown are mean ± s.e.m. of biological duplicates.

(B) Fold change in expression levels of *Nanog* in OSKM + Nr5a2 reprogramming cells as compared to OSKM reprogramming cells based on time-course (3, 7 and 11 dpi) biological triplicate microarray data (mean ± s.e.m). Fold change in expression levels of ESC-relevant genes, *Gdf3* and *Zic3* were also included in the graph.

(C) Time-course fold change of endogenous *Pou5f1* mRNA levels in OSKM + Nr5a2 or OSKM-infected MEFs with respect to uninfected MEFs.

(D) Time-course fold change of endogenous *Nanog* mRNA levels in OSKM + Nr5a2 or OSKM-infected MEFs with respect to uninfected MEFs. Real-time quantitative PCR data in C-D are mean ± s.e.m of biological triplicate samples..

(E) Real-time quantitative PCR verification of Nr5a2 mRNA level in ESCs after *Nr5a2* shRNA knockdown. Control ESCs were transfected with a shRNA construct targeting the *luciferase* gene.

(F) Western analyses of Nr5a2 protein expression in ESCs after introduction of knockdown construct targeting *Nr5a2*. Nr5a2 protein was targeted with an antibody specific to Nr5a2.

(G) shRNA knockdown of *Nr5a2* in OSKM reprogramming. *Pou5f1* RNAi with OSKM is used as a positive knockdown control while *luciferase* RNAi is used as a negative knockdown control. Nanog or Mtf2 was introduced to investigate their ability to rescue the knockdown effects.

(H) Reprogramming with OSKM in addition to both Nr5a2 and Nanog. Graph depicts fold change of number of GFP-positive colonies with respect to control. Data in E, G and H are mean ± s.e.m. of three independent experiments (n=3).

Figure 16. Nr5a2 reprograms MEFs with Sox2, Klf4 and c-Myc

(A) Schematic representation of the transgenic *Pou5f1-Enhanced Green Fluorescent Protein* (*EGFP*) reporter construct in MEFs. Expression of *EGFP* is under the control of *Pou5f1* regulatory regions, which include the *Pou5f1* distal enhancer and *Pou5f1* promoter (Szabo et al., 2002).

(B) Tunel assay of Nr5a2 and Nr1i2-infected MEFs. Graph shows percentage of tunel-positive cells after fluorescence activated cell sorting (FACS) analysis. MEFs were infected with retroviruses encoding either no gene (pMX), Nr1i2 (pMX-Nr1i2) or Nr5a2 (pMX-Nr5a2). For positive control, uninfected MEFs were subjected to DNase 1 treatment prior to Tunel labeling. Data represent mean ± s.e.m. of three retrovirus-mediated transduction experiments (n=3).

(C) Phase contrast image of iPSC colonies derived from the retroviral transduction of *Pou5f1-GFP* MEFs with Nr5a2, Sox2, Klf4 and c-Myc.

(D) Fluorescence image of C shows the restoration of endogenous *Pou5f1* in Nr5a2-reprogrammed cells.

(E) Alkaline phosphatase expression in N_2SKM iPSCs.

(F) Nanog expression in N_2SKM iPSCs.

(G) Nuclei in F are counterstained with Hoechst.

(H) Expression of SSEA-1 in N_2SKM iPSCs.

(I) Cells in H are stained with Hoechst to mark nuclei. Scale bars represent 200 μm in C-E and 50 μm in F-I.

(J) Screen of other nuclear receptors for their ability to replace Oct4. MEFs were co-transduced with SKM viruses and viruses encoding each of the nuclear receptor. SKM + Nr5a2 was used as a positive control. Control experiment represents transduction of MEFs with only SKM viruses. Number of GFP-positive colonies was counted on 14 dpi. Data represent mean ± s.e.m. of three retrovirus-mediated transduction experiments (n=3).

(K) Karyotypic analysis of N_2SKM #A5, N_2SK #B3 and N_2SK #B11 iPSC lines.

(L) Genotypic analysis of Nr5a2-reprogrammed cells. PCR verification of genomic integration of retroviral genes, *Nr5a2*, *Sox2*, *Klf4* and *c-Myc* in Nr5a2-reprogrammed cells was performed on genomic DNA harvested from ESCs, MEFs and iPSCs with a viral-specific primer and a gene-specific primer. OSKM iPSCs were derived from the viral transduction of MEFs with Oct4, Sox2, Klf4 and c-Myc. PCR amplification of a region of the *p21* gene was performed on all samples and is shown in the control panel.

(M) Adult mouse chimaera generated from the microinjection of N_2SKM #A5 iPSCs derived from F1 (129S2/SV x *Pou5f1-GFP*) MEFs into C57BL/6J embryos.

Figure 17. Nr5a2-reprogrammed cells differentiate into lineages of the three major germ layers in the *in* vitro and *in vivo* differentiation assays, and global expression profiling and epigenetic state of Nr5a2-reprogrammed cells

(A) Embryoid body (EB)-mediated *in vitro* differentiation assay showed that Nr5a2-reprogrammed cells could differentiate into cells of the three major embryonic lineages. Cells differentiated from Nr5a2-reprogramemd cells stained positive for Gata-4 (endoderm), Nestin (ectoderm) and α-Smooth Muscle Actin (mesoderm).

Differentiation markers were stained red and Hoechst dye counterstained the nuclei blue.

(B) Nr5a2-reprogrammed cells differentiated into tissues of the three primary germ layers in the teratoma assay. Teratomas sectioned and stained with Mallory's tetrachrome revealed ectodermal tissue (neural ectoderm), mesodermal tissue (muscle and cartilage) and endodermal tissue (gut epithelium and pancreatic cells). Scale bars represent 100 μm in A and 50 μm in B.

(C) Correlation analysis (46,643 probes) was carried out to cluster the transcriptome of ESCs, iPSCs (OSKM, N_2SKM #A5, N_2SK #B3 and N_2SK #B11) and MEFs (*actin-GFP* and *Pou5f1-GFP*).

(D) Heatmap generated from the microarray data in C displays the expression profile of 1,000 ESC-associated and MEF-associated genes. Genes were selected based on fold differences of expression in ESCs and *Pou5f1-GFP* MEFs and were sorted by average expression ratio and mean-centered to the *Pou5f1-GFP* MEF signal. Green represents downregulation of gene expression while red represents upregulation of gene expression with respect to *Pou5f1-GFP* MEFs.

(E) *Pou5f1* and *Nanog* promoter methylation analysis of Nr5a2-reprogrammed cells. Bisulfite genomic sequencing was performed to analyze methylation status of the promoter region of *Pou5f1* and *Nanog* in ESCs, MEFs and Nr5a2-reprogrammed cells. For each cell line, ten random clones were sequenced and the results are displayed in circles in which open circles represent unmethylated CpG dinucleotides while red circles represent methylated CpG dinucleotides.

(F) Bivalent chromatin marks in Nr5a2-reprogrammed cells. Following ChIP assay, quantitative real-time PCR was performed to analyze the enrichment of trimethylated histone H3K4 and H3K27 chromatin marks in ESCs, MEFs and Nr5a2-reprogrammed cells. Data represents Log_2 enrichment for reported bivalent gene loci (*Zfpm2, Sox21, Pax5, Lbx1h, Evx1* and *Dlx*). Data shown are mean ± s.e.m. of three independent experiments (n=3).

Figure 18. ChIP-seq binding profiles of Nr5a2, Sox2 and Klf4 to common target genes

(A) Cell lysate of ESCs was loaded into lane 1 and endogenous Nr5a2 protein was targeted by an Nr5a2-specific antibody. In lane 2, cell lysate of Nr5a2 3HA-tagged (three HA tags in tandem) stable cell line was loaded. Upper band represents 3HA-tagged Nr5a2 protein whereas lower band represents endogenous Nr5a2 protein.

(B) Binding profiles of Nr5a2, Sox2 and Klf4 to common target genes. The transcription factor trio, Nr5a2, Sox2 and Klf4, binds to pluripotency and self-renewal genes such as *Pou5f1*, *Nanog*, *Klf2*, Tbx3. These transcription factors also bind to cell proliferation genes such as *c-Myc*, *N-Myc* and genes involved in oxidative stress-induced cellular senescence such as *Bach1*. The binding profiles of each of the transcription factor to these target genes from our current and previous ChIP-seq analyses (Chen et al., 2008) are depicted in the plot as shown.

Detailed Disclosure

[00017]. The present invention derives from our discovery that nuclear receptors, preferably members of the nuclear receptor subfamily 5 are able to replace Oct4 in the derivation of iPSCs from somatic cells *in vitro*. Further orphan nuclear receptor Nr5a2 (also known as Lrh-1) is also able to enhance the efficiency of reprogramming with OSKM. Hence, we were interested in testing the reprogramming capacity of Nr5a2 with mutated lysine residues, using a mutant construct with two lysine residues mutated (2KR) and another with five lysine residues mutated (5KR). Western analysis showed that the WT and mutant constructs expressed similar levels of protein (Figure 13R). Strikingly, the OSKM reprogramming assay revealed that the 2KR mutant boosted reprogramming efficiency to at least 7-fold as compared to the 4-fold enhancement achieved by the WT (Figure 13S). When the 5KR mutant was introduced, reprogramming efficiency was further augmented to almost 11-fold (Figure 13S). These results suggest that the concomitant prevention of subcellular localization and the enhanced transcriptional activity brought about by the SUMO site mutations could trigger a greater induction of reprogramming by Nr5a2. The nuclear receptor subfamily 5 -reprogrammed cells are positive for ESC-specific markers, are able to form teratomas comprising tissues of the three lineages and give rise to chimaeras. Taken together, our study shows that transcription

factors unrelated to Oct4 can replace Oct4 and highlights the roles of nuclear receptors as important factors in reprogramming.

[00018]. On the basis of the above, the present invention provides a method for inducing pluripotent stem cells in vitro comprising the steps of: culturing cells in vitro; introducing a polynucleotide that encodes a transcription factor into the cell in the culture, wherein the polynucleotide encodes a transcription factor comprises a nuclear receptor and one or more transcription factor selected from a Sox gene, Krüppel-like factor gene or a gene from the myc family to induce the cell to be a pluripotent cell.

[00019]. Nuclear receptors have the ability to directly bind to DNA and regulate the expression of adjacent genes. Nuclear receptors are modular in structure and contain specific domains such as DNA binding domain (DBD) and Ligand binding domain (LBD). In a preferred embodiment the nuclear receptor comprises one of the nuclear receptors listed in table 1. Preferably the nuclear receptor comprises a nuclear receptor from subfamily 5. The nuclear receptors in subfamily 5 include Nr5a1 and Nr5a2. In a preferred embodiment the nuclear receptor comprises Nr5a2 or a sumoylated mutant thereof.

[00020]. There are 20 human SOX genes and around 30 Sox genes in total have been identified. A Sox gene is a transcription factor that binds to the minor groove in DNA. Sox stand for Sry-related HMG box. A Sox gene is characterized by a sequence called the HMG (high mobility group) box. This HMG box is a DNA binding domain that is highly conserved throughout eukaryotic species. The Sox family has no singular function, and many members possess the ability to regulate several different aspects of development. Sox genes include SOX1 involved in early development of the central nervous system, Sox2 and Sox3 involved in the transition of epithelial granule cells in the cerebellum to their migratory state, Sox 5 involved in the regulation of embryonic development and in the determination of the cell fate as well as many other Sox genes known to those skilled in the art. Preferably the Sox gene comprises Sox 2, Sox 1 and Sox 5. In a preferred embodiment the Sox gene is selected from the group of Sox 2, Sox 1 and Sox 5.

[00021]. The Krüppel-like factor family of transcription factors (Klfs), are characterised by their three Cys2 His2 zinc fingers located at the C terminus separated by a highly conserved link. The following human genes encode Kruppel-like factors: KLF1, KLF2, KLF3, KLF4, KLF5, KLF6, KLF7, KLF8, KLF9, KLF10, KLF11, KLF12, KLF13, KLF14, KLF15, KLF16, or KLF17. In a preferred embodiment the Krüppel-like factor comprises klf4, klf2 or klf5. In a preferred embodiment the Krüppel-like factor is selected from the group of klf4, klf2 and klf5.

[00022]. Myc family of genes comprises transcription factors, which contain the bHLH/LZ (basic Helix-Loop-Helix/ Leucine Zipper) domain. Myc family of genes includes N-Myc and L-Myc genes. In a preferred embodiment the gene from the Myc-family comprises N-Myc, L-Myc or C-Myc. In a preferred embodiment the gene from the Myc-family is selected from the group of N-Myc, L-Myc and C-Myc.

Vectors

[00023]. The present invention also provides a vector comprising a polynucleotide of the invention, for example an expression vector comprising a polynucleotide of the invention, operably linked to regulatory sequences capable of directing expression of said polynucleotide in a host cell.

[00024]. Any nuclear receptor nucleic acid specimen, in purified or non-purified form, can be utilised as the starting nucleic acid or acids.

[00025]. PCR is one such process that may be used to amplify isolated nuclear receptor sequences. This technique may amplify, for example, DNA or RNA, including messenger RNA, wherein DNA or RNA may be single stranded or double stranded. In the event that RNA is to be used as a template, enzymes, and/or conditions optimal for reverse transcribing the template to DNA would be utilized. In addition, a DNA-RNA hybrid that contains one strand of each may be utilized. A mixture of nucleic acids may also be employed, or the nucleic acids produced in a previous amplification reaction described herein, using the same or different primers may be so utilised.

[00026]. The specific nucleic acid sequence to be amplified, may be a fraction of a nucleic acid or can be present initially as a discrete nucleic acid, so that the specific sequence constitutes the entire nucleic acid. It is not necessary that the sequence to be amplified is present initially in a pure form; it may be a minor fraction of a complex mixture, such as contained in whole human DNA.

[00027]. DNA utilized herein may be extracted from a body sample, such as blood, tissue material, lung tissue and the like by a variety of techniques known in the art. If the extracted sample has not been purified, it may be treated before amplification with an amount of a reagent effective to open the cells, or animal cell membranes of the sample, and to expose and/or separate the strand(s) of the nucleic acid(s). This lysing and nucleic acid denaturing step to expose and separate the strands will allow amplification to occur much more readily.

[00028]. The deoxyribonucleotide triphosphates dATP, dCTP, dGTP and dTTP are added to the synthesis mixture, either separately or together with the primers, in adequate amounts and the resulting solution is heated to about 90 degrees – 100 degrees C from about 1 to 10 minutes, preferably from 1 to 4 minutes. After this heating period, the solution is allowed to cool, which is preferable for the primer hybridization. To the cooled mixture is added an appropriate agent for effecting the primer extension reaction (called herein "agent for polymerization"), and the reaction is allowed to occur under conditions known in the art. The agent for polymerization may also be added together with the other reagents if it is heat stable. This synthesis (or amplification) reaction may occur at room temperature up to a temperature above which the agent for polymerization no longer functions. Thus, for example, if DNA polymerase is used as the agent, the temperature is generally no greater than about 40 degree C. Most conveniently the reaction occurs at room temperature.

[00029]. Primers direct amplification of a target polynucleotide (eg nuclear receptor such as subfamily 5). Primers used should be of sufficient length and appropriate sequence to provide initiation of polyrmerisation. Environmental conditions conducive to synthesis include the presence of nucleoside triphosphates and an

agent for polymerisation, such as DNA polymerase, and a suitable temperature and pH.

