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(54) **Title:** AVIAN INDUCED PLURIPOTENT STEM CELLS AND THEIR USE

(57) **Abstract:** Avian species are important to animal agriculture around the world and for developmental and disease models; however, unlike in mice, where clonal lines of pluripotent stem cells have enabled researchers to study mammalian gene function, clonal highly proliferative pluripotent avian cell lines have been an elusive goal. In this report we demonstrate for the first time the successful generation of induced pluripotent stem cells in a non-mammalian species. Quail and chicken embryonic fibroblast cells were transfected or transduced with pluripotency genes OCT4, NANOG, SOX2, LIN28, C-MYC and KLF4 in reprogramming vectors. Avian induced pluripotent stem cells (AiPSC) showed iPSC morphology, high telomerase activity, rapid proliferation and have been maintained for >50 passages without loss of pluripotent phenotype. AiPSCs were highly positive for AP, PAS staining, POU5F1 and SOX2 and were capable of clonal expansion post-genetic manipulation. After in vitro embryoid body formation and directed differentiation, aiPSCs formed all three germ layers and all three lineages of neural cells: neuronal, astrocyte and oligodendrocyte. Ultimately, aiPSCs, even late passage cells, were capable of generating live chimeric birds when introduced into stage X-XV embryos and incorporated into tissues from all three germ layers, extraembryonic tissues and potentially the germline.



Avian Induced Pluripotent Stem Cells and Their Use

Related Applications

This application claims the benefit of priority of provisional application serial number 61/495,499, filed June 10, 2011 entitled "Avian Induced Pluripotent Stem Cells Generate Live Quail-chicken Chimeras and Undergo Advanced *In Vitro* Differentiation" the contents of which application is incorporated herein in its entirety.

Field of the Invention

The present invention relates to the production of avian induced pluripotent stem cells from non-pluripotent somatic cells, including embryonic fibroblasts and adult somatic cells. In this method, avian (including quail or chicken) somatic cells are reprogrammed into a state closely resembling embryonic stem cells including the expression of key stem cell markers alkaline phosphatase, etc. by transfecting/transducing the non-stem cells with genes (preferably using a non-integrating vector as otherwise described herein or alternatively an integrating vector, such a lentiviral vector, retroviral vector or inducible lentiviral vector, among others) which express at least nanog, Lin28 and cMyc. In preferred aspects of the invention, the transfected/transduced vectors express nanog, Lin28, cMyc, Oct 4 (POU5F1 or PouV), SOX2 and KLF4. The induced stem cells which are produced contribute to all 3 germ layers, the trophectoderm and in certain aspects, the gonad in chimeric offspring. The experiments which are presented herein evidence that the induced quail and chicken pluripotent stem cells according to the present invention are bona fide stem cells that exist in a pluripotent state where they can be differentiated or turned into any cell type in the body or even a chimeric cell or mature bird. The present invention represents the first time that avian iPSCs were demonstrated to be capable of contributing to chimeric offspring, an essential component in their use as a biotechnology tool, including the preparation of vaccines.

Introduction/Background of the Invention

In mammals, embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are highly proliferative populations capable of differentiating into multiple cell types and tissues representing all 3 germ layers and the germline (1-5). Similar to ESCs, mammalian iPSCs exhibit high levels of plasticity in both in vitro and in vivo environments (1). After the first derivation of mouse iPSCs in 2006, this technology has firmly proven its potency in generating iPSCs from various mammals and is advancing many scientific fields, from regenerative medicine to basic developmental biology (1, 2, 5-10). Given the diverse applications of mammalian iPSCs, avian iPSCs would have similar utility for developmental biologists, facilitating gene function and tissue transplant studies.

Although avian ESC and primordial germ cell (PGC) lines have been established (11, 12), they have not been used in gene targeting studies mostly likely because they have not been clonally isolated nor are they highly proliferative in extended cultures. Additionally, many of these avian lines have not been shown to robustly undergo directed in vitro differentiation into multiple lineages with phenotypic characteristic similar to mammalian pluripotent cells. Given some of the issues that plague the development of avian ESCs, the probability that avian iPSCs could be generated and show diverse differentiation potential in vitro and in vivo would seem unlikely. Generating non-mammal iPSCs faces other challenges and difficulties. Intuitively species specific or at least related reprogramming factors may be required for proper reprogramming and little is known of the required reprogramming factors in phylogenetically diverse species.

Avian embryonic models have a long history of providing critical new insights into developmental biology including organ function (13, 14), disease progression (e.g. Pompe disease) (15), eye disorders (16, 17) and many others (18, 19). The advantage that avian species have is their relative size and ease of access to the embryo for manipulation. Cells and tissues including whole sections of the spinal column can be transplanted into the avian embryo and can be monitored in real time during development (20). This is not possible in

mammalian species. Moreover, the quail-chicken chimera is an attractive and widely used model for developmental patterning and cell fate studies given that cells can be readily tracked in this model (21, 22). The quail also has a short generation interval (3-4 generation per year)(23), facilitating genetic selection studies and experiments requiring multiple generational observations (24). Coupling a robust and clonal feeder free iPSC lines and derived committed cell lines or tissues with these model systems offers new opportunities to manipulate and study developmental process both in vitro and in vivo.