[00030]. Primers are preferably single stranded for maximum efficiency in amplification, but may be double stranded. If double stranded, primers may be first treated to separate the strands before being used to prepare extension products. Primers should be sufficiently long to prime the synthesis of nuclear receptor of the invention, into extension products in the presence of the inducing agent for polymerization. The exact length of a primer will depend on many factors, including temperature, buffer, and nucleotide composition. Oligonucleotide primers will typically contain 12-20 or more nucleotides, although they may contain fewer nucleotides.

[00031]. Primers should be designed to be substantially complementary to each strand of the nuclear receptor genomic gene sequence. This means that the primers must be sufficiently complementary to hybridise with their respective strands under conditions that allow the agent for polymerisation to perform. In other words, the primers should have sufficient complementarity with the 5' and 3' sequences flanking the mutation to hybridise therewith and permit amplification of the nuclear receptor genomic gene sequence.

[00032]. Oligonucleotide primers of the invention employed in the PCR amplification process that is an enzymatic chain reaction that produces exponential quantities of CD166 gene sequence relative to the number of reaction steps involved. Typically, one primer will be complementary to the negative (-) strand of the nuclear receptor gene sequence and the other is complementary to the positive (+) strand. Annealing the primers to denatured nucleic acid followed by extension with an enzyme, such as the large fragment of DNA polymerase I (Klenow) and nucleotides, results in newly synthesised + and - strands containing the target nuclear receptor gene sequence. Because these newly synthesized sequences are also templates, repeated cycles of denaturing, primer annealing, and extension results in exponential production of the region (i.e., the nuclear receptor gene sequence) defined by the primers. The product of the chain reaction is a discreet

nucleic acid duplex with termini corresponding to the ends of the specific primers employed.

[00033]. Oligonucleotide primers may be prepared using any suitable method, such as conventional phosphotriester and phosphodiester methods or automated embodiments thereof. In one such automated embodiment, diethylphosphoramidites are used as starting materials and may be synthesized as known in the art.

[00034]. The agent for polymerisation may be any compound or system which will function to accomplish the synthesis of primer extension products, including enzymes. Suitable enzymes for this purpose include, for example, E. coli DNA polymerase I, Klenow fragment of E. coli DNA polymerase, polymerase muteins, reverse transcriptase, other enzymes, including heat-stable enzymes (ie, those enzymes which perform primer extension after being subjected to temperatures sufficiently elevated to cause denaturation), such as Taq polymerase. Suitable enzyme will facilitate combination of the nucleotides in the proper manner to form the primer extension products that are complementary to each nuclear receptor gene sequence nucleic acid strand. Generally, the synthesis will be initiated at the 3' end of each primer and proceed in the 5' direction along the template strand, until synthesis terminates, producing molecules of different lengths.

[00035]. The newly synthesised nuclear receptor strand and its complementary nucleic acid strand will form a double-stranded molecule under hybridizing conditions described above and this hybrid is used in subsequent steps of the process.

[00036]. The steps of denaturing, annealing, and extension product synthesis can be repeated as often as needed to amplify the target polymorphic gene sequence nucleic acid sequence to the extent necessary. The amount of the specific nucleic acid sequence produced will accumulate in an exponential fashion. This may also be achieve via real time PCR as known in the art.

[00037]. Preferably, the method of amplifying nuclear receptor is by PCR, as described herein or real time PCR and as is commonly used by those of ordinary

skill in the art. Alternative methods of amplification have been described and can also be employed as long as the nuclear receptor sequence amplified by PCR using primers of the invention is similarly amplified by the alternative means. Such alternative amplification systems include but are not limited to self-sustained sequence replication, which begins with a short sequence of RNA of interest and a T7 promoter. Reverse transcriptase copies the RNA into cDNA and degrades the RNA, followed by reverse transcriptase polymerizing a second strand of DNA. Another nucleic acid amplification technique is nucleic acid sequence-based amplification (NASBA) which uses reverse transcription and T7 RNA polymerase and incorporates two primers to target its cycling scheme. NASBA can begin with either DNA or RNA and finish with either, and amplifies to 10^8 copies within 60 to 90 minutes. Alternatively, nucleic acid can be amplified by ligation activated transcription (LAT). LAT works from a single-stranded template with a single primer that is partially single-stranded and partially double-stranded. Amplification is initiated by ligating a cDNA to the promoter oligonucleotide and within a few hours, amplification is 10^8 to 10^9 fold. The QB replicase system can be utilized by attaching an RNA sequence called MDV-1 to RNA complementary to a DNA sequence of interest. Upon mixing with a sample, the hybrid RNA finds its complement among the specimen's mRNAs and binds, activating the replicase to copy the tag-along sequence of interest. Another nucleic acid amplification technique, ligase chain reaction (LCR), works by using two differently labeled halves of a sequence of interest that are covalently bonded by ligase in the presence of the contiguous sequence in a sample, forming a new target. The repair chain reaction (RCR) nucleic acid amplification technique uses two complementary and target-specific oligonucleotide probe pairs, thermostable polymerase and ligase, and DNA nucleotides to geometrically amplify targeted sequences. A 2-base gap separates the oligonucleotide probe pairs, and the RCR fills and joins the gap, mimicking normal DNA repair. Nucleic acid amplification by strand displacement activation (SDA) utilizes a short primer containing a recognition site for hincII with short overhang on the 5' end that binds to target DNA. A DNA polymerase fills in the part of the primer opposite the overhang with sulfur-containing adenine analogs. HincII is

added but only cuts the unmodified DNA strand. A DNA polymerase that lacks 5' exonuclease activity enters at the site of the nick and begins to polymerize, displacing the initial primer strand downstream and building a new one which serves as more primer. SDA produces greater than 10^7 -fold amplification in 2 hours at 37 degrees C. Unlike PCR and LCR, SDA does not require instrumented temperature cycling. Another amplification system useful in the method of the invention is the QB Replicase System. Although PCR is the preferred method of amplification if the invention, these other methods can also be used to amplify the nuclear receptor sequences as described in the method of the invention.

[00038]. Polynucleotides of the invention may be incorporated into a recombinant replicable vector for introduction into a host cell. Such vectors may typically comprise a replication system recognized by the host, including the intended polynucleotide encoding the desired polypeptide, and will preferably also include transcription and translational initiation regulatory sequences operably linked to the polypeptide encoding segment. Expression vectors may include, for example, an origin of replication or autonomously replicating sequence (ARS) and expression control sequences, a promoter, an enhancer and necessary processing information sites, such as ribosome-binding sites, RNA splice sites, polyadenylation sites, transcriptional terminator sequences, and mRNA stabilizing sequences. localization signals may also be included where appropriate, whether from a native nuclear receptor protein or from other receptors or from secreted polypeptides of the same or related species, which allow the protein to move across cell membranes, and thus attain its functional topology. Such vectors may be prepared by means of standard recombinant techniques well known in the art.

[00039]. An appropriate promoter and other necessary vector sequences will be selected so as to be functional in the host, and may include, when appropriate, those naturally associated with nuclear receptor genes. Examples of workable combinations of cell lines and expression vectors are known in the art. Many useful vectors are known in the art and may be obtained from such vendors as Stratagene, New England Biolabs, Promega Biotech, and others. Promoters such as the trp, lac

and phage promoters, tRNA promoters and glycolytic enzyme promoters may be used in prokaryotic hosts. Useful yeast promoters include promoter regions for metallothionein, 3-phosphoglycerate kinase or other glycolytic enzymes such as enolase or glyceraldehyde-3-phosphate dehydrogenase, enzymes responsible for maltose and galactose utilization, and others. Vectors and promoters suitable for use in yeast expression are known. Appropriate non-native mammalian promoters might include the early and late promoters from SV40 or promoters derived from murine Moloney leukemia virus, mouse tumour virus, avian sarcoma viruses, adenovirus II, bovine papilloma virus or polyoma. In addition, the construct may be joined to an amplifiable gene (e.g., DHFR) so that multiple copies of the gene may be made. For appropriate enhancer and other expression control sequences.

[00040]. While such expression vectors may replicate autonomously, they may also replicate by being inserted into the genome of the host cell, by methods well known in the art.

[00041]. Expression and cloning vectors will likely contain a selectable marker, a gene encoding a protein necessary for survival or growth of a host cell transformed with the vector. The presence of this gene ensures growth of only those host cells that express the inserts. Typical selection genes encode proteins that a) confer resistance to antibiotics or other toxic substances, e.g. ampicillin, neomycin, methotrexate, etc.; b) complement auxotrophic deficiencies, or c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for *Bacilli.* The choice of the proper selectable marker will depend on the host cell, and appropriate markers for different hosts are well known in the art.

[00042]. The vectors containing the nucleic acids of interest can be transcribed in vitro, and the resulting RNA introduced into the host cell by well-known methods, e.g., by injection, or the vectors can be introduced directly into host cells by methods well known in the art, which vary depending on the type of cellular host, including electroporation; transfection employing calcium chloride, rubidium chloride, calcium phosphate, DEAE-dextran, or other substances; microprojectile bombardment; lipofection; infection (where the vector is an infectious agent, such as a retroviral

genome); and other methods. The introduction of the polynucleotides into the host cell by any method known in the art, including, inter alia, those described above, will be referred to herein as "transformation." The cells into which have been introduced nucleic acids described below are meant to also include the progeny of such cells.

Polynucleotides

[00043]. An isolated nuclear receptor nucleic acid molecule is disclosed which molecule typically encodes a nuclear receptor polypeptide. The nucleic acid molecule comprises any nucleic acid capable of encoding a functional nuclear receptor polypeptide listed in table 1. Preferably the nucleic acid molecule comprises a nucleic acid capable of encoding a nuclear receptor from subfamily 5, an allelic variant, or analog, including fragments, thereof. The nuclear receptors of subfamily 5 may include any one of Nr5a2, Nr5a1 or an allelic variant, or analog, including fragments, thereof that includes the DNA binding domain (DBD) and or an activation domain. Specifically provided are DNA molecules selected from the group consisting of: (a) DNA molecules set out in SEQ ID NOS: 1, 3, 5, 8 or encode fragments thereof such as SEQ ID NO: 10; (b) DNA molecules that hybridize to the DNA molecules defined in (a) or hybridisable fragments thereof; and (c) DNA molecules that encode an expression for the amino acid sequence encoded by any of the foregoing DNA molecules.

[00044]. Preferred DNA molecules according to the invention include DNA molecules comprising the sequence set out in SEQ ID NOS: 1, 3, 5 8 or that encode fragments thereof such as SEQ ID NO: 10.

[00045]. A polynucleotide is said to "encode" a polypeptide if, in its native state or when manipulated by methods well known to those skilled in the art, it can be transcribed and/or translated to produce the mRNA for and/or the polypeptide or a fragment thereof. The anti-sense strand is the complement of such a nucleic acid, and the encoding sequence can be deduced there-from.

[00046]. An "isolated" or "substantially pure" nucleic acid (e.g., an RNA, DNA or a mixed polymer) is one which is substantially separated from other cellular

components which naturally accompany a native human sequence or protein, e.g., ribosomes, polymerases, many other human genome sequences and proteins. The term embraces a nucleic acid sequence or protein that has been removed from its naturally occurring environment, and includes recombinant or cloned DNA isolates and chemically synthesized analogs or analogs biologically synthesized by heterologous systems.

[00047]. "Nuclear receptor gene sequence," "nuclear receptor gene," "nuclear receptor nucleic acids" or " nuclear receptor polynucleotide" each refer to a polynucleotide that contains specific domains such as DNA binding domain (DBD) and the activation domain. Further, they refer to a polynucleotide thatencode proteins listed in table 1. Preferably the nucleic acid molecule comprises a nucleic acid capable of encoding a nuclear receptor from subfamily 5, an allelic variant, or analog, including fragments, or mutants thereof. The nuclear receptors of subfamily 5 may include any one of Nr5a2, Nr5a1 or an allelic variant, or analog, including fragments, or mutants thereof that includes the DNA binding domain (DBD) and an activation domain. A sumoylated mutant may refer to an Nr5a2 or Nr5a1 mutant construct with lysine resides mutated for example Nr5a2 2KR set out in SEQ ID No. 1 or SEQ ID No. 3.

[00048]. These terms, when applied to a nucleic acid, refer to a nucleic acid that encodes a nuclear receptor polypeptide, fragment, homologue mutant or variant, including, e.g., protein fusions, sumoylated mutant or deletions. The nucleic acids of the present invention will possess a sequence that is either derived from, or substantially similar to a natural nuclear receptor encoding gene or one having substantial homology with a natural nuclear receptor encoding gene or a portion thereof. The coding sequences for mouse nuclear receptor polypeptide from subfamily 5 are shown in SEQ ID NOS: 5 and 8 with the amino acid sequence shown in SEQ ID NOS: 6, 7 and 9 to 11. The coding sequences for sumoylated nuclear receptor polypeptide from subfamily 5 are shown in SEQ ID NOS: 1 and 3 with the amino acid sequence shown in SEQ ID NOS: 2 and 4.

[00049]. A nucleic acid or fragment thereof is "substantially homologous" ("or substantially similar") to another if, when optimally aligned (with appropriate nucleotide insertions or deletions) with the other nucleic acid (or its complementary strand), there is nucleotide sequence identity in at least about 60% of the nucleotide bases, usually at least about 70%, more usually at least about 80%, preferably at least about 90%, and more preferably at least about 95-98% of the nucleotide bases. Examples of coding sequence for working substantially homologous fragments are shown in SEQ ID NOS: 1, 3, 5, and 8 with the amino acid sequence shown in SEQ ID NOS: 2, 4, 6, 7 and 9 to 11.

[00050]. Alternatively, substantial homology or (identity) exists when a nucleic acid or fragment thereof will hybridise to another nucleic acid (or a complementary strand thereof) under selective hybridisation conditions, to a strand, or to its complement. Selectivity of hybridisation exists when hybridisation that is substantially more selective than total lack of specificity occurs. Typically, selective hybridisation will occur when there is at least about 55% identity over a stretch of at least about 14 nucleotides, preferably at least about 65%, more preferably at least about 75%, and most preferably at least about 90%. The length of homology comparison, as described, may be over longer stretches, and in certain embodiments will often be over a stretch of at least about nine nucleotides, usually at least about 20 nucleotides, more usually at least about 24 nucleotides, typically at least about 28 nucleotides, more typically at least about 32 nucleotides, and preferably at least about 36 or more nucleotides.

[00051]. Thus, polynucleotides of the invention preferably have at least 75%, more preferably at least 85%, more preferably at least 90% homology to the sequences shown in the sequence listings herein. More preferably there is at least 95%, more preferably at least 98%, homology. Nucleotide homology comparisons may be conducted as described below for polypeptides. A preferred sequence comparison program is the GCG Wisconsin Bestfit program described below. The default scoring matrix has a match value of 10 for each identical nucleotide and -9 for each

mismatch. The default gap creation penalty is -50 and the default gap extension penalty is -3 for each nucleotide.