In the present application it is demonstrated for the first time in a non-mammalian species the successful generation of highly proliferative quail and chicken iPSC populations amenable to genetic manipulation and capable of generating live chimeric offspring and undergoing advanced directed neural differentiation in vitro. These first iPSCs in a non-mammalian species demonstrate the highly conserved nature of the reprogramming genes and provide for future mechanistic developmental studies in well characterized avian models.

Brief Description of the Figures

Figure 1 shows the derivation of quail induced pluripotent stem cells (qiPSCs) from quail embryonic fibroblast cells (QEFs). QEFs prior to addition of reprogramming factors (A). Incomplete reprogrammed QEFs maintain a fibroblast-like morphology at day 6 post-transduction (B), while qiPSC colonies at day 17 showed defined borders (C) and at the single cell level a high nuclear to cytoplasm ratio, clear cell borders and prominent nucleoli (D, E). qiPSCs were positive for Alkaline Phosphatase (AP; F) and Periodic Acid Schiff's (PAS) staining (G). 5 out of 6 human pluripotent stem cell factors were integrated and expressed in qiPSCs (H).

Figure 2 shows qiPSCs demonstrate rapid proliferation, high levels of telomerase activity and clonal expansion after genetic manipulation. qiPSCs doubling time was 16.6 hr (n=3), significantly faster than the QEF cells (36.9 hr; $P < 0.01$) (A). Telomerase activity in qiPSC was higher than QEF (>11 fold, $*P < 0.01$) and comparable to HeLa cells ($P = 0.07$) (B). 8 days post transduction a subpopulation

qiPSC expressed green fluorescent protein (GFP). (C, D, E). After 9 days post-fluorescence activated cell sorting (FACS), clonally expanded GFP⁺ qiPSCs generated colonies (F, G) that have maintained GFP expression long term (H).

Figure 3 shows qiPSCs express pluripotent genes. Immunocytochemistry demonstrated that QEFs were negative for POU5F1 (A) and SOX2 (B), while qiPSCs were POU5F1 (D) and SOX2 (E) positive. Scale bars are 50 μ m.

Figure 4 shows that qiPSCs generate EBs that form all 3 germ layers. Compact EBs were formed after culture for 6 days (A). EBs were replated for further differentiation for 2 days (B). Ectoderm (*TUJ1*, *PAX6*), endoderm (*Vimentin*) and mesoderm (*Brachyury*) genes were expressed in EBs (C). Immunocytochemistry demonstrated that EB derived cells were positive for ectoderm (TUJ1, D), endoderm (SOX17, E) and mesoderm (α SMA, F) proteins. Scale bars are 50 μ m.

Figure 5 shows directed differentiation of qiPSCs to 3 neural lineages. qiPSCs were subjected to a 3 step neural differentiation process, with cells first cultured in neural derivation medium for 12 days, then in proliferation medium for 7 days, followed by continual maintenance in differentiation medium. Neurite extensions could be found after culture in differentiation medium for 48 days. Neuron-like cells expressing Hu C/D⁺ (A) and MAP2 (B) were present after 10 days of differentiation and astrocytes (C) and oligodendrocyte (D) like cells after 23 and 39 days of differentiation respectively. Scale bars in E, F and G are 50 μ m. Scale bars in H are 100 μ m.

Figure 6 shows chimeric chicken embryos derived from qiPSC. GFP⁺ qiPSCs were incorporated in brain (A, ectoderm), eye (B, ectoderm), trachea/lung (C, endoderm), heart (D, mesoderm) and yolk sac (F, extraembryonic tissue) of quail-chicken chimeric embryos. PCR results demonstrated that various tissues were positive for the *hPOU5F1* transgene (E, G).

Figure 7 also shows chimeric chickens derived from qiPSC Two chicks produced by low passage qiPSC (P7) determined to be at day 14 (A) and 19 (B) developmental stages exhibited significant levels of feather chimerism (black arrow), yet failed to hatch. High passage qiPSC (P45) contributed to live chimeras as indicated by the presents of the human *POU5F1* gene in the brain, liver and gonad of two individuals (N=15; C).

Figure 8 shows the transduction efficiency in QEF. QEFs were transduced with lentiviral eGFP gene constructs. Flow cytometry was carried out 48 hours after transduction. Utilizing GeneJammer, at 10 MOI 14.6 % of QEFs were transduced (A) and at 20 MOI 25.3% of QEFs were transduced and (B) expressed GFP. While using TransDux, at 10 MOI 31.4% of QEFs were transduced (C) and at 20 MOI 40.5% QEFs were transduced and (D) expressed GFP.

Figure 9 shows the injection of qiPSC into stage X chicken embryo. To inject qiPSCs into chicken embryos, a single window was drilled into the shell of stage X White Leghorn chicken egg (A). qiPSC were then injected into the subgerminal cavity with a micropipette (B). Windows were sealed with hot glue and injected eggs were then transferred to incubators (C).

Figure 10 shows the derivation of ciPSCs. Chicken embryonic fibroblasts (CEFs) were transduced with lentiviral vectors containing human stem cell factor (A) and then replated onto MEF feeder. Colonies of reprogrammed CEFs were observed at day 5 (B) and an enlarged view of the typical ciPSC-like colony demonstrating stem cell morphology (C). The reprogrammed cells were around 50% confluent at day 7 after transduction and subjected to serial passages (D) and finally plated on Matrigel coated plates (E). The ciPSCs were positive for Alkaline Phosphatase (F) and Periodic Acid Schiff's Staining (G) were also positive for pluripotent markers SSEA1 (H) and germ cell marker DDX4 (I) in immunostaining.

Figure 11 shows pluripotent genes expression in ciPSCs. PCR showed that human

stem cell genes hNANOG, hLIN28 and hC-MYC were incorporated in ciPSCs (A). Quantitative PCR showed that the chicken endogenous gene cPOUV, cSOX2, cC-MYC, Telomerase reverse transcriptase (cTERT) and chicken VASA Homologue (cVH) were significantly upregulated in ciPSC BA3 and BA4 line over the CEF (B, C, D, E, F).

Figure 12 shows ciPSCs differentiated into cells from all 3 germ layer in vitro.

To perform the differentiation through embryoid body formation, ciPSC were plated in AggreWell plates for 24 hours and the aggregates were then harvested and cultured in suspension for 6 days (A). RT-PCR revealed that the ciPSCs differentiated into all 3 germ layers.

Figure 13 shows the screening for NDV-resistant ciPSC The NDV virus

screening was initiated at 50 MOI using GFP-NDV, with GFP indicating successful infection. An infection efficiency of >99% was seen in either CEFs (E) or ciPSCs (M). CEFs were used as control cell line for virus screening (A) and they grew to confluence in the mock infection dish (B, F) while mostly died in the infected dish 48 hours after infection (C, G). The infected CEFs could not recover from the infection 9 days after infection. ciPSCs plated on Matrigel dish (I) were used for NDV screening and they grew to confluence in the mock infection dish (J, N) while mostly died in the infected dish 48 hours after infection (K, O). 9 days after infection, ciPSC survived the NDV infection formed colonies and some of the cell were clear of the NDV virus.

Figure 14 shows the established ciPSC line demonstrates significant resistance to NDV. 48 after NDV infection, significant cell death was seen in infected CEF (E) and ciPSC I0 (F) dishes but not in ciPSC I12 (G) and ciPSC I14 (H), while in the mock infection control CEF, ciPSC I0, ciPSC I12 and ciPSC I14 showed rapid growth and confluence (A, B, C, D). The results of cell counts (I) 48 hours after NDV infection (normalized to mock control) showed significant resistant to NDV in ciPSC I12 and ciPSC I14 over their parent/original cell line CEF and ciPSC I0. (a,b: $P < 0.01$)

Figure 15 shows that more pluripotent ciPSCs were derived by a 2nd round of reprogramming. The ciPSCs are positive for OCT4, SOX2, NANOG and SSEA1 after a 2nd round of reprogramming (Fig. 6A-D). The SSEA1+ ciPSCs was enriched by MACS sorting and additional selection by manual picking yielded a population of ciPSCs >82% positive for SSEA1 (Fig. 15E-G).

Figure 16 shows the generation of chicken induced pluripotent stem cells (ciPSCs) from CEFs. (A) CEFs prior to addition of reprogramming factors (B) GFP expressed 72 hours post-transfection (C) Transfection efficiency comparison between different transfection reagents (D) Lipofectamine showed highest transfection efficiency.

Figure 17 shows the typical ciPSC colony formed in culture. (A) Reprogrammed CEFs showed defined borders at day 20 post-transfection (B) Typical ciPSC colonies were present in culture. (C) colony formation number counted and comparison between 4 different transfection reagents. *P<0.05 between 4 groups using different transfection reagents.

Figure 18 shows that the rate of doubling the population of ciPSCs was only slightly greater than the doubling time for the somatic cell from the ciPSCs were derived.

Figure 19 shows that ciPSCs injected into gonads evidenced migration consistent with formation of gametes.

Figure 20 shows the viPSTM vector kit lentiviral vector which expresses human Oct4 (POU5F1, Sox2, Nanog, Klf4, c-Myc, and Lin28 under control of the human elongation factor-1 alpha promoter (EF1 α).