[00052]. In the context of the present invention, a homologous sequence is taken to include a nucleotide sequence which is at least 60, 70, 80 or 90% identical, preferably at least 95 or 98% identical at the amino acid level over at least 20, 50, 100, 200, 300, 500 or 1000 nucleotides with the nucleotides sequences set out in SEQ ID. NOS: 1, 3, 5, and 8. In particular, homology should typically be considered with respect to those regions of the sequence that encode contiguous amino acid sequences known to be essential for the function of the protein rather than non-essential neighbouring sequences. Preferred polypeptides of the invention comprise a contiguous sequence having greater than 50, 60 or 70% homology, more preferably greater than 80, 90, 95 or 97% homology, to one or more of the nucleotides sequences encoding polypeptide sequences SEQ ID NOS: 10, or 11 which encode amino acids 1 to 499, 1 to 560, 1 to 462, of SEQ ID NOS: 6, 7 or 9 respectively. Preferred polynucleotides may alternatively or in addition comprise a contiguous sequence having greater than 80, 90, 95 or 97% homology to the sequence of SEQ ID NOS: 5, or 8 that encodes amino acids 1 to 499, 1 to 560, 1 to 462, 100 to 187 or 317 to 560 of SEQ ID NOS: 6, 7, 9, 10 or 11 respectively. Preferred nucleic acids preferably contain specific domains such as DNA binding domain (DBD) and the activation domain.

[00053]. Nucleotide sequences are preferably at least 15 nucleotides in length, more preferably at least 20, 30, 40, 50, 100 or 200 nucleotides in length. Generally, the shorter the length of the polynucleotide, the greater the homology required to obtain selective hybridization. Consequently, where a polynucleotide of the invention consists of less than about 30 nucleotides, it is preferred that the % identity is greater than 75%, preferably greater than 90% or 95% compared with the nuclear receptor nucleotide sequences set out in the sequence listings herein. Conversely, where a polynucleotide of the invention consists of, for example, greater than 50 or 100 nucleotides, the % identity compared with the nuclear receptor nucleotide

sequences set out in the sequence listings herein may be lower, for example greater than 50%, preferably greater than 60 or 75%.

[00054]. Nucleic acid hybridisation will be affected by such conditions as salt concentration, temperature, or organic solvents, in addition to the base composition, length of the complementary strands, and the number of nucleotide base mismatches between the hybridizing nucleic acids, as will be readily appreciated by those skilled in the art. Stringent temperature conditions will generally include temperatures in excess of 30 degrees C., typically in excess of 37 degrees C., and preferably in excess of 45 degrees C. Stringent salt conditions will ordinarily be less than 1000 mM, typically less than 500 mM, and preferably less than 200 mM. However, the combination of parameters is much more important than the measure of any single parameter. An example of stringent hybridization conditions is 65°C and 0.1xSSC (1xSSC = 0.15 M NaCl, 0.015 M sodium citrate pH 7.0).

[00055]. The "polynucleotide" compositions of this invention include RNA, cDNA, genomic DNA, synthetic forms, and mixed polymers, both sense and antisense strands, and may be chemically or biochemically modified or may contain non-natural or derivatized nucleotide bases, as will be readily appreciated by those skilled in the art. Such modifications include, for example, labels, methylation, substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, etc.), charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), pendent moieties (e.g., polypeptides), intercalators (e.g., acridine, psoralen, etc.), chelators, alkylators, sumoylated site mutants and modified linkages (e.g., alpha anomeric nucleic acids, etc.). Also included are synthetic molecules that mimic polynucleotides in their ability to bind to a designated sequence via hydrogen bonding and other chemical interactions. Such molecules are known in the art and include, for example, those in which peptide linkages substitute for phosphate linkages in the backbone of the molecule.

Polypeptides

[00056]. Full length nuclear receptor polypeptides of the present invention have at least 87 amino acids, encode a nuclear receptor in an animal, particularly a mammal, and include allelic variants, mutants or homologues. Nuclear receptor polypeptides of the invention also include fragments and derivatives of full length nuclear receptor polypeptides, particularly fragments or derivatives having substantially the same or enhanced biological activity. The nuclear receptor polypeptides include those comprising the amino acid sequences of SEQ ID NOS: 2, 4, 6, 7, 9, 10, 11 or allelic variants, mutants or homologues, including fragments, thereof such as SEQ ID NOS: 10, or 11. A particularly preferred polypeptide consists of amino acids 1 to 560, 1 to 560, 1 to 499, 1 to 560, 1 to 462, of the amino acid sequence shown as SEQ ID NOS: 2, 4, 6, 7 or 9 respectively or allelic variants, homologues or fragments, thereof such as SEQ ID NOS: 10, or 11.

[00057]. The term "polypeptide" refers to a polymer of amino acids and its equivalent and does not refer to a specific length of the product; thus, peptides, oligopeptides and proteins are included within the definition of a polypeptide. This term also does not refer to, or exclude modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations, and the like. Included within the definition are, for example, polypeptides containing one or more analog of an amino acid (including, for example, natural isolated amino acids, etc.), polypeptides with substituted linkages as well as other modifications known in the art, both naturally and non-naturally occurring.

[00058]. In the context of the present invention, a homologous sequence is taken to include an amino acid sequence which is at least 60, 70, 80 or 90% identical, preferably at least 95 or 98% identical at the amino acid level over at least 20, 50, 100, 200, 300 or 400 amino acids with the amino acid sequences set out in SEQ ID. Nos 2, 4, 6, 7, 9, 10 or 11. In particular, homology should typically be considered with respect to those regions of the sequence known to be essential for the function of the protein such as the DNA binding domain an example of which is SEQ ID NO: 10 or the activation domain an example of which is SEQ ID NO: 11 rather than less important neighbouring sequences such as the ligand binding domain (LBD).

Preferred polypeptides of the invention comprise a contiguous sequence having greater than 50, 60 or 70% homology, more preferably greater than 80 or 90% homology, to one or more of amino acids of SEQ ID NOS: 2, 4, 6, 7, 9, 10 or 11.

[00059]. Other preferred polypeptides comprise a contiguous sequence having greater than 40, 50, 60, or 70% homology, of SEQ ID No: 2, 4, 6, 7, 9, 10 or 11. Although homology can also be considered in terms of similarity (i.e. amino acid residues having similar chemical properties/functions), in the context of the present invention it is preferred to express homology in terms of sequence identity. The terms "substantial homology" or "substantial identity", when referring to polypeptides, indicate that the polypeptide or protein in question exhibits at least about 70% identity with an entire naturally-occurring protein or a portion thereof, usually at least about 80% identity, and preferably at least about 90 or 95% identity.

[00060]. Homology comparisons can be conducted by eye, or more usually, with the aid of readily available sequence comparison programs. These commercially available computer programs can calculate % homology between two or more sequences.

[00061]. Percentage (%) homology may be calculated over contiguous sequences, i.e. one sequence is aligned with the other sequence and each amino acid in one sequence directly compared with the corresponding amino acid in the other sequence, one residue at a time. This is called an "ungapped" alignment. Typically, such ungapped alignments are performed only over a relatively short number of residues (for example less than 50 contiguous amino acids).

[00062]. Although this is a very simple and consistent method, it fails to take into consideration that, for example, in an otherwise identical pair of sequences, one insertion or deletion will cause the following amino acid residues to be put out of alignment, thus potentially resulting in a large reduction in % homology when a global alignment is performed. Consequently, most sequence comparison methods are designed to produce optimal alignments that take into consideration possible insertions and deletions without penalising unduly the overall homology score. This

is achieved by inserting "gaps" in the sequence alignment to try to maximise local homology.

[00063]. However, these more complex methods assign "gap penalties" to each gap that occurs in the alignment so that, for the same number of identical amino acids, a sequence alignment with as few gaps as possible - reflecting higher relatedness between the two compared sequences - will achieve a higher score than one with many gaps. "Affine gap costs" are typically used that charge a relatively high cost for the existence of a gap and a smaller penalty for each subsequent residue in the gap. This is the most commonly used gap scoring system. High gap penalties will of course produce optimised alignments with fewer gaps. Most alignment programs allow the gap penalties to be modified. However, it is preferred to use the default values when using such software for sequence comparisons. For example when using the GCG Wisconsin Bestfit package (see below) the default gap penalty for amino acid sequences is -12 for a gap and -4 for each extension.

[00064]. Calculation of maximum % homology therefore firstly requires the production of an optimal alignment, taking into consideration gap penalties. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package (University of Wisconsin, U.S.A.; Devereux *et al.,* 1984, Nucleic Acids Research 12:387). Examples of other software that can perform sequence comparisons include, but are not limited to, the BLAST package (see Ausubel *et al.*, 1999 *ibid* – Chapter 18), FASTA (Atschul *et al.,* 1990, J. Mol. Biol., 403-410) and the GENEWORKS suite of comparison tools. Both BLAST and FASTA are available for offline and online searching (see Ausubel *et al.*, 1999 *ibid*, pages 7-58 to 7-60). However it is preferred to use the GCG Bestfit program.

[00065]. Although the final % homology can be measured in terms of identity, the alignment process itself is typically not based on an all-or-nothing pair comparison. Instead, a scaled similarity score matrix is generally used that assigns scores to each pairwise comparison based on chemical similarity or evolutionary distance. An example of such a matrix commonly used is the BLOSUM62 matrix - the default matrix for the BLAST suite of programs. GCG Wisconsin programs generally use

either the public default values or a custom symbol comparison table if supplied (see user manual for further details). It is preferred to use the public default values for the GCG package, or in the case of other software, the default matrix, such as BLOSUM62.

[00066]. Once the software has produced an optimal alignment, it is possible to calculate % homology, preferably % sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result.

[00067]. Nuclear receptor polypeptide homologues include those having the amino acid sequences, wherein one or more of the amino acids is substituted with another amino acid which substitutions do not substantially alter the biological activity of the molecule to reprogram a differentiated cell to a pluripotent state. A nuclear receptor polypeptide homologue according to the invention preferably has 80 percent or greater with any of the amino acids listed in table 1. Preferably the nuclear receptor is from subfamily 5 and preferably has 80 percent or greater homology with any one of the sequence identity to a amino acid sequence set out in SEQ ID NO: 2, 4, 6, 7, 9, 10 or 11. Examples of nuclear receptor polypeptide homologues within the scope of the invention include the amino acid sequence of SEQ ID NOS: 2, 4, 6, 7, 9, 10 or 11 wherein: (a) one or more aspartic acid residues is substituted with glutamic acid; (b) one or more isoleucine residues is substituted with leucine; (c) one or more glycine or valine residues is substituted with alanine; (d) one or more arginine residues is substituted with histidine; or (e) one or more tyrosine or phenylalanine residues is substituted with tryptophan.

[00068]. Preferably "nuclear receptor protein" or " nuclear receptor polypeptide" refers to a protein or polypeptide encoded by a nuclear receptor gene sequence, variants or fragments thereof. Preferably the "nuclear receptor protein" or "nuclear receptor polypeptide" refers to a protein or polypeptide listed in table 1. More preferably the "nuclear receptor protein" or " nuclear receptor polypeptide" refers to a protein or polypeptide of the subfamily 5. The nuclear receptors of subfamily 5 may include any one of Nr5a2, Nr5a1 or any variants mutants or fragments thereof. Also included are proteins encoded by DNA that hybridize under high or low stringency

conditions, to nuclear receptor encoding nucleic acids and closely related polypeptides or proteins retrieved by antisera to the nuclear receptor protein(s).

[00069]. "Protein modifications or fragments" are provided by the present invention for nuclear receptor polypeptides or fragments thereof which are substantially homologous to primary structural sequence but which include, e.g., *in vivo* or *in vitro* chemical and biochemical modifications or which incorporate unusual amino acids. Such modifications include, for example, sumoylated site mutations acetylation, carboxylation, phosphorylation, glycosylation, ubiquitination, labeling, e.g., with radionuclides, and various enzymatic modifications, as will be readily appreciated by those well skilled in the art. A variety of methods for labeling polypeptides and of substituents or labels useful for such purposes are well known in the art, and include radioactive isotopes such as ^{32}P, ligands which bind to labeled antiligands (e.g., antibodies), fluorophores, chemiluminescent agents, enzymes, and antiligands which can serve as specific binding pair members for a labeled ligand. The choice of label depends on the sensitivity required, ease of conjugation with the primer, stability requirements, and available instrumentation. Methods of labeling polypeptides are well known in the art.

[00070]. A polypeptide "fragment," "portion" or "segment" is a stretch of amino acid residues of at least about five to seven contiguous amino acids, often at least about seven to nine contiguous amino acids, typically at least about nine to 13 contiguous amino acids and, most preferably, at least about 20 to 30 or more contiguous amino acids.

[00071]. Preferred polypeptides of the invention have substantially similar function to wild type full length nuclear transcription factor. Preferred polynucleotides of the invention encode polypeptides having substantially similar function to wild type full length nuclear transcription factor. "Substantially similar function" refers to the function of a nucleic acid or polypeptide homologue, variant, derivative or fragment of nuclear receptor with reference to the wild-type nuclear receptor nucleic acid or wild-type nuclear receptor polypeptide to reprogramme somatic cells in accordance with the assays described herein.

Compositions of the Invention

[00072]. Polyeptides produced according to the invention can be administered to somatic cells for reprogramming in the form of compositions.

[00073]. Thus, the present invention also relates to compositions including pharmaceutical compositions comprising a therapeutically effective amount of a a nuclear receptor polypeptide and one or more transcription factor polypeptide selected from a Sox polypeptide, Krüppel-like factor polypeptide or a polypeptide from the myc family.. As used herein a compound will be therapeutically effective if it is able to reprogramming somatic cells *in vitro*.

[00074]. Compositions of the invention suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions and or one or more carrier. Alternatively, injectable solutions may be delivered encapsulated in liposomes to assist their transport across cell membrane. Alternatively or in addition such preparations may contain constituents of self-assembling pore structures to facilitate transport across the cellular membrane. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating/destructive action of microorganisms such as, for example, bacteria and fungi.

[00075]. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as, for example, lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Preventing the action of microorganisms in the compositions of the invention is achieved by adding antibacterial and/or antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the

injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

[00076]. Sterile injectable solutions are prepared by incorporating the active polypeptides in the required amount in the appropriate solvent with several of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying, to yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

[00077]. Biologically acceptable carriers and/or diluents may also include any and all solvents, dispersion media, antibacterials and/or antifungals, isotonic and absorption delaying agents and the like. The use of such media and agents for culture is well known in the art. Except insofar as any conventional media or agent is incompatible with the active polypeptide, use thereof in the compositions is contemplated.

[00078]. Polypeptides can also be delivered by protein delivery methods known in the art such as transfection as described above, macromolecule delivery vehicles and other methods known to those skilled in the art.

[00079]. The compositions may be for use in reprogramming somatic cells. Use includes use of a composition of the invention for the preparation of a pluripotent cell as a medicament for the treatment of degenerative diseases where new cells are required.

Method of inducing pluripotent cells for use in treatment

[00080]. An embodiment of the present invention resides in a method for inducing pluripotent stem cells *in vitro* in the manufacture of a medicament for treating a patient in need of a pluripotent stem cell treatment comprising the steps of: isolating

cells from an individual donor; culturing the cells in vitro; introducing a polynucleotide that encodes a transcription factor into the cell in the culture, wherein the polynucleotide encodes a transcription factor comprises a nuclear receptor and one or more transcription factor selected from the group Sox2, Klf4, c-Myc, Klf2, Klf5, Sox1, Sox5, N-myc and L-myc to induce the cell to be a pluripotent cell; introducing the pluripotent cell to the patient in need of a pluripotent stem cell treatment.

[00081]. "Treatment" and "treat" and synonyms thereof refer to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent or slow down (lessen) a degenerative condition. Those in need of such treatment include those already diagnosed with stroke, cancer, diabetes, neurological disorders such as Parkinson's disease, Huntington's disease, Alzheimer's, dementia, as well as cardiac failure and muscle damage, along with many others.

[00082]. As used herein a "therapeutically effective amount" of a compound will be an amount of cells that are capable of preventing or at least slowing down (lessening) a degenerative condition, in particular increasing the lifespan of the patent. Dosages and administration of cells of the invention may be determined by one of ordinary skill in the art. An effective amount of the cells to be employed therapeutically will depend, for example, upon the therapeutic objectives, the route of administration, and the condition of the mammal. Accordingly, it will be necessary for the therapist to adjust the dosage and modify the route of administration as required to obtain the optimal therapeutic effect.

[00083]. Preferably, the pluripotent cells of the invention are used in neurological disorders such as Parkinson's disease, Huntington's disease, Alzheimer's, dementia or stroke.

Induced pluripotent stem cell lines

[00084]. In one embodiment the method of making pluripotent stem cell lines comprises: culturing cells *in vitro*; introducing a polynucleotide that encodes a

transcription factor into the cell in the culture, wherein the polynucleotide encodes a transcription factor comprises a nuclear receptor and one or more transcription factor selected from the group Sox2, Klf4, c-Myc, Klf2, Klf5, Sox1, Sox5, N-myc and L-myc to induce the cell to be a pluripotent cell; passaging the pluripotent cells to maintain the cell line.

Cell Preparation

[00085]. A "cell", as used herein, refers to a biological sample obtained from a tissue in the body, or from body fluid. Frequently the cell will be a "clinical sample," which is a sample derived from a patient such as a fine needle biopsy sample. A "cell" may also include cells isolated from fluids such as blood, serum and the like. Cell samples can be isolated and obtained from tissues from lung, bladder, brain, uterus, cervix, colon, rectum, esophagus, mouth, head, muscle, heart, skin, kidney, breast, ovary, neck, pancreas, prostate, testis, liver gonads, stomach or from any other organ or tissue known to those skilled in the art.

[00086]. Cell samples are obtained from the body and include cells and extracellular matter. Cell samples may be from humans or non human animals. Cell samples can be from any organ or fluid. Cell samples can be obtained using known procedures, such as excision, a needle biopsy, blood extraction or the like. The cells are to be processed in a manner that allows culturing and reprogramming of the cells. Accordingly, cells obtained from a subject, donor or individual are ideally washed then immediately cultured.

Cell culture

[00087]. The iPSC's may be cultured as known in the art on a relevant culture media such as an artificial medium to grow the cells *in vitro* for research or medical treatment. The cells may be passaged though several generations as known in the art to keep the cells continuous. The culture media may contain nutrients to nourish and support the cells. Culture medium may also include growth factors added to produce desired changes in the cells.

Examples of preferred embodiments

Reprogramming capacity of nuclear receptors

[00088]. A screen of 18 nuclear receptors (Table 1) was performed to identify nuclear receptors that could enhance the efficiency of reprogramming. Mouse Embryonic Fibroblasts (MEFs) which contain an endogenous *Pou5f1*-GFP reporter were used in the screen. Reprogrammed MEFs were positively identified by the expression of GFP as a result of reactivation of the silenced *Pou5f1*-GFP reporter. The screen was conducted with each nuclear receptor retrovirally transduced with the Oct4 (O), Sox2 (S), Klf4 (K), and c-Myc (M) viruses. The frequency of *Pou5f1*-GFP-positive colonies was registered at 14 days post infection (dpi). Transcript expression of all the nuclear receptor constructs was also verified (Fig. 5). From this screen we found that both orphan nuclear receptor s Nr1i2 (also known as pregnane X receptor, Pxr) and Nr5a2 (also known as liver receptor homolog-1, Lrh-1), were able to enhance the efficiency of reprogramming (as compared to the OSKM control) by 2.7 and 4.0 fold, respectively (Fig. 1a).

Table 1:

List of nuclear receptor s screened for enhancers of reprogramming.

Nuclear receptor s (NRs)	**Functions**
Nr0b1 (Dax1)	Transcription factor, transcriptional repressor of several nuclear receptors
Nr1b1 (Rara)	Transcription factor, regulator of Oct4
Nr1b3 (Rarg)	Transcription factor, regulator of Oct4
Nr1d1 (Rev-erba)	Transcription factor, regulator of circardian and metabolic pathways
Nr1h2 (Lxrb)	Transcription factor, regulator of lipid and cholesterol homeostasis
Nr1h3 (Lxra)	Transcription factor, regulator of lipid and cholesterol homeostasis

Nr1i1 (Vdr)	Transcription factor, mediator of vitamin D
Nr1i2 (Pxr)	Transcription factor, regulator of cytochrome P450
Nr1i3 (Car)	Transcription factor, regulator of cytochrome P450
Nr1f1 (Rora)	Transcription factor, regulator of metabolic homeostasis
Nr2a1 (Hnf4a)	Transcription factor, regulator of liver-specific genes
Nr2b1 (Rxra)	Transcription factor, role in ESC differentiation to cardiomyocyte
Nr2e1 (Tlx)	Transcription factor, role in neurogenesis
Nr2e3 (Pnr)	Transcription factor, transcriptional repressor of cone-specific genes
Nr2f6 (Ear2)	Transcription factor, transcriptional repressor of IL-17 in T-cells
Nr3b1 (Esrra)	Transcription factor, role in osteoblast development
Nr3b2 (Esrrb)	Transcription factor, self-renewal regulator, reprogramming factor
Nr3b3 (Esrrg)	Transcription factor, reprogramming factor
Nr5a2 (Lrh-1)	Transcription factor, activator of Oct4

[00089]. We next sought to investigate if these enhancers of reprogramming could replace the core reprogramming factors. As c-Myc was previously shown to be dispensable for reprogramming we did not investigate the replaceability of c-Myc but instead investigated the ability of these two nuclear receptors in replacing any of the OSK trio. Strikingly, when *Pou5f1*-GFP MEFs were transduced with Nr5a2 and SKM viruses, *Pou5f1*-GFP-positive colonies (23.7 ± 3.5 per 100,000 MEFs plated) were observed by 14 dpi (Fig. 1b and Fig. 6a-b). This demonstrates that besides augmenting reprogramming efficiency, exogenous Nr5a2 could also replace

exogenous Oct4 in the reprogramming of MEFs. We refer to these cells that have been reprogrammed with Nr5a2, Sox2, Klf4 and c-Myc as N_2SKM iPSCs. These colonies could be stably passaged long-term and stained positive for alkaline phosphatase (Fig. 6c), Nanog (Fig. 6d-e) and SSEA-1 (Fig. 6f-g).

[00090]. Given that c-Myc is dispensable for reprogramming, we were also able to generate iPSCs from *Pou5f1*-MEFs that were transduced with just Nr5a2 and SK viruses albeit at a lower efficiency (2.3 ± 0.6 per 100,000 MEFs plated) than that of N_2SKM iPSCs (Fig. 1c-e). These three-factor Nr5a2-reprogrammed cells are referred to as N_2SK iPSCs. Similar to N_2SKM iPSCs, N_2SK iPSCs stained positive for alkaline phosphatase (Fig. 1f), Nanog (Fig. 1g-h) and SSEA-1 (Fig 1i-j).

[00091]. Nr5a2-reprogrammed cells were cytogenetically analyzed and shown to be karyotypically normal (Fig. 7a). In addition, genomic integrations of the respective viruses were tested and absence of Oct4-retroviruses in the genomic DNA of Nr5a2-reprogrammed cells was verified (Fig. 7b).

Characterisation of cells reprogrammed with Nr5a2

[00092]. Global gene expression profiling of Nr5a2-reprogrammed cells was performed to study if the genetic expression of these iPSCs was akin to ESCs. The transcriptome of Nr5a2-reprogrammed cell lines (N_2SKM and N_2SK) as well as MEFs (*actin*-GFP and *Pou5f1*-GFP), ESCs and an OSKM iPSC line were characterized. Cluster analysis revealed that Nr5a2-reprogrammed cells were more similar to ESCs and OSKM iPSCs than MEFs (Fig. 2a). In addition, the expression profiling showed a concomitant upregulation of ESC-associated genes and a downregulation of MEF-associated genes in Nr5a2-reprogrammed cells (Fig. 2b). Taken together, the expression profiles of Nr5a2-reprogrammed cells are similar to both ESC and conventional OSKM iPSCs.

[00093]. Next, bisulfite sequencing was performed to investigate the methylation status of the *Pou5f1* and *Nanog* promoters in Nr5a2-reprogrammed cells. Promoter methylation analysis revealed that the *Pou5f1* and *Nanog* promoters of Nr5a2-reprogrammed cells were largely unmethylated (Fig. 3a) and were similar to that of

ESCs, whereas the *Pou5f1* and *Nanog* promoter regions of MEFs were hypermethylated. We also explored the bivalent domain patterns of Nr5a2-reprogrammed cells. Our results showed that Nr5a2-reprogrammed cells possessed both active H3K4me3 and repressive H3K27me3 chromatin modifications on six genes (*Zfpm2*, *Sox21*, *Pax5*, *Lbx1h*, *Evx1* and *Dlx1*) (Fig. 3b). These results are consistent with that of ESCs, which harbor both chromatin modifications, unlike differentiated cells which have resolved to either chromatin mark.

[00094]. Both embryoid body (EB)-mediated differentiation and teratoma formation assays were carried out to test the pluripotency of the Nr5a2-reprogrammed cells. Nr5a2-reprogrammed cells were indeed pluripotent as they could be *in vitro* differentiated into cells of the three major germ layers (endoderm, ectoderm and mesoderm) (Fig. 8a) and form teratomas that consisted of differentiated tissue originating from the three major germ layers (Fig. 8b).

[00095]. A more stringent assay for pluripotency was performed whereby Nr5a2-reprogrammed cells were microinjected into 8-cell stage wild-type C57BL/6J or B6(Cg)-Tyr^{c-2J}/J (B6-albino) embryos. As the Nr5a2-reprogrammed cells were derived from *Pou5f1*-GFP MEFs, GFP expression should be observed in the gonads due to high levels of endogenous Oct4 expression in the gonads. As expected, E13.5 embryo displayed GFP-expression in the gonads (Fig. 4a-b). More importantly, live-born chimaeras were generated from both N_2SKM (Fig. 6i) and N_2SK lines (Fig. 4c).

Characterisation of cells reprogrammed with Nr5a1

[00096]. Nr5a1, also known as steroidogenic factor 1 (Sf1), belongs to the same nuclear receptor subfamily 5 as Nr5a2. Hence, we were interested to examine if Nr5a1 was able to both enhance the efficiency of reprogramming and replace Oct4. Nr5a1 enhanced reprogramming efficiency (Fig. 9a) but to a lesser extent than Nr5a2. Next, we investigated if Nr5a1 could replace any of the core reprogramming factors (O, S and K). Similar to Nr5a2, Nr5a1 was unable to replace Sox2 and Klf4. Interestingly, MEFs transduced with Nr5a1 and SKM viruses generated *Pou5f1*-GFP

positive iPSC colonies (Fig. 9b-d). We refer to these reprogrammed MEFs as N_1SKM iPSCs. These Nr5a1-reprogrammed cells express alkaline phosphatase (Fig. 9e), Nanog (Fig. 9f-g) and SSEA-1 (Fig. 9h-i). In addition, these karyotypically normal N_1SKM iPSCs (Fig 9j) could be *in vitro* differentiated to lineages of the three different germ layers (Fig. 9k). The independent demonstration of reprogramming with Nr5a1 shows that both members of the Nr5a subfamily indeed possess similar reprogramming properties.

[00097]. Next, we examined if the addition of both Nr5a2 and Nr5a1 would boost the efficiency of reprogramming without Oct4. Hence, Pou5f1-GFP MEFs were co-transduced with Nr5a2, Nr5a1 and SKM viruses. Interestingly, the addition of both Nr5a2 and Nr5a1 with SKM was able to increase the number of *Pou5f1*-GFP positive colonies by about 3-fold with respect to Nr5a2 and SKM (Fig. 10). This result shows that both factors indeed had an additive effective on reprogramming efficiency when introduced together.

Design of nuclear receptor fragments

[00098]. Similar to other nuclear receptors, Nr5a2 possesses a ligand binding domain (LBD) and a DNA binding domain (DBD). However, being an orphan nuclear receptor, the endogenous ligands of Nr5a2 remains unknown. Unlike most nuclear receptors which function as dimers, Nr5a2 is able to bind DNA in its monomeric state. We investigated the functional importance of the LBD and DBD of Nr5a2 in the reprogramming of MEFs without Oct4. We mutated a specific residue to a bulkier residue (A368M) that fills the cavity of Nr5a2 LBD so as to disrupt the binding of putative ligands. Next, we created a DBD mutant with a double mutation (G190V, P191A) in the conserved Ftz-F1 domain that would result in a marked decrease in Nr5a2 DNA binding activity. A reprogramming assay was hence performed whereby *Pou5f1*-GFP MEFs transduced with SKM viruses were also transduced with viruses encoded with either wildtype (WT) Nr5a2, A368M Nr5a2 mutant or G190V, P191A Nr5a2 mutant. Western analysis was carried out to ensure that the retroviral vectors expressed equivalent level of Nr5a2 protein (Fig. 11a). Our results show that the Nr5a2 LBD mutant did not decrease the number of formed *Pou5f1*-GFP positive

colonies as compared to the WT (Fig. 11b). This suggests that Nr5a2 functions as a reprogramming factor independent of ligand binding. In contrast, there was a dramatic reduction in the number of *Pou5f1*-GFP positive colonies when Nr5a2 DBD mutant was introduced with SKM (Fig. 11b). This shows that the integrity of Nr5a2 DBD is important for proper binding of the nuclear receptor to promoter/enhancer regions of target genes to initiate the reprogramming process in MEFs. Taken together, we show that the DBD is crucial for the reprogramming function of Nr5a2 while ligand binding is dispensable for its role in reprogramming.

[00099]. Reprogramming with Nr5a2 or Nr5a1 is the first reported instance of transcription factors that are able to bypass the need for exogenous Oct4. Nr5a2 is responsible for the maintenance of Oct4 expression in early mouse embryonic development. Nr5a2 has been shown to be able to bind to both the proximal enhancer and proximal promoter regions of *Pou5f1* and regulate Oct4 in the epiblast stage of mouse embryonic development. Hence, as an Oct4-regulator, exogenous Nr5a2 may be sufficient to induce endogenous Oct4 expression and substitute for exogenous Oct4 in the reprogramming process of MEFs. Although Nr5a1 is not expressed in mouse ESCs, it activates Oct4 expression in mouse embryonal carcinoma cells and this is consistent with its ability to replace Oct4 in reprogramming. In this regard, it is conceivable that factors which activate Oct4 expression may also replace Oct4 in the reprogramming process. Sall4 is a known transcriptional regulator of Oct4. However, when Sall4 retrovirus was introduced with SKM viruses no *Pou5f1*-GFP positive colonies were observed (data not shown). Hence, it is noteworthy that not all Oct4 regulators are able to replace Oct4 in the generation of iPSCs.