Brief Description of the Invention

The present invention relates to a method of inducing embryonic and/or mature avian, in particular, quail or chicken, somatic cells, including fibroblasts, to a pluripotent stem

cell state (expressing the pluripotent stem cell markers Nanog, PouV, Oct 4 and preferably also SSEA1) comprising transfecting or transducing (often transfecting) said somatic cells (which can be from any number of avian species, especially including quail and chickens, preferably avian fibroblast cells, more preferably avian embryonic fibroblast cells) with at least three reprogramming genes consisting of Nanog, Lin28 and c-Myc and optionally, at least one additional reprogramming gene selected from the group consisting of Oct 4 (POU5F1 or PouV), SOX2 and KLF4 such that the somatic cell, after reprogramming, exhibits characteristics of an induced pluripotent stem cell. In the present invention, at least one reprogramming vector population expressing the reprogramming genes is used to reprogram the cells, preferably more than one reprogramming vector population is used, preferably as many reprogramming vector populations as there are reprogramming genes to be transfected or transduced are used such that each reprogramming vector population comprises no more than one reprogramming gene (e.g., Nanog, Lin28, c-Myc and optionally one or more of Oct4 (POU5F1 or PouV), SOX2 and KLF4). In various aspects of the invention, the reprogramming vector is a non-integrating or integrating vector preferably selected from the group consisting of a circle DNA vector (episomal DNA), a lentiviral vector, an inducible lentiviral vector or a retroviral vector, preferably, a circle DNA vector or a lentiviral vector, and each vector preferably comprises only one reprogramming gene per vector). The somatic cells are transfected/transduced with the reprogramming vectors and reprogrammed under conditions wherein said reprogramming genes optionally integrate with the genome of the somatic cells, thus producing an avian induced pluripotent stem cells according to the present invention. By introducing the reprogramming genes from the transfected/transduced vectors into the somatic cells, the somatic cells are thus converted into an induced pluripotent stem cell state, which closely resembles embryonic stem cells (as evidenced by their expressed biomarkers, as well as their differentiation capacity and other physical and functional characteristics). The induced pluripotent cells may be expanded/propagated in nutrient medium (e.g., stem cell expansion medium or related enriched culture medium), optionally stored (e.g. by cryopreservation) and used in differentiation processes to produce differentiated cells such as neural cells or muscle cells or to genetically manipulate phenotypic changes in an avian population, preferably including

chickens.

The quail or chicken induced pluripotent stem cells (iPSCs) according to the present invention can be used in a number of ways to genetically manipulate chickens or other commercially relevant avian species, including turkeys. Genes (expressing a desired/modified phenotype) can be introduced into either the somatic cells which are used to derive iPSCs or directly into iPSCs which have been produced. These cells can then be utilized in a variety of differentiation methods to form differentiated cells such as neural cells or other differentiated cells or to form chimeras (e.g. by injecting genetically manipulated iPSC into embryos) which can be used to generate offspring with the modified phenotype. The phenotypes could include, but are not limited to, animals that exhibit improved production and disease resistance (in particular, Avian flu and/or Newcastle disease) or the development of animals to study specific diseases and/or injury.

Another aspect of the invention relates to chimeric embryos which are produced by injecting aiPSCs into avian embryos (generally contained within eggs) in an effective amount, and allowing the chimeric embryo to grow to maturity. The resulting matured embryos exhibit phenotypic characteristics of the wild-type or native embryo as well as those of the aiPSCs. In a method aspect of the invention, chimeric embryos and/or mature chimeric chickens according to the invention are produced by injecting an effective population of aiPSCs (generally from about 1,000 to 50,000 or more, preferably about 8500-15,000, about 10,000 cells) into an avian, preferably chicken embryo in an egg shell anytime during stage X to XV, preferably at stage X soon after incubation begins (preferably within the first few hours of incubation), by providing an opening in the shell and injecting the cells (preferably, those cells exhibiting disease resistance) into the subgerminal cavity of an avian, preferably chicken egg. The chimeric embryo produced by injecting the aiPSCs into the embryo is allowed to grow and mature, thus producing a chimeric embryo soon after injection (generally, between two and tens days after injection, depending upon the stage X-XV when the aiPSC is injected into the embryo, exhibiting at least in part, the characteristics of the injected aiPSCs. The resulting chimeric embryo may be grown to maturity producing

chimeric adults exhibiting characteristics of the aiPSCs, including resistance to disease, other desired characteristics or they may be used in vaccine production or research.