[000100]. In summary, our study provides an Oct4-independent code for reprogramming of somatic cells. In addition, we also show that both Nr5a2 and Nr5a1 are able to enhance the efficiency of reprogramming with the conventional four factors. Altogether, we have uncovered an unexpected dual role of nuclear receptor s in both enhancing and mediating reprogramming.

METHODS

[000101]. Cell culture and transfection. iPSCs were cultured on mitomycin C-treated MEF feeders in Dulbecco's modified Eagle medium (DMEM; Gibco), supplemented with 15% heat-inactivated fetal bovine serum (FBS; Gibco) or 15% knockout serum replacement (KSR; Gibco), 0.055 mM β-mercaptoethanol (Gibco), 2 mM L-glutamine (Gibco), 0.1 mM MEM non-essential amino acid (Gibco), 20 μg ml^{-1} gentamicin (Gibco) and 1000 U ml^{-1} of LIF (homemade) and passaged every 2-3 days. MEFs were isolated from E13.5 embryos and cultured as described previously[7]. 293-T cells on 10 cm plates were transfected with 25 μg of each PMX retroviral vector using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

[000102]. Mouse molecular genetics. MEFs were isolated from *Pou5f1*-GFP transgenic mice and *actin*-GFP transgenic mice (Jackson's lab, stock no. 004654 and 003516). *Pou5f1*-GFP and *actin*-GFP MEFs were harvested from E13.5 embryos derived from the intercross between male *Pou5f1*-GFP male mice and female wild-type 129S2/SV and the intercross between actin-GFP mice and female wild type CD1 mice, respectively. 8-12 iPSCs were microinjected into C57BL/6J and B6(Cg)-Tyr^{c-2J}/J embryos that were obtained at the 8-cell stage. Microinjected embryos were transferred to the oviduct of E0.5 pseudopreognant F1 (CBA x C57BL/6J) females. Chimaeric embryos were harvested at E13.5 and assayed for GFP expression in the gonads with a fluorescence microscope.

[000103]. Retrovirus packaging and infection. cDNA sequences of Nr5a2 and other factors were PCR amplified from either mouse ESC cDNA or commercial plasmids (Open Biosystems). Nr5a2 mutants were PCR amplified with the appropriate primers. Amplified coding sequences were verified by sequencing and cloned into MMLV-based pMXs retroviral vector. Retroviruses were generated as described previously[1]. For iPSC generation, equal amounts of viruses encoding the different factors were introduced to MEFs at 70% confluence in DMEM containing 15% FBS and 6 ng ml^{-1} polybrene. At 1 dpi, medium was changed to fresh MEF medium. At 2 dpi, cells were passaged to MEF feeders and cultured for 6 days in culture medium

containing FBS as described previously followed by an additional 5-15 days in culture medium containing KSR as described previously.

[000104]. RNA extraction, reverse transcription and quantitative real-time PCR. As described above.

[000105]. Bisulphite genomic sequencing. Genomic DNA was bisulphite-treated with the Imprint™ DNA modification kit (Sigma) according to the manufacturer's instructions. Promoter regions of *Pou5f1* and *Nanog* were amplified by PCR and cloned into the pCR2.1-TOPO vector (Invitrogen) and sequenced with the M13 forward and M13 reverse primers.

[000106]. Primer sequences used in the PCR amplification of the *Pou5f1 and Nanog* promoter regions are

5'-ATGGGTTGAAATATTGGGTTTATTTA,

5'CCACCCTCTAACCTTAACCTCTAAC and

5'-GATTTTGTAGGTGGGATTAATTGTGAATTT,

5'-ACCAAAAAAACCCACACTCATATCAATATA respectively.

[000107]. Karyotyping. iPSCs were treated with colcemid (Invitrogen) and harvested by standard hypotonic treatment and fixed with methanol:acetic acid (3:1). Slides were air-dried before G-band karyotyping.

[000108]. Genotyping. Each PCR amplification reaction was performed with 300 ng of genomic DNA harvested from either iPSCs, ESCs, MEFs or embryo.

Sense primer sequence: 5'-GACGGCATCGCAGCTTGGATACAC.

Antisense primer sequences are:

Nr5a2: 5'-GACGCAATAGCTGTAAGTCCATG

Sox2: 5'-GCTTCAGCTCCGTCTCCATCATGTT

Klf4: 5'-GCCATGTCAGACTCGCCAGG

c-Myc: 5'-TCGTCGCAGATGAAATAGGGCTG

Oct4: 5'-CCAATACCTCTGAGCCTGGTCCGAT.

[000109]. EB-mediated *in vitro* differentiation. For EB formation, iPSCs were trypsinized and cultured in Petri-dish for 4-5 days in iPSC culture medium in the absence of LIF and β-mercaptoethanol. EBs were transferred to gelatin-coated plates and cultured for 5-6 days with the addition of 1 μM retinoic acid (Sigma). Samples were fixed in 4% paraformadehyde, permeabilized with 1% triton X-100, blocked with 8% FBS, and stained with anti-Gata-4 (1:100, sc-25310, Santa Cruz), anti-Nestin (1:100, sc-58813, Santa Cruz) or anti-α-Smooth Muscle Actin (1:100, ab18460, Abcam). Samples were then stained with the secondary antibody, Alexa Fluor 546 conjugated anti-mouse (1:1000, Invitrogen) followed by staining of the nuclei with Hoechst (1:4000, Invitrogen).

[000110]. Teratoma assay. iPSCs were harvested by trypsinization and resuspended to a concentration of $1x10^7$ cells ml^{-1} in 0.9% saline. 100 μl of the cell suspension was injected subcutaneously into each dorsal flank of avertin-anesthetized SCID mice. Teratomas were dissected after 3-4 weeks, weighted and fixed in Bouin's solution, before embedding in parafilm. Parafilm-embedded tissue was sectioned and stained with Mallory's Tetrachrome as previously described[26].

[000111]. Immunofluorescence microscopy and alkaline phosphatase staining. iPSCs cultured on gelatin-coated cover slips were fixed with 4% paraformadehyde, permeablized in 1% triton X-100, blocked with 8% FBS. After blocking, samples were stained with anti-Nanog (1:50, RCAB0002PF, CosmoBio) or anti-SSEA-1

(1:200, MAB4301, Chemicon), before staining with Alexa Fluor 568 conjugated anti rabbit (1:300, Invitrogen) or Alexa Fluor 546 conjugated anti-mouse IgM (1:2000, Invitrogen), respectively. Nuclei were then counterstained with Hoechst (Invitrogen). Alkaline phosphatase detection was performed using a commercial ESC characterization kit (Chemicon).

[000112]. Western analysis. After 48 h transfection, 293-T cells were lysed with RIPA buffer (Pierce) supplemented with protease inhibitor cocktail (Roche). Protein concentration was measured with a Bradford assay kit (Bio-Rad). 50 μg of cell lysate was resolved on a 10% SDS-polyacrylamide gel and transferred to a polyvinylidine difluoride membrane (Millipore). The membrane was blocked with 5% skim milk. After blocking, the blot was incubated with either anti-Nr5a2 (1:2000, ab18293, Abcam) or anti-actin (1:2000, sc-1616, Santa-Cruz) primary antibodies for 1 h, washed with PBST and incubated with either horse-radish peroxidase (HRP)-conjugated anti-rabbit IgG (1:5000, sc-2004, Santa Cruz) or HRP-conjugated anti-goat IgG (1:5000, sc-2768, Santa Cruz), respectively. After washing with PBST, signals were detected using the Western Blotting Luminol Reagents (Santa Cruz).

[000113]. ChIP assay. ChIP assays were performed as described previously[27]. In short, cells were crosslinked with 1% formaldehyde for 10 min at room temperature and the formaldehyde was quenched with 125 mM glycine. Cell lysates were sonicated and chromatin extracts were immunoprecipitated with anti-H3K4me3 (ab8580, Abcam) or anti-H3K27me3 (07-449, Millipore) antibodies. Quantitative PCR analyses were performed as previously described[7].

[000114]. Microarray analysis. Reverse transcription of mRNAs harvested from mouse ESCs, iPSCs (OSKM, N_2SKM #A5, N_2SK #B3 and #B11) and MEFs *(actin*-GFP and *Pou5f1*-GFP*)* was performed. Two biological replicate microarray data was generated for each cell line. Arrays (Sentrix Mouse-6 Expression BeadChip version 1.1) processed according to the manufacturer's instructions were scanned with the Illumina microarray platform. Differentially expressed genes were selected based on Significance Analysis of Microarrays (SAM) criteria: fold change (FC)<0.6 for

downregulated, FC>1.5 for upregulated; q value<2%; and detection probability greater than 0.95 in all samples.

Screen of nuclear receptors reveals that Nr1i2 and Nr5a2 can enhance reprogramming efficiency

[000115]. We carried out a screen of 19 nuclear receptors (Table 1) for their ability to enhance reprogramming efficiency. MEFs containing a *Pou5f1-GFP* reporter (Figure 12A) (Feng et al., 2009a) were used to identify putative iPSC colonies, based on the reactivation of the *Pou5f1* gene. We transfected each nuclear receptor retrovirally along with Oct4 (O), Sox2 (S), Klf4 (K), and c-Myc (M) retroviruses. The frequency of GFP-positive colonies was determined at 14 days post infection (dpi). Transcript expression of all the nuclear receptor constructs was verified (data not shown). From this screen, we found that both orphan nuclear receptors, Nr1i2 (also known as pregnane X receptor, Pxr) and Nr5a2 (also known as liver receptor homolog-1, Lrh-1), can enhance the efficiency of reprogramming (as compared to OSKM control) by 2.7 and 4.0-fold, respectively (Figure 12A). Addition of Nr5a2 also enhanced the kinetics of OSKM reprogramming with GFP expression detectable three days earlier than in the case of conventional four factor reprogramming (Figure 12B). Cell viability assays confirmed that both Nr1i2 and Nr5a2 do not induce cell death (Figure 16B).

Nr5a2 can replace Oct4 in the reprogramming of MEFs to iPSCs

[000116]. We next investigated if Nr1i2 and Nr5a2 could replace the core reprogramming factors in addition to enhancing reprogramming efficiencies. As c-Myc has already been demonstrated to be dispensable for reprogramming (Nakagawa et al., 2008; Wernig et al., 2008), we did not investigate the replaceability of c-Myc but instead tested the ability of these two nuclear receptors in replacing any of the OSK trio. Nr1i2 was unable to replace O, S or K, and Nr5a2 was unable to replace S or K (Figure 12C). Strikingly, when *Pou5f1*-GFP MEFs were transduced with Nr5a2 and SKM viruses, GFP-positive colonies (23.7 ± 3.5 per 100,000 MEFs plated) were observed by 14 dpi (Figure 12C; Figures 16C and 16D).

This demonstrates that besides augmenting reprogramming efficiency, exogenous Nr5a2 could also replace exogenous Oct4. We refer to these cells that have been reprogrammed with Nr5a2, Sox2, Klf4 and c-Myc as N_2SKM iPSCs. These colonies could be stably passaged long-term and stained positive for alkaline phosphatase (Figure 16E), Nanog (Figures 16F and 16G) and SSEA-1 (Figures 16H and 16I). The other 18 nuclear receptors were also tested for their ability to replace Oct4. However, unlike Nr5a2, none were able to replace Oct4 (Figure 16J).

[000117]. Given that c-Myc is dispensable for reprogramming, we were also able to generate iPSCs from *Pou5f1-GFP* MEFs that were transduced with Nr5a2 and SK viruses, albeit at a lower efficiency (2.3 ± 0.6 per 100,000 MEFs plated) than that of N_2SKM combination (Figures 12D-12F). These three-factor Nr5a2-reprogrammed cells are referred to as N_2SK iPSCs. Similar to N_2SKM iPSCs, N_2SK iPSCs stained positive for alkaline phosphatase (Figure 12G), Nanog (Figures 12H and 12I) and SSEA-1 (Figures 12J and 12K).

[000118]. Nr5a2-reprogrammed cells were karyotypically normal (Figure 16K) and genomic integrations of the respective viruses into the genomic DNA were verified and showed no evidence of Oct4 transgene integration (Figure 16L). Both embryoid body-mediated differentiation and teratoma formation assays were carried out to test the pluripotency of the Nr5a2-reprogrammed cells. Nr5a2-reprogrammed cells were indeed pluripotent as they could be *in vitro* differentiated into cells of the three major germ layers (Figure 17A) and form teratomas that consisted of differentiated tissue originating from the three major germ layers (Figure 17B).

[000119]. A more stringent assay for pluripotency was performed whereby Nr5a2-reprogrammed cells were microinjected into 8-cell stage wild-type C57BL/6J or B6(Cg)-Tyr^{c-2J}/J (B6-albino) embryos. As the Nr5a2-reprogrammed cells were derived from *Pou5f1-GFP* MEFs, E13.5 embryos displayed GFP expression in the gonads due to high levels of endogenous Oct4 expression (Figures 12L and 12M). In addition, live-born chimaeras were generated from both N_2SKM (Figure 16M) and N_2SK lines (Figure 12N). More importantly, the N_2SK line is germline competent (Figure 12O, Table 2).

Table 2. Pluripotency assays of Nr5a2-reprogrammed cells.

Lines	#A5 (N_2SKM)	#B3 (N_2SK)	#B11 (N_2SK)
EB formation	yes	yes	yes
EB differentiation to cells of three germ	yes	yes	yes
Teratoma formation consisting of tissues from three germ	yes	yes	yes
Gonad incorporation	yes	yes	yes
Chimaeras	yes	yes	yes
Germline transmission	no	no	yes

Table Legend yes denotes iPSC line passing assay and no denotes no germline transmission was observed.

Expression and epigenetic profiling of Nr5a2-reprogrammed cells closely resemble ESCs

[000120]. Global gene expression profiling of Nr5a2-reprogrammed cells was performed and hierarchical clustering of the microarray data revealed that Nr5a2-reprogrammed cells were more similar to ESCs and OSKM iPSCs than MEFs (Figure 17C). In addition, expression profiling showed a concomitant upregulation of ESC-associated genes and a downregulation of MEF-associated genes in Nr5a2-reprogrammed cells (Figure 17D).

[000121]. Next, promoter methylation analysis revealed that the *Pou5f1* and *Nanog* promoters of Nr5a2-reprogrammed cells were largely unmethylated (Figure 17E) and were similar to that of ESCs. We also explored the bivalent domain patterns of

Nr5a2-reprogrammed cells. Our results indicated that Nr5a2-reprogrammed cells possessed both active H3K4me3 and repressive H3K27me3 chromatin modifications (Figure 17F) which were similar to that of ESCs.

The close family member Nr5a1 can also enhance reprogramming efficiency and replace Oct4

[000122]. Closely related members of the same family of transcription factors can replace each other in the context of reprogramming (Feng et al., 2009a; Nakagawa et al., 2008). As both Nr5a1 (also known as steroidogenic factor 1, Sf1) and Nr5a2 belong to the same nuclear receptor subfamily V, we were interested to examine if Nr5a1 was able to both enhance the efficiency of reprogramming and replace Oct4. Nr5a1 enhanced reprogramming efficiency (Figure 13A) but to a lesser extent than Nr5a2 (Figure 12A). Next, we investigated if Nr5a1 could replace any of the core reprogramming factors (O, S and K). Similar to Nr5a2, Nr5a1 was unable to replace S and K but it was able to replace Oct4 (Figure 13B). We refer to these GFP-positive iPSC colonies (Figures 13C and 13D) as N_1SKM iPSCs. These Nr5a1-reprogrammed cells express alkaline phosphatase (Figure 13E), Nanog (Figures 13F and 13G) and SSEA-1 (Figures 13H and 13I). We verified the genomic integration of viral *Nr5a1* in N_1SKM iPSCs and found no evidence of viral *Pou5f1* and viral *Nr5a2* genomic integrations (Figure 13J). These karyotypically normal N_1SKM iPSCs (Figure 13K) could be differentiated *in vitro* to lineages of the three different germ layers (Figure 13L) and form teratomas comprising tissues of the three different lineages (Figure 13M). The demonstration of reprogramming with Nr5a1 shows that both members of the Nr5a subfamily indeed possess similar reprogramming properties.

Other transcription factors that bind *Pou5f1* regulatory regions are unable to replace exogenous Oct4 in reprogramming

[000123]. Nr5a2 has been shown to bind both the proximal enhancer and proximal promoter regions of *Pou5f1* and regulate *Pou5f1* in the epiblast stage of mouse embryonic development (Gu et al., 2005). *Nr5a2*-null embryos display a loss of Oct4

expression in the epiblasts (Gu et al., 2005) and die between E6.5 and E7.5 (Gu et al., 2005; Pare et al., 2004). Therefore, part of the mechanism of Nr5a2 in replacing exogenous Oct4 may be explained by the findings that Nr5a2 directly regulates *Pou5f1* and acts upstream of *Pou5f1*.

[000124]. We went on to investigate if other transcription factors that bind to the *Pou5f1* promoter or enhancer region could also replace Oct4 in the reprogramming of MEFs. Hence, we tested nine other transcription factors (Nanog, Sall4, Stat3, Zfx, Tcfcp2l1, Klf2, Klf5, N-Myc, Esrrb) that bind to the *Pou5f1* regulatory regions (Chen et al., 2008). Expression of the respective viral transcripts was verified (Figure 13N). Our results revealed that none of these transcription factors was able to replace Oct4 in the SKM combination (Figure 13O). This result shows that not all transcription factors that bind to the *Pou5f1* regulatory regions can replace Oct4 in reprogramming. Hence, Nr5a2 and its close family member, Nr5a1 are unique in their ability to replace Oct4.

DNA binding ability of Nr5a2 is important for its role in reprogramming whereas ligand binding is less so

[000125]. Similar to other nuclear receptors, Nr5a2 possesses a ligand binding domain (LBD) and a DNA binding domain (DBD). However, being an orphan nuclear receptor, the endogenous ligands of Nr5a2 remain unknown. We investigated the functional importance of ligand binding and DNA binding of Nr5a2 in reprogramming without Oct4. We mutated a specific residue to a bulkier residue (A368M) that fills the cavity of Nr5a2 LBD so as to disrupt the binding of putative ligands (Sablin et al., 2003). Next, we created a DNA binding mutant with a double mutation (G190V, P191A) in the conserved Ftz-F1 domain that would result in a marked decrease in the DNA binding activity of Nr5a2 (Solomon et al., 2005). Western analysis was performed to ensure that these retroviral vectors expressed equivalent levels of Nr5a2 protein (Figure 13P). Our reprogramming assays show that the Nr5a2 ligand binding mutant did not decrease the number of formed GFP-positive colonies as compared to wildtype (WT) Nr5a2 (Figure 13Q). This suggests that the ability of Nr5a2 to function as a reprogramming factor is independent of ligand binding. In

contrast, there was a dramatic reduction in the number of GFP-positive colonies when the Nr5a2 DNA binding mutant was introduced with SKM (Figure 13Q). Taken together, we show that the DNA binding is crucial for the reprogramming function of Nr5a2 whereas ligand binding is dispensable.

Nr5a2 sumoylation site mutants exhibit enhanced reprogramming capacity

[000126]. We tested the reprogramming capacity of Nr5a2 with mutated lysine residues, using a mutant construct with two lysine residues mutated (2KR) and another with five lysine residues mutated (5KR). Western analysis showed that the WT and mutant constructs expressed similar levels of protein (Figure 13R). Strikingly, the OSKM reprogramming assay revealed that the 2KR mutant boosted reprogramming efficiency to at least 7-fold as compared to the 4-fold enhancement achieved by the WT (Figure 13S). When the 5KR mutant was introduced, reprogramming efficiency was further augmented to almost 11-fold (Figure 13S). These results suggest that the concomitant prevention of subcellular localization and the enhanced transcriptional activity brought about by the SUMO site mutations could trigger a greater induction of reprogramming by Nr5a2.

Genome-wide binding analysis of Nr5a2 in ESCs

[000127]. Other than *Pou5f1* (Gu et al., 2005), there is no known target gene for Nr5a2 in pluripotent cells. To this end, we performed a genome-wide mapping study of Nr5a2 in ESCs by employing chromatin immunoprecipitation sequencing (ChIP-seq) technology (Table 3). We created a stable ESC cell line expressing HA-tagged Nr5a2 and the expression of HA-tagged Nr5a2 protein was verified by western blot using a Nr5a2-specific antibody (Figure 18A). Nr5a2-bound chromatin was enriched with an anti-HA-tag antibody. We used the *de novo* motif discovery algorithm MEME and uncovered a known Nr5a2 motif enriched in our dataset (Figure 14A). More importantly, our pairwise co-occurrence analyses revealed that Nr5a2 tends to co-localize with Nanog, Oct4, Sox2, Smad1 and Esrrb (Figure 14B). This result associates Nr5a2 with the previously reported Nanog-Oct4-Sox2 cluster (Chen et al., 2008). As Nr5a2 works in concert with Sox2 and Klf4 to reprogram MEFs to iPSCs,

we investigated if these three transcription factors share similar binding targets. Interestingly, we found that all three transcription factors bind target genes that are pivotal for maintenance of ESC identity such as *Pou5f1*, *Nanog*, *Tbx3*, *Klf2* and *Klf5* (Figure 14C; Figure 18B).

***Nanog* is a target of Nr5a2**

[000128]. Nanog is important in ESCs as it governs the gateway to a ground state level of pluripotency (Silva et al., 2009). To confirm that *Nanog* is a target of Nr5a2 during reprogramming, we introduced exogenous HA-tagged Nr5a2 into MEFs. ChIP experiment showed that Nr5a2 was indeed bound to the *Nanog* enhancer during reprogramming (Figure 15A). As *Nanog* is a target of Nr5a2 in both ESCs and MEFs (Figure 18B; Figure 15A), we investigated the role of Nanog in the context of reprogramming that involves Nr5a2. We found that *Nanog* expression increases when Nr5a2 was introduced to reprogramming MEFs (Figure 4B). As expected, endogenous *Pou5f1* increases in a similar trend as *Nanog* when Nr5a2 is introduced with OSKM during reprogramming (Figure 15C and 15D). As Nanog is important in the transition towards the pluripotent state (Silva et al., 2009), the enhancement of reprogramming efficiency brought about by the introduction of Nr5a2 may be in part facilitated by Nanog.

[000129]. Next, we wanted to know if Nr5a2 was important in the reprogramming of MEFs. We performed a knockdown of *Nr5a2* concurrently with the introduction of OSKM to MEFs. The *Nr5a2* shRNA knockdown construct was able to reduce the *Nr5a2* mRNA and protein expression in mouse ESCs (Figure 15E and 15F). Depletion of Nr5a2 during reprogramming resulted in a reduction in the number of colonies (Figure 15G). Importantly, exogenous Nanog was able to rescue the reduction of colonies caused by the knockdown of *Nr5a2* (Figure 15G). Unlike Nanog, Mtf2, an independent factor was not able to rescue the reduction in colonies (Figure 15G). Though Nanog was able to rescue the reduction in reprogramming efficiency brought about by Nr5a2 knockdown, Nanog was unable to rescue the effects of *Pou5f1* knockdown (Figure 15G). Interestingly, addition of both Nanog and Nr5a2 with OSKM was able to produce more GFP-positive colonies than Nr5a2

alone with OSKM (Figure 15H). Taken together, these results suggest that *Nanog* is one of the important downstream targets of Nr5a2 in the reprogramming of MEFs in which it mediates the enhancement of reprogramming efficiency.

[000130]. Herein, we show that reprogramming with Nr5a2 or Nr5a1 is able to bypass the need for exogenous Oct4. Our data indicate that Nr5a2 functions synergistically with Sox2 and Klf4 to replace exogenous Oct4 to mediate the successful reprogramming of MEFs. Other than MEFs, we were also able to reprogram mouse NPCs with exogenous Nr5a2 together with Klf4 and c-Myc (data not shown). Besides being an upstream activator of *Pou5f1* (Gu et al., 2005), Nr5a2 also works in part through Nanog, an important mediator of ground state pluripotency in ESCs (Silva et al., 2009), and *Nanog* induction by Nr5a2 facilitates the acquisition of pluripotency. Recently, it was found that chemicals that inhibit Tgf-β signaling (Ichida et al., 2009; Maherali and Hochedlinger, 2009) induces Nanog to replace exogenous Sox2 in the reprogramming of MEFs (Ichida et al., 2009). Hence, Nanog is indeed an important target of reprogramming.

[000131]. In summary, our study provides an example of exogenous Oct4-free code for the reprogramming of somatic cells. We also show that both Nr5a2 and Nr5a1 are able to enhance the efficiency of reprogramming with the conventional four factors. Altogether, we have uncovered an unexpected dual role of nuclear receptors in both enhancing and mediating reprogramming.

Cell culture and transfection

[000132]. iPSCs were cultured on mitomycin C-treated MEF feeders as previously described (Feng et al., 2009a). MEFs were isolated from E13.5 embryos and cultured as described previously (Feng et al., 2009a). 293-T cells were transfected with each pMX retroviral vector using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. For RNAi experiments, shRNA constructs that were cloned into the pSUPER.puro vector were transfected with lipofectamine into ESCs. Cells were selected with 1 μg ml^{-1} of puromycin 16 h post-transfection. shRNA

sequences are *Pou5f1*: 5' -GAAGGATGTGGTTCGAGTA-3', *luciferase*: 5'-GATGAAATGGGTAAGTACA -3', and *Nr5a2*: 5' GCAAGTGTCTCAATTTAAA-3'.

ChIP-seq analysis

[000133]. Peak calling of the Nr5a2 ChIP-seq data (8,023 427 uniquely mapped tags) was carried out using MACS with a p value cutoff of 1e-9 and 3,346 peaks were generated. The control anti-HA ChIP-seq library contained 13,001,272 uniquely mapped tags. Enriched motifs were identified by the *de novo* motif discovery tool MEME using 200-bp sequences centered on the ChIP-seq peaks. Co-occurrence analysis to study overlap of Nr5a2 binding sites with binding sites of other important transcription factors was performed with Nr5a2 ChIP-seq data and data set generated from our previous study (Chen et al., 2008).

Microarray analysis

[000134]. Reverse transcription of mRNA harvested from mouse ESCs, iPSCs (OSKM, N_2SKM #A5, N_2SK #B3 and #B11) and MEFs *(actin-GFP* and *Pou5f1-GFP)* was performed. Two biological replicate microarray data was generated for each cell line. For microarray of OSKM + Nr5a2 and OSKM samples, biological triplicates were used. Arrays (Sentrix Mouse-6 Expression BeadChip version 1.1) processed according to the manufacturer's instructions were scanned with the Illumina microarray platform. Differentially expressed genes were selected based on Significance Analysis of Microarrays criteria: fold change (FC)<0.6 for downregulated, FC>1.5 for upregulated; q value < 0.02; and detection probability greater than 0.95 in all samples.

GEO accession codes

[000135]. Microarray and ChIP-seq data are accessible at the GEO database under accession numbers GSE19023 and GSE19019, respectively.

Mouse molecular genetics

[000136]. MEFs were isolated from *Pou5f1-GFP* transgenic mice and *actin-GFP* transgenic mice (JAX laboratory, stock no. 004654 and 003516). *Pou5f1-GFP* and

actin-GFP MEFs were harvested from E13.5 embryos derived from the intercross between male *Pou5f1-GFP* male mice and female wild-type 129S2/SV and the intercross between actin-*GFP* mice and female wild type CD1 mice, respectively. 8-12 iPSCs were microinjected into C57BL/6J and B6(Cg)-Tyr^{c-2J}/J embryos that were obtained at the 8-cell stage. Microinjected embryos were transferred to the oviduct of E0.5 pseudopregnant F1 (CBA x C57BL/6J) females. Chimaeric embryos were harvested at E13.5 and assayed for GFP expression in the gonads with a fluorescence microscope. Chimaeric mice were mated with albino B6(Cg)-Tyr^{c-2J}/J mice to assay for germline contribution. All animal work was performed according to IACUC guidelines.

Retrovirus constructs, packaging and infection

[000137]. cDNA sequences of *Nr5a2* and other factors were PCR amplified from either mouse ESC cDNA or commercial plasmids (Open Biosystems). cDNA sequences of *Nr5a2* SUMO mutants (2KR: K173R, K289R and 5KR: K173R, K213R, K289R, K329R, K389R) were amplified from donated constructs (Yang et al., 2009). *Nr5a2* ligand and DNA binding mutants were PCR amplified with the appropriate primers. Amplified coding sequences were verified by sequencing and cloned into MMLV-based pMX retroviral vector. shRNA knockdown constructs with their respective promoter regions were transferred from the pSUPER.puro vector to the pMX vector. Retroviruses were generated as described previously (Takahashi and Yamanaka, 2006). 3T3 cells were infected with pMX retroviruses harboring the *GFP* gene. After 48 h of infection, FACs was perform to quantify the proportion of GFP-positive cells. Number of transducing units was calculated as previously described (Tiscornia et al., 2006). Number of transducing units was used to calculate the amount of virus needed to achieve a multiplicity of infection (MOI) of 5 (Park et al., 2008). For iPSC generation, viruses encoding the different factors each with a MOI of 5 were introduced to MEFs at 70% confluence in DMEM containing 15% FBS and 6 ng ml^{-1} polybrene. At 1 dpi, medium was changed to fresh MEF medium. At 2 dpi, cells were passaged to MEF feeders and cultured for 6 days in culture medium

containing FBS followed by an additional 5-15 days in culture medium containing KSR.

RNA extraction, reverse transcription and quantitative real-time PCR

[000138]. Methods are as described previously (Feng et al., 2009).

Bisulfite genomic sequencing

[000139]. Genomic DNA was bisulfite-treated with the Imprint™ DNA modification kit (Sigma) according to the manufacturer's instructions. Promoter regions of *Pou5f1* and *Nanog* were amplified by PCR and cloned into the pCR2.1-TOPO vector (Invitrogen) and sequence-verified with the M13 forward and M13 reverse primers. Primer sequences used in the PCR amplification of the *Pou5f1 and Nanog* promoter regions are 5'-ATGGGTTGAAATATTGGGTTTATTTA-3', 5'-CCACCCTCTAACCTTAACCTCTAAC-3' and

5'-GATTTTGTAGGTGGGATTAATTGTGAATTT-3', 5'-ACCAAAAAAACCCACACTCATATCAATATA-3', respectively.

Karyotyping

[000140]. iPSCs were treated with colcemid (Invitrogen), harvested by standard hypotonic treatment, and fixed with methanol:acetic acid (3:1). Slides were air-dried before G-band karyotyping.