In an alternative embodiment, the aiPSCs can be used to produce a chimeric germline cell, by introducing the aiPSCs into a germline cell population, thus producing a chimeric germline cell, which can mature into gametes such that any selected trait which is introduced into the aiPSCs, including disease resistance, may be transmitted to offspring through the gamete. In an alternative method, the aiPSCs (which may advantageously exhibit advantageous selected traits such as disease resistance) are injected into the reproductive organs, including the testes of an adult bird and in the environment of the testes or ovaries, differentiate into sperm cells or ova.

In an alternative embodiment according to the present invention aiPSCs are selected for disease resistance (Cellular Adaptive Resistance or CAR) by exposing the naïve cells (i.e., those cells not exposed to a disease agent), generally about 10×10^9 or more cells) to a disease agent for which resistance as a trait is desired (e.g. Newcastle disease, Avian influenza) in a growth medium for a period sufficient to induce cell death in a large majority of cells (often approaching 100% of the naïve cells), generally at least about 48 hours to about 72 hours, preferably at least about 72 hours where almost all cells (generally, greater than 99%, and often close to 100% of the naïve cells) die from infection. A small percentage of the original naïve cells that survive are then isolated/collected, expanded and utilized to produce disease resistant differentiated cells and/or tissue or chimeric embryos which will allow the maturation of the embryos into disease resistant adults (especially commercially relevant chickens, turkeys, quail or geese). In this aspect of the invention, the resistant cells which are isolated may be reprogrammed pursuant to methods according to the present invention to increase the number of aiPSCs in the resistant cell population. Given that species specific cell lines offer potential for species specific vaccines, these resistant cells may be particularly useful for providing a better host for species specific vaccines, especially where vaccines could be generated from a specific genetic or tissue specific genetic or epigenetic background that is shown to be a better host for the production of vaccines, for

example, an animal infected with a virus, but cells from it demonstrate resistance to cell death.

In another embodiment, the present invention is directed to a cell viability assay for identifying cells which are viable and resistant after exposure to a disease agent as described above. In this assay, a measurement of metabolic activity of the cells exposed to disease agent after an appropriate time (e.g. 72 hours) is taken by measuring the conversion of MTS tetrazolium ([3-(4,5-dimethylthiazol-2-yl)- 5-(3-carboxymethonyphenol)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt]) to formazan (colorimetric) due to the production of NADPH and NADH during normal cellular respiration and comparing the decline with a standard (usually a population of cells which are resistant or non-resistant to the disease agent). A decline in the conversion of MTS tetrazolium to formazan as evidenced by a reduction in the intensity or the absence of color from formazan formation indicates non-viability, whereas a conversion to formazan which is approximately the same as or slightly higher than the viable resistant cells is indication of viability. This method is preferably conducted using flow cytometry. In certain aspects of the invention, viable resistant aiPSCs demonstrate metabolic activity which is at least about 10 times, at least about 20 times, at least about 25 times, at least about 30 times and at least about 35-40 times the metabolic activity of naïve cells after exposure to ND virus which are not resistant.

In a further embodiment, a method referred to as the aiPSC virus exposure survival and recovery assay is used to determine the resistance of cells in a sample. In this assay a sample of resistant aiPSCs which has been exposed previously to a disease agent (e.g. ND virus) is plated, exposed to the disease agent and then measured to determine viability. Resistant cells evidence higher cell numbers at earlier points in time and a more rapid increase in cell numbers over time at later points in time evidencing a faster recovery rate in comparison to naïve cells which have not been exposed to the disease agent. In the present invention, resistant ciPSCs demonstrated a higher survival than naïve ciPSCs at early (about 12-14 fold or more higher than naïve ciPSCs) and later (20 to 26 fold or more high than naïve ciPSCs) time points, evidencing a resistant population of ciPSCs which exhibits increased

survivability and rapid recovery of resistant cell lines.

Thus, in one aspect, the present invention is directed to a method for producing avian induced pluripotent stem cells comprising providing a population of somatic cells, preferably quail, chicken, turkey, duck or geese somatic cells (including fibroblast cells, especially embryonic fibroblast cells), to be reprogrammed to pluripotent stem cells (PSCs), transfecting or transducing these somatic cells with at least one population of reprogramming vectors (preferably more than one reprogramming vector) comprising reprogramming genes selected from at least Nanog, Lin28 and c-Myc and optionally one or more of Oct4 (POU5F1 or PouV), SOX2 and KLF4, allowing said reprogramming genes to reprogram said somatic cells into pluripotent stem cells and optionally, separating and/or isolating said pluripotent stem cells.

The present invention is directed to embodiments related to one or more of the following specific embodiments of the invention, among others.

A population of avian induced pluripotent stem cells reprogrammed from avian somatic cells, said somatic cells being reprogrammed with at least the three reprogramming genes Nanog, LIN28 and c-MYC and optionally, at least one reprogramming gene selected from the group consisting of OCT4, SOX2 and KLF4.

The population of cells wherein the cells have been reprogrammed with Nanog, LIN28, c-MYC, Oct4 and optionally SOX 2, KLF4 or a mixture of SOX 2 and KLF4.

The population of cells wherein the cells have been reprogrammed with Nanog, LIN28, c-MYC, SOX2 and optionally Oct4 and/or KLF4.

The population of cells above wherein the cells have been reprogrammed with Nanog, LIN28, c-MYC and SOX2.

The population of cells above wherein the cells have been reprogrammed with Nanog, LIN28, c-MYC, KLF4 and optionally, SOX2 and/or Oct4.

The population of cells above wherein the cells have been reprogrammed with Nanog, LIN28, c-MYC, Oct4, SOX2 and KLF4.

The population of cells above wherein the cells have been reprogrammed with Nanog,

LIN28 and c-MYC in the absence of Oct4, SOX2 and KLF4.

The population of cells above wherein the somatic cells are embryonic somatic cells, preferably, embryonic fibroblast cells.

The population of cells above wherein the somatic cells are adult somatic cells.

The population of cells above wherein the somatic cells are adult fibroblast cells.

The population of cells above wherein the somatic cells are reprogrammed by transfecting or transducing the cells with at least one vector comprising the reprogramming genes.

The population of cells above wherein the reprogramming genes are avian or mammalian genes each of which has a sequence homology of at least about 50% of the identical gene of the avian species from which the somatic cells are reprogrammed.

The population of cells above wherein the reprogramming genes are chicken, mouse, human, pig or cow genes.

The population of cells above wherein the cells are transfected or transduced with vectors wherein each of said vectors comprises a single reprogramming gene.

The population of cells above wherein the avian induced pluripotent cell is a quail, chicken, turkey, duck or goose pluripotent cell.

A population of induced avian pluripotent stem cells exhibiting the following four characteristics:

- form colonies with cells with a high nuclear to cytoplasmic ratio ranging from about 4.1 to about 1:2;
- express alkaline phosphatase;
- are developmentally identical to avian embryonic stem cells (aESCs); and
- are capable of *in vitro* differentiation to all three germ layer types (mesoderm, endoderm and ectoderm) as determined by gene expression.

The population of pluripotent stem cells above wherein the cells exhibit one or more of the following characteristics:

- express the following genes and proteins: Nanog, PouV, Sox2;

- exhibit a normal karyotype;
- express high levels of telomerase; and
- express Periodic Acid Schiff (PAS).

The population of pluripotent stem cells described above wherein the cells exhibit one or more of the following characteristics:

- express at least one of the cell surface markers SSEA1, SSEA3, SSEA4, TRA 1-61 and TRA 1-81;
- exhibit stable/robust pluripotency and can differentiate or contribute to chimeras even after more than 25 passages;
- fast cell cycle with high population doubling;
- when injected into avian embryos, contribute to all germ cell lineages during development;
- chimera generate protein and morphologically distinct cells *in vitro* such as neurons and muscle cells, among numerous others; and
- the cells are clonogenic.

The population of pluripotent stem cells described above which have been instilled with disease resistance from a disease agent.

The population of pluripotent stems cells described above wherein the disease agent is an agent against which a vaccine is to be made.

The population of pluripotent stem cells described above wherein the disease agent is Newcastle disease virus or an influenza virus, especially avian influenza virus.

The population of pluripotent stem cells described above wherein the cells are reprogrammed after an initial reprogramming.

The population of cells reprogrammed after an initial reprogramming which cells express SSEA1.

A chimeric bird produced from pluripotent stem cells described above.

The chimeric bird described above which is a quail, chicken, turkey, duck or goose,

often a chicken or turkey, most often a chicken.

The chimeric bird described above which exhibits resistance to a disease agent, including Newcastle disease virus or avian influenza virus.

A method comprising reprogramming an avian embryonic or adult somatic cell to an avian induced pluripotent stem cell, the method comprising transfecting or transducing the somatic cell with the three reprogramming genes Nanog, Lin28 and c-Myc and optionally, at least one reprogramming gene selected from the group consisting of Oct 4, SOX2 and KLF4, allowing the genes to reprogram the somatic cells into pluripotent stem cells and optionally, isolating and/or expanding the stem cells, wherein each of the reprogramming genes is an avian or mammalian gene which exhibits at least about 50% sequence homology with the identical gene from the species from which the somatic cells are reprogrammed.

The method described above wherein the avian somatic cell is reprogrammed with the reprogramming genes Nanog, Lin28, c-Myc, Oct4, SOX2 and KLF4 to produce avian induced pluripotent stem cells.

The method described above wherein the reprogramming genes are human, chicken, mouse, pig or cow genes.

The method described above wherein the reprogramming genes are transduced into the somatic cells via a viral vector and integrated into the genome of the somatic cell.

The method described above wherein the reprogramming genes are transfected into the somatic cells via a non-integrating reprogramming vector.

The method described above wherein the somatic cells have been reprogrammed with Nanog, LIN28, c-MYC, Oct4 and optionally SOX 2, KLF4 or a mixture of SOX 2 and KLF4.

The method described above wherein the cells have been reprogrammed with Nanog, LIN28, c-MYC, SOX2 and optionally Oct4 and/or KLF4.

The method described above wherein the somatic cells have been reprogrammed with Nanog, LIN28, c-MYC and SOX2.

The method described above wherein the stomatic cells have been reprogrammed with Nanog, LIN28, c-MYC, KLF4 and optionally, SOX2 and/or Oct4.

The method described above wherein the cells have been reprogrammed with Nanog,

LIN28, c-MYC, Oct4, SOX2 and KLF4.

The method described above wherein the cells have been reprogrammed with Nanog, LIN28 and c-MYC in the absence of Oct4, SOX2 and KLF4.

The method described above wherein the somatic cells are embryonic somatic cells, often embryonic fibroblast cells or adult somatic cells, including adult fibroblast cells.

The method described above wherein the somatic cells are reprogrammed by transfecting or transducing the cells with at least one vector comprising the reprogramming genes.

The method described above wherein the vector is a viral vector and the reprogramming genes integrate into the genome of the somatic cell to produce the induced pluripotent stem cell.

The method described wherein the vector is a non-viral vector and the reprogramming genes are non-integrated with the genome of the somatic cells to produce the induced pluripotent stem cell.

The method described above wherein the somatic cells are transfected or transduced with vectors wherein each of the vectors comprises a single reprogramming gene.

The method described above wherein the avian induced pluripotent cell is a quail, chicken, turkey, duck or goose pluripotent cell, often a chicken pluripotent cell.

The method described above wherein the pluripotent stem cells once produced are reprogrammed at least one further time and express SSEA1.

A method of genetically manipulating avian species, especially chickens, comprising selecting and introducing genes into somatic cells to be reprogrammed into avian iPSCs or directly into avian iPSCs to produce genetically manipulated iPSCs, introducing the genetically manipulated avian iPSCs cells into avian embryos to form chimeric embryos; and generating offspring with the chimeric embryos, wherein said offspring exhibit a phenotype consistent with the introduced genes.

The method described above wherein the genes are introduced directly into said iPSCs.

The method described above wherein the genes are introduced into the somatic cells before being reprogrammed to iPSCs.

The method described above wherein the genetically manipulated iPSCs are introduced into a subgerminal cavity of the embryos and the embryos are allowed to mature.

The method described above wherein the embryo is a chicken, turkey or duck embryo, often a chicken embryo.

A method of instilling disease resistance in an avian induced pluripotent stem cell, the method comprising producing an avian induced pluripotent stem cell from somatic cells according to any of the methods described above, exposing the pluripotent cells to a disease agent for a period of time sufficient to produce resistance to the disease agent in the cells so that more than 99% of the population of the pluripotent stem cells succumb to the disease agent, isolating the remaining viable cells, optionally testing the cells for resistance to the disease agent and optionally, propagating the resistant cells to provide a population of avian induced pluripotent stem cells exhibiting resistance to the disease agent.

A method of instilling disease resistance into a population of avian induced pluripotent stem cell from disease resistant somatic cells, the method comprising producing disease resistant somatic cells by exposing somatic cells to a disease agent for a period of time sufficient to produce resistance to the disease agent in the cells, more than 99% of the population of which succumb to the disease agent, isolating the resistant somatic cells, propagating the cells to provide a population of resistant somatic and producing a population of disease resistant avian induced pluripotent stem cells from the disease resistant somatic cells according to any of the methods described above.

The method described above wherein the resistant aiPSCs are injected into an avian embryo to produce a chimeric bird, the chimeric bird exhibiting resistance to the disease agent.

The method described above wherein the bird is a chicken and the disease agent is Newcastle disease virus or avian influenza virus.

A method of producing a chimeric avian gamete comprising administering into a germline cell an avian induced pluripotent cell described above to produce a chimeric germline cell, introducing the chimeric germline cell into the testes or ovaries of an adult bird and isolated a chimeric gamete after a period sufficient to produce the chimeric gamete from the germline in the adult bird.

The method described above wherein said chimeric gamete is an ovum or a sperm.

A method of producing immunogenic material for a vaccine comprising providing a population of aiPSCs resistant to a disease agent described above, growing the disease agent in the population of aiPSCs and removing the disease agent from the population of aiPSCs after said growing step.

The method described above wherein the disease agent is influenza virus or Newcastle disease virus.

The method described above wherein said disease agent is inactivated after the removing step.

The method described above wherein the disease agent is inactivated before being grown in the aiPSCs.

A vaccine produced with immunogenic material prepared according to the method described above.

aiPSCs according to the present invention exhibit a robust clonal rate of 20% (i.e. a single cell will produce a colony of cells 20% of the time). This unique feature of aiPSCs according to the present invention allows these cells to be particularly useful in studies that require multiple clonal isolation, such as complex genetic manipulation or virus screening, among others.

Detailed Description of the Invention

Unless otherwise noted, the terms used herein are to be understood according to conventional usage by those of ordinary skill in the relevant art. In addition to the definitions

of terms provided below, definitions of common terms in molecular biology may also be found in Rieger *et al.*, 1991 Glossary of genetics: classical and molecular, 5th Ed., Berlin: Springer-Verlag; and in Current Protocols in Molecular Biology, F.M. Ausubel *et al.*, Eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1998 Supplement). It is to be understood that as used in the specification and in the claims, "a" or "an" can mean one or more, depending upon the context in which it is used. Thus, for example, reference to "a cell" can mean that at least one cell can be utilized.

The present invention may be understood more readily by reference to the following detailed description of the preferred embodiments of the invention and the Examples included herein. However, before the present compositions and methods are disclosed and described, it is to be understood that this invention is not limited to specific conditions, or specific methods, etc., as such may, of course, vary, and the numerous modifications and variations therein will be apparent to those skilled in the art.

Standard techniques for growing cells, separating cells, and where relevant, cloning, DNA isolation, amplification and purification, for enzymatic reactions involving DNA ligase, DNA polymerase, restriction endonucleases and the like, and various separation techniques are those known and commonly employed by those skilled in the art. A number of standard techniques are described in Sambrook *et al.*, 1989 Molecular Cloning, Second Edition, Cold Spring Harbor Laboratory, Plainview, New York; Maniatis *et al.*, 1982 Molecular Cloning, Cold Spring Harbor Laboratory, Plainview, New York; Wu (Ed.) 1993 Meth. Enzymol. 218, Part I; Wu (Ed.) 1979 Meth. Enzymol. 68; Wu et al., (Eds.) 1983 Meth. Enzymol. 100 and 101; Grossman and Moldave (Eds.) 1980 Meth. Enzymol. 65; Miller (ed.) 1972 Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York; Old and Primrose, 1981 Principles of Gene Manipulation, University of California Press, Berkeley; Schleif and Wensink, 1982 Practical Methods in Molecular Biology; Glover (Ed.) 1985 DNA Cloning Vol. I and II, IRL Press, Oxford, UK; Hames and Higgins (Eds.) 1985 Nucleic Acid Hybridization, IRL Press, Oxford, UK; and Setlow and Hollaender 1979

Genetic Engineering: Principles and Methods, Vols. 1-4, Plenum Press, New York.

Abbreviations and nomenclature, where employed, are deemed standard in the field and commonly used in professional journals such as those cited herein.

The term "subject", or in some instances, "patient" is used throughout the specification within context to describe an animal, generally an animal which is a member of an avian species, especially, for example, a quail, a chicken, a turkey or other commercially relevant species which is exposed to methods and/or compositions which are described herein (including vectors and/or cells) according to the present invention. For treatment of those infections, conditions or disease states which are specific for a specific animal including a human patient, the term patient refers to that specific animal, including numerous avian species, especially quail, chicken, turkeys, ducks and geese, among others.

The terms "transfect", "transfecting", "transduce", "transduction" are used (in many instances synonymously) to describe a process of introducing nucleic acids into cells pursuant to the present invention. The term transfecting is used notably for introducing non-viral DNA (generally plasmids, although naked DNA, including supercoiled naked DNA and RNA including modified mRNA and MicroRNA may also be used) into eukaryotic cells, but the term may also refer to other methods and cell types, although other terms may also be used. Transfection or transduction of animal cells typically involves opening transient pores or "holes" in the cell membrane, to allow the uptake of material into the cells to be transfected. Transfection is often carried out using an agent such as calcium phosphate or other agent to assist transfection into the target cell, by electroporation, or by mixing a cationic lipid with the material to produce liposomes, which fuse with the cell membrane and deposit cargo within the cell. Transduction is a term which describes the process by which foreign DNA is introduced or transferred from one bacterium to another by a virus. This term also refers to the process whereby foreign DNA is introduced into another cell via a viral vector, as are preferred aspects of the present invention. Transduction does not require cell-to-cell contact (which occurs in conjugation), and it is DNAase resistant, but it often is benefitted by the inclusion of a transduction factor such as GeneJammer[™] or TransDux[™] transduction

reagents. Transduction is a relatively common tool used by those of skill to stably introduce or integrate a foreign gene into a host cell's genome.

The term "somatic cell" is used to describe any cell which forms the body of a multicellular organism that is other than a gamete, germ cell, gametocytes or undifferentiated stem cell. In contrast to somatic cells, gametes are cells that are involved in sexual reproduction, germ cells are gamete precursory cells and stem cells are cells that can divide (mitosis) and differentiate into a variety