Genotyping

[000141]. Each PCR amplification reaction was performed with 300 ng of genomic DNA harvested from either iPSCs, ESCs, MEFs or embryo.

Sense primer sequence: 5'-GACGGCATCGCAGCTTGGATACAC-3'.

Antisense primer sequences are:

Nr5a2: 5'-GACGCAATAGCTGTAAGTCCATG-3'

Sox2: 5'-GCTTCAGCTCCGTCTCCATCATGTT-3'

Klf4: 5'-GCCATGTCAGACTCGCCAGG-3'

c-Myc: 5'-TCGTCGCAGATGAAATAGGGCTG-3'

Pou5f1: 5'-CCAATACCTCTGAGCCTGGTCCGAT-3'.

EB-mediated *in vitro* differentiation

[000142]. For EB formation, iPSCs were trypsinized and cultured in Petri-dish for 4-5 days in iPSC culture medium in the absence of LIF and β-mercaptoethanol. EBs were transferred to gelatin-coated plates and cultured for 5-6 days with the addition of 1 μM retinoic acid (Sigma). Samples were fixed in 4% paraformadehyde, permeabilized with 1% triton X-100, blocked with 8% FBS, and stained with anti-Gata-4 (1:100, sc-25310, Santa Cruz), anti-Nestin (1:100, sc-58813, Santa Cruz) or anti-α-Smooth Muscle Actin (1:100, ab18460, Abcam). Samples were then stained with the secondary antibody, Alexa Fluor 546 conjugated anti-mouse (1:1000, Invitrogen) followed by staining of the nuclei with Hoechst (1:4000, Invitrogen).

Teratoma assay

[000143]. iPSCs were harvested by trypsinization and resuspended to $1x10^7$ cells ml^{-1} in 0.9% saline. 100 μl of the cell suspension was injected subcutaneously into each dorsal flank of avertin-anesthetized SCID mice. Teratomas were dissected after 3-4 weeks, weighted and fixed in Bouin's solution, before embedding in paraffin. Paraffin-embedded tissue was sectioned and stained with Mallory's Tetrachrome as previously described (Wang and Lufkin, 2000).

Immunofluorescence microscopy and alkaline phosphatase staining

[000144]. iPSCs cultured on gelatin-coated cover slips were fixed with 4% paraformadehyde, permeablized in 1% triton X-100 and blocked with 8% FBS. After blocking, samples were stained with anti-Nanog (1:50, RCAB0002PF, CosmoBio) or anti-SSEA-1 (1:200, MAB4301, Chemicon), before staining with Alexa Fluor 568 conjugated anti rabbit (1:300, Invitrogen) or Alexa Fluor 546 conjugated anti-mouse IgM (1:2000, Invitrogen), respectively. Nuclei were then counterstained with Hoechst (Invitrogen). Alkaline phosphatase detection was performed using a commercial ESC characterization kit (Chemicon) according to the manufacturer's protocol.

Western analysis

[000145]. Cells were lysed with RIPA buffer (Pierce) supplemented with protease inhibitor cocktail (Roche). Protein concentration was measured with a Bradford assay kit (Bio-Rad). 50 μg of cell lysate was resolved on a 10% SDS-polyacrylamide gel and transferred to a polyvinylidine difluoride membrane (Millipore). The membrane was blocked with 5% skim milk. After blocking, the blot was incubated with either anti-HA (1:2000, sc-7392, Santa Cruz), anti-Nr5a2 (1:2000, ab18293, Abcam) or anti-actin (1:2000, sc-1616, Santa-Cruz) primary antibodies for 1 h, washed with PBST and incubated with either horse-radish peroxidase (HRP)-conjugated anti-mouse IgG (1:5000, 1858413, Pierce), HRP-conjugated rabbit IgG (1:5000, sc-2004, Santa Cruz) or HRP-conjugated anti-goat IgG (1:5000, sc-2768, Santa Cruz), respectively. After washing with PBST, signals were detected using the Western Blotting Luminol Reagents (Santa Cruz).

Tunel assay

[000146]. MEFs were infected with respective viruses and infection media was replaced with fresh MEF media after 24 h. Cells were harvested for tunel assay 78 h after infection. For positive control, uninfected MEFs were subjected to DNase 1 (Ambion) treatment before cell labeling. Tunel labeling was performed using a commercial kit according to the manufacturer's protocol (Roche). After labeling, cells were subjected to FACS analysis.

ChIP assay

[000147]. ChIP assays were performed as described previously (Loh et al., 2006). In general, cells were crosslinked with 1% formaldehyde for 10 min at room temperature and the formaldehyde was quenched with 125 mM glycine. Cell lysates were sonicated and chromatin extracts were immunoprecipitated with anti-H3K4me3 (ab8580, Abcam), anti-H3K27me3 (07-449, Millipore) or anti-HA (sc-7392, Santa Cruz) antibodies. Quantitative PCR analyses were performed as previously described (Feng et al., 2009).

[000148]. Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. The invention includes all such variation and modifications. The invention also includes all of the steps, features, formulations and compounds referred to or indicated in the specification, individually or collectively and any and all combinations or any two or more of the steps or features.

[000149]. Each document, reference, patent application or patent cited in this text is expressly incorporated herein in their entirety by reference, which means that it should be read and considered by the reader as part of this text. That the document, reference, patent application or patent cited in this text is not repeated in this text is merely for reasons of conciseness.

[000150]. Any manufacturer's instructions, descriptions, product specifications, and product sheets for any products mentioned herein or in any document incorporated by reference herein, are hereby incorporated herein by reference, and may be employed in the practice of the invention.

[000151]. The present invention is not to be limited in scope by any of the specific embodiments described herein. These embodiments are intended for the purpose of exemplification only. Functionally equivalent products, formulations and methods are clearly within the scope of the invention as described herein.

[000152]. The invention described herein may include one or more range of values (eg size, concentration etc). A range of values will be understood to include all values within the range, including the values defining the range, and values adjacent to the range which lead to the same or substantially the same outcome as the values immediately adjacent to that value which defines the boundary to the range.

[000153]. Throughout this specification, unless the context requires otherwise, the word "comprise" or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers. It is also noted that in this disclosure and particularly in the claims and/or paragraphs, terms such as

"comprises", "comprised", "comprising" and the like can have the meaning attributed to it in U.S. Patent law; e.g., they can mean "includes", "included", "including", and the like; and that terms such as "consisting essentially of" and "consists essentially of" have the meaning ascribed to them in U.S. Patent law, e.g., they allow for elements not explicitly recited, but exclude elements that are found in the prior art or that affect a basic or novel characteristic of the invention.

[000154]. Other definitions for selected terms used herein may be found within the detailed description of the invention and apply throughout. Unless otherwise defined, all other scientific and technical terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which the invention belongs.

[000155]. While the invention has been described with reference to specific methods and embodiments, it will be appreciated that various modifications and changes may be made without departing from the invention.

Claims:

1. A method for inducing pluripotent stem cells *in vitro* comprising the steps of:

a) culturing cells *in vitro*;

b) introducing a polynucleotide that encodes a transcription factor into the cell in the culture, wherein the polynucleotide encoding the transcription factor consists of a nuclear receptor from subfamily 5 or a sumoylated mutant thereof and two or more transcription factor selected from a Sox gene, Krüppel-like factor gene or a gene from the myc family to induce the cell to be a pluripotent cell.

2. The method of claim 1 wherein the nuclear receptor from subfamily 5 comprises SEQ ID NO. 10.

3. The method of claim 1 wherein the nuclear receptor from subfamily 5 comprises SEQ ID NO. 1, SEQ ID NO. 3, SEQ ID NO. 5 or SEQ ID NO. 8.

4 The method of claim 1 wherein the nuclear receptor from subfamily 5 encodes a polypeptide selected from SEQ ID NO. 2, SEQ ID NO. 4, SEQ ID NO. 6 SEQ ID NO. 7, .SEQ ID NO. 9, SEQ ID NO. 10 or SEQ ID NO. 11.

5. An expression vector comprising a polynucleotide consisting of a nuclear receptor from subfamily 5 or a sumoylated mutant thereof and two or more transcription factor selected from a Sox gene, Krüppel-like factor gene or a gene from the myc family operably linked to a regulatory sequence capable of directing expression of said polynucleotide in a host cell for inducing pluripotent stem cells.

6. The expression vector of claim 5 wherein the nuclear receptor from subfamily 5 comprises SEQ ID NO. 10.

7. The expression vector of claim 5 wherein the nuclear receptor from subfamily 5 comprises SEQ ID NO. 1, SEQ ID NO. 3, SEQ ID NO. 5 or SEQ ID NO. 8..

8. The expression vector of claim 5 wherein the nuclear receptor from subfamily 5 encodes a polypeptide selected from SEQ ID NO. 2, SEQ ID NO. 4, SEQ ID NO. 6 SEQ ID NO. 7, .SEQ ID NO. 9, SEQ ID NO. 10 or SEQ ID NO. 11.

9. The expression vector of any one of claims 5 to 8 for use in the treatment of a degenerative disorder.

10. The method of claim 1 wherein the polynucleotide is introduced to the cell in culture by the vector of any one of claims 5 to 8.

11. A method for inducing pluripotent stem cells for treating a patient in need of a pluripotent stem cell treatment comprising the steps of:

a) isolating cells from an individual donor;

b) culturing the cells *in vitro*;

c) introducing a polynucleotide that encodes a transcription factor into the cell in the culture, wherein the polynucleotide encodes a transcription factor consisting of a nuclear receptor from subfamily 5 or a sumoylated mutant thereof and two or more transcription factor selected from a Sox gene, Krüppel-like factor gene or a gene from the myc family to induce the cell to be a pluripotent cell;

d) introducing the pluripotent cell to the patient in need of a pluripotent stem cell treatment.

12. The method of claim 11 wherein the nuclear receptor from subfamily 5 comprises a polynucleotide expressing SEQ ID NO. 10.

13. The method of claim 11 wherein the nuclear receptor from subfamily 5 comprises a polynucleotide of SEQ ID NO. 1, SEQ ID NO. 3, SEQ ID NO. 5 or SEQ ID NO. 8..

14. The method of claim 11 wherein the nuclear receptor from subfamily 5 encodes a polypeptide selected from SEQ ID NO. 2, SEQ ID NO. 4, SEQ ID NO. 6 SEQ ID NO. 7, .SEQ ID NO. 9, SEQ ID NO. 10 or SEQ ID NO. 11.

15. A use of the vector of any one of claims 5 to 8 for the preparation of a medicament for the treatment of a degenerative disorder.

16. A method of making pluripotent stem cell lines comprising:

a) culturing cells *in vitro*;

b) introducing a polynucleotide that encodes a transcription factor into the cell in the culture, wherein the polynucleotide encodes a transcription factor consisting of a nuclear receptor from subfamily 5 or a sumoylated mutant thereof and two or more transcription factor selected from a Sox gene, Krüppel-like factor gene or a gene from the myc family to induce the cell to be a pluripotent cell; and

c) passaging the pluripotent cells to maintain the cell line.

17. A composition for reprogramming somatic cells consisting of a nuclear receptor polypeptide from subfamily 5 or a sumoylated mutant thereof and two or more transcription factor polypeptide selected from a Sox polypeptide, Krüppel-like factor polypeptide or a polypeptide from the myc family.

18. The composition of claim 17 wherein the nuclear receptor polypeptide from subfamily 5 is selected from SEQ ID NO. 2, SEQ ID NO. 4, SEQ ID NO. 6 SEQ ID NO. 7, .SEQ ID NO. 9, SEQ ID NO. 10 or SEQ ID NO. 11.

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Arg Asn Asn Leu Leu Ile Glu Met Leu Gln Ala Lys Gln Thr
450 455 460

<210> 6
<211> 88
<212> PRT
<213> Artificial Sequence

<220>
<223> from Nuclear receptor subfamily 5 sequences

<400> 6

Asp Glu Asp Leu Glu Glu Leu Cys Pro Val Cys Gly Asp Lys Val Ser
1 5 10 15

Gly Tyr His Tyr Gly Leu Leu Thr Cys Glu Ser Cys Lys Gly Phe Phe
20 25 30

Lys Arg Thr Val Gln Asn Gln Lys Arg Tyr Thr Cys Ile Glu Asn Gln
35 40 45

Asn Cys Gln Ile Asp Lys Thr Gln Arg Lys Arg Cys Pro Tyr Cys Arg
50 55 60

Phe Lys Lys Cys Ile Asp Val Gly Met Lys Leu Glu Ala Val Arg Ala
65 70 75 80

Asp Arg Met Arg Gly Gly Arg Asn

<210> 7
<211> 244
<212> PRT
<213> Artificial Sequence

<220>
<223> from Nuclear receptor subfamily 5 proteins

<400> 7

Pro Ala Ser Ile Pro His Leu Ile Leu Glu Leu Leu Lys Cys Glu Pro
1 5 10 15

Asp Glu Pro Gln Val Gln Ala Lys Ile Met Ala Tyr Leu Gln Gln Glu
20 25 30

Gln Ser Asn Arg Asn Arg Gln Glu Lys Leu Ser Ala Phe Gly Leu Leu
35 40 45

Cys Lys Met Ala Asp Gln Thr Leu Phe Ser Ile Val Glu Trp Ala Arg
50 55 60

Ser Ser Ile Phe Phe Arg Glu Leu Lys Val Asp Asp Gln Met Lys Leu
65 70 75 80

Leu Gln Asn Cys Trp Ser Glu Leu Leu Ile Leu Asp His Ile Tyr Arg
85 90 95

Gln Val Ala His Gly Lys Glu Gly Thr Ile Phe Leu Val Thr Gly Glu
100 105 110

His Val Asp Tyr Ser Thr Ile Ile Ser His Thr Glu Val Ala Phe Asn
115 120 125

Asn Leu Leu Ser Leu Ala Gln Glu Leu Val Val Arg Leu Arg Ser Leu
130 135 140

Gln Phe Asp Gln Arg Glu Phe Val Cys Leu Lys Phe Leu Val Leu Phe
145 150 155 160

Ser Ser Asp Val Lys Asn Leu Glu Asn Leu Gln Leu Val Glu Gly Val
165 170 175

Gln Glu Gln Val Asn Ala Ala Leu Leu Asp Tyr Thr Val Cys Asn Tyr
180 185 190

Pro Gln Gln Thr Glu Lys Phe Gly Gln Leu Leu Leu Arg Leu Pro Glu
195 200 205

Ile Arg Ala Ile Ser Lys Gln Ala Glu Asp Tyr Leu Tyr Tyr Lys His
210 215 220

Val Asn Gly Asp Val Pro Tyr Asn Asn Leu Leu Ile Glu Met Leu His
225 230 235 240

Ala Lys Arg Ala

<210> 8
<211> 1683
<212> DNA
<213> Artificial Sequence

<220>
<223> sumoylated mutant of nuclear receptor construct with two lysine residues mutated

<400> 8
atgtctgcta gtttggatac tggagatttt caagaatttc ttaagcatgg acttacagct 60

attgcgtctg caccagggtc agagactcgc cactccccca aacgtgagga acaactccgg 120

gaaaaacgtg ctgggcttcc ggaccgacac cgacgcccca ttcccgcccg cagccgcctt 180

gtcatgctgc ccaaagtgga gacggaagcc ccaggactgg tccgatcgca tggggaacag 240

gggcagatgc cagaaaacat gcaagtgtct caatttaaaa tggtgaatta ctcctatgat 300

gaagatctgg aagagctatg tcctgtgtgt ggcgataaag tgtctgggta ccattacggt 360

ctcctcacgt gcgaaagctg caagggtttt tttaagcgaa ctgtccaaaa ccaaaaaagg 420

tacacgtgca tagagaacca gaattgccaa attgacaaaa cgcagagaaa acgatgtccc 480

tactgtcgat tcaaaaaatg tatcgatgtt gggatgaggc tggaagccgt aagagccgac 540

cgcatgcgag ggggcagaaa taagtttggg ccaatgtaca agagagacag ggctttgaag 600

cagcagaaga aagccctcat tcgagccaat ggacttaagc tggaagccat gtctcaggtg 660

atccaagcaa tgccctcaga cctgacctct gcaattcaga acattcattc cgcctccaaa 720

ggcctacctc tgagccatgt agccttgcct ccgacagact atgacagaag tccctttgtc 780

acatctccca ttagcatgac aatgccacct cacagcagcc tgcatggtta ccaaccctat 840

ggtcactttc ctagtcgggc catcaggtct gagtacccag acccctactc cagctcacct 900

gagtcaatga tgggttactc ctacatggat ggttaccaga caaactcccc ggccagcatc 960

ccacacctga tactggaact tttgaagtgt gaaccagatg agcctcaagt tcaagcgaag 1020

atcatggctt acctccagca agagcagagt aaccgaaaca ggcaagaaaa gctgagcgca 1080

tttgggcttt tatgcaaaat ggcggaccag accctgttct ccattgttga gtgggccagg 1140

agtagtatct tcttcaggga actgaaggtt gatgaccaaa tgaagctgct tcaaaactgc 1200

tggagtgagc tcttgattct cgatcacatt taccgacaag tggcgcatgg gaaggaaggg 1260

acaatcttcc tggttactgg agaacacgtg gactactcca ccatcatctc acacacagaa 1320

gtcgcgttca acaacctcct gagtctcgca caggagctgg tggtgaggct ccgttccctt 1380

cagttcgatc agcgggagtt tgtatgtctc aagttcctgg tgctgttcag ctcagatgtg 1440

aagaacctgg agaacctgca gctggtggaa ggtgtccaag agcaggtgaa tgccgccctg 1500

ctggactaca cggtttgcaa ctacccacaa cagactgaga aattcggaca gctacttctt 1560

cggctacccg agatccgggc aatcagcaag caggcagaag actacctgta ctataagcac 1620

gtgaacgggg atgtgcccta taataacctc ctcattgaga tgctgcatgc caaaagagcc 1680

taa 1683

<210> 9
<211> 560
<212> PRT
<213> Artificial Sequence

<220>
<223> A sumoylated mutant of Nr5a2 expression product with two lysine residues mutated

<400> 9

Met Ser Ala Ser Leu Asp Thr Gly Asp Phe Gln Glu Phe Leu Lys His
1 5 10 15

Gly Leu Thr Ala Ile Ala Ser Ala Pro Gly Ser Glu Thr Arg His Ser
20 25 30

Pro Lys Arg Glu Glu Gln Leu Arg Glu Lys Arg Ala Gly Leu Pro Asp
35 40 45

Arg His Arg Arg Pro Ile Pro Ala Arg Ser Arg Leu Val Met Leu Pro
50 55 60

Lys Val Glu Thr Glu Ala Pro Gly Leu Val Arg Ser His Gly Glu Gln
65 70 75 80

Gly Gln Met Pro Glu Asn Met Gln Val Ser Gln Phe Lys Met Val Asn
85 90 95

Tyr Ser Tyr Asp Glu Asp Leu Glu Glu Leu Cys Pro Val Cys Gly Asp
100 105 110

Lys Val Ser Gly Tyr His Tyr Gly Leu Leu Thr Cys Glu Ser Cys Lys
115 120 125

Gly Phe Phe Lys Arg Thr Val Gln Asn Gln Lys Arg Tyr Thr Cys Ile
130 135 140

Glu Asn Gln Asn Cys Gln Ile Asp Lys Thr Gln Arg Lys Arg Cys Pro
145 150 155 160

Tyr Cys Arg Phe Lys Lys Cys Ile Asp Val Gly Met Arg Leu Glu Ala
165 170 175

Val Arg Ala Asp Arg Met Arg Gly Gly Arg Asn Lys Phe Gly Pro Met
180 185 190

Tyr Lys Arg Asp Arg Ala Leu Lys Gln Gln Lys Lys Ala Leu Ile Arg
195 200 205

Ala Asn Gly Leu Lys Leu Glu Ala Met Ser Gln Val Ile Gln Ala Met
210 215 220

Pro Ser Asp Leu Thr Ser Ala Ile Gln Asn Ile His Ser Ala Ser Lys
225 230 235 240

Gly Leu Pro Leu Ser His Val Ala Leu Pro Pro Thr Asp Tyr Asp Arg
245 250 255

Ser Pro Phe Val Thr Ser Pro Ile Ser Met Thr Met Pro Pro His Ser
260 265 270

Ser Leu His Gly Tyr Gln Pro Tyr Gly His Phe Pro Ser Arg Ala Ile
275 280 285

Arg Ser Glu Tyr Pro Asp Pro Tyr Ser Ser Ser Pro Glu Ser Met Met
290 295 300

Gly Tyr Ser Tyr Met Asp Gly Tyr Gln Thr Asn Ser Pro Ala Ser Ile
305 310 315 320

Pro His Leu Ile Leu Glu Leu Leu Lys Cys Glu Pro Asp Glu Pro Gln
325 330 335

Val Gln Ala Lys Ile Met Ala Tyr Leu Gln Gln Glu Gln Ser Asn Arg
340 345 350

Asn Arg Gln Glu Lys Leu Ser Ala Phe Gly Leu Leu Cys Lys Met Ala
355 360 365

Asp Gln Thr Leu Phe Ser Ile Val Glu Trp Ala Arg Ser Ser Ile Phe
370 375 380

Phe Arg Glu Leu Lys Val Asp Asp Gln Met Lys Leu Leu Gln Asn Cys
385 390 395 400

Trp Ser Glu Leu Leu Ile Leu Asp His Ile Tyr Arg Gln Val Ala His
405 410 415

Gly Lys Glu Gly Thr Ile Phe Leu Val Thr Gly Glu His Val Asp Tyr
420 425 430

Ser Thr Ile Ile Ser His Thr Glu Val Ala Phe Asn Asn Leu Leu Ser
435 440 445

Leu Ala Gln Glu Leu Val Val Arg Leu Arg Ser Leu Gln Phe Asp Gln
450 455 460

Arg Glu Phe Val Cys Leu Lys Phe Leu Val Leu Phe Ser Ser Asp Val
465 470 475 480

Lys Asn Leu Glu Asn Leu Gln Leu Val Glu Gly Val Gln Glu Gln Val
485 490 495

Asn Ala Ala Leu Leu Asp Tyr Thr Val Cys Asn Tyr Pro Gln Gln Thr
500 505 510

Glu Lys Phe Gly Gln Leu Leu Leu Arg Leu Pro Glu Ile Arg Ala Ile
515 520 525

Ser Lys Gln Ala Glu Asp Tyr Leu Tyr Tyr Lys His Val Asn Gly Asp
530 535 540

Val Pro Tyr Asn Asn Leu Leu Ile Glu Met Leu His Ala Lys Arg Ala
545 550 555 560

<210> 10
<211> 1683
<212> DNA
<213> Artificial Sequence

<220>
<223> A sumoylation construct of Nr5a2 with five lysine residues mutated

<400> 10
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attgcgtctg caccagggtc agagactcgc cactccccca aacgtgagga acaactccgg 120

gaaaaacgtg ctgggcttcc ggaccgacac cgacgcccca ttcccgcccg cagccgcctt 180

gtcatgctgc ccaaagtgga gacggaagcc ccaggactgg tccgatcgca tggggaacag 240

gggcagatgc cagaaaacat gcaagtgtct caatttaaaa tggtgaatta ctcctatgat 300

gaagatctgg aagagctatg tcctgtgtgt ggcgataaag tgtctgggta ccattacggt 360

ctcctcacgt gcgaaagctg caagggtttt tttaagcgaa ctgtccaaaa ccaaaaaagg 420

tacacgtgca tagagaacca gaattgccaa attgacaaaa cgcagagaaa acgatgtccc 480

tactgtcgat tcaaaaaatg tatcgatgtt gggatgaggc tggaagccgt aagagccgac 540

cgcatgcgag ggggcagaaa taagtttggg ccaatgtaca agagagacag ggctttgaag 600

cagcagaaga aagccctcat tcgagccaat ggacttaggc tggaagccat gtctcaggtg 660

atccaagcaa tgccctcaga cctgacctct gcaattcaga acattcattc cgcctccaaa 720

ggcctacctc tgagccatgt agccttgcct ccgacagact atgacagaag tccctttgtc 780

acatctccca ttagcatgac aatgccacct cacagcagcc tgcatggtta ccaaccctat 840

ggtcactttc ctagtcgggc catcaggtct gagtacccag acccctactc cagctcacct 900

gagtcaatga tgggttactc ctacatggat ggttaccaga caaactcccc ggccagcatc 960

ccacacctga tactggaact tttgaggtgt gaaccagatg agcctcaagt tcaagcgaag 1020

atcatggctt acctccagca agagcagagt aaccgaaaca ggcaagaaaa gctgagcgca 1080

tttgggcttt tatgcaaaat ggcggaccag accctgttct ccattgttga gtgggccagg 1140

agtagtatct tcttcaggga actgagggtt gatgaccaaa tgaagctgct tcaaaactgc 1200

tggagtgagc tcttgattct cgatcacatt taccgacaag tggcgcatgg gaaggaaggg 1260

acaatcttcc tggttactgg agaacacgtg gactactcca ccatcatctc acacacagaa 1320

gtcgcgttca acaacctcct gagtctcgca caggagctgg tggtgaggct ccgttccctt 1380

cagttcgatc agcgggagtt tgtatgtctc aagttcctgg tgctgttcag ctcagatgtg 1440

aagaacctgg agaacctgca gctggtggaa ggtgtccaag agcaggtgaa tgccgccctg 1500

ctggactaca cggtttgcaa ctacccacaa cagactgaga aattcggaca gctacttctt 1560

cggctacccg agatccgggc aatcagcaag caggcagaag actacctgta ctataagcac 1620

gtgaacgggg atgtgcccta taataacctc ctcattgaga tgctgcatgc caaaagagcc 1680

taa 1683

<210> 11
<211> 560
<212> PRT
<213> Artificial Sequence

<220>
<223> An expression product of a sumoylation mutant of Nr5a2 with 5 lysine residues mutated

<400> 11

Met Ser Ala Ser Leu Asp Thr Gly Asp Phe Gln Glu Phe Leu Lys His
1 5 10 15

Gly Leu Thr Ala Ile Ala Ser Ala Pro Gly Ser Glu Thr Arg His Ser
20 25 30

Pro Lys Arg Glu Glu Gln Leu Arg Glu Lys Arg Ala Gly Leu Pro Asp
35 40 45

Arg His Arg Arg Pro Ile Pro Ala Arg Ser Arg Leu Val Met Leu Pro
50 55 60

Lys Val Glu Thr Glu Ala Pro Gly Leu Val Arg Ser His Gly Glu Gln
65 70 75 80

Gly Gln Met Pro Glu Asn Met Gln Val Ser Gln Phe Lys Met Val Asn
85 90 95

Tyr Ser Tyr Asp Glu Asp Leu Glu Glu Leu Cys Pro Val Cys Gly Asp
100 105 110

Lys Val Ser Gly Tyr His Tyr Gly Leu Leu Thr Cys Glu Ser Cys Lys
115 120 125

Gly Phe Phe Lys Arg Thr Val Gln Asn Gln Lys Arg Tyr Thr Cys Ile
130 135 140

Glu Asn Gln Asn Cys Gln Ile Asp Lys Thr Gln Arg Lys Arg Cys Pro
145 150 155 160

Tyr Cys Arg Phe Lys Lys Cys Ile Asp Val Gly Met Arg Leu Glu Ala
165 170 175

Val Arg Ala Asp Arg Met Arg Gly Gly Arg Asn Lys Phe Gly Pro Met
180 185 190

Tyr Lys Arg Asp Arg Ala Leu Lys Gln Gln Lys Lys Ala Leu Ile Arg
195 200 205

Ala Asn Gly Leu Arg Leu Glu Ala Met Ser Gln Val Ile Gln Ala Met
210 215 220

Pro Ser Asp Leu Thr Ser Ala Ile Gln Asn Ile His Ser Ala Ser Lys
225 230 235 240

Gly Leu Pro Leu Ser His Val Ala Leu Pro Pro Thr Asp Tyr Asp Arg
245 250 255

Ser Pro Phe Val Thr Ser Pro Ile Ser Met Thr Met Pro Pro His Ser
260 265 270

Ser Leu His Gly Tyr Gln Pro Tyr Gly His Phe Pro Ser Arg Ala Ile
275 280 285

Arg Ser Glu Tyr Pro Asp Pro Tyr Ser Ser Ser Pro Glu Ser Met Met
290 295 300

Gly Tyr Ser Tyr Met Asp Gly Tyr Gln Thr Asn Ser Pro Ala Ser Ile
305 310 315 320

Pro His Leu Ile Leu Glu Leu Leu Arg Cys Glu Pro Asp Glu Pro Gln
325 330 335

Val Gln Ala Lys Ile Met Ala Tyr Leu Gln Gln Glu Gln Ser Asn Arg
340 345 350

Asn Arg Gln Glu Lys Leu Ser Ala Phe Gly Leu Leu Cys Lys Met Ala
355 360 365

Asp Gln Thr Leu Phe Ser Ile Val Glu Trp Ala Arg Ser Ser Ile Phe
370 375 380

Phe Arg Glu Leu Arg Val Asp Asp Gln Met Lys Leu Leu Gln Asn Cys
385 390 395 400

Trp Ser Glu Leu Leu Ile Leu Asp His Ile Tyr Arg Gln Val Ala His
405 410 415

Gly Lys Glu Gly Thr Ile Phe Leu Val Thr Gly Glu His Val Asp Tyr
420 425 430

Ser Thr Ile Ile Ser His Thr Glu Val Ala Phe Asn Asn Leu Leu Ser
435 440 445

Leu Ala Gln Glu Leu Val Val Arg Leu Arg Ser Leu Gln Phe Asp Gln
450 455 460

Arg Glu Phe Val Cys Leu Lys Phe Leu Val Leu Phe Ser Ser Asp Val
465 470 475 480

Lys Asn Leu Glu Asn Leu Gln Leu Val Glu Gly Val Gln Glu Gln Val
485 490 495

Asn Ala Ala Leu Leu Asp Tyr Thr Val Cys Asn Tyr Pro Gln Gln Thr
500 505 510

Glu Lys Phe Gly Gln Leu Leu Leu Arg Leu Pro Glu Ile Arg Ala Ile
515 520 525

Ser Lys Gln Ala Glu Asp Tyr Leu Tyr Tyr Lys His Val Asn Gly Asp
530 535 540

Val Pro Tyr Asn Asn Leu Leu Ile Glu Met Leu His Ala Lys Arg Ala
545 550 555 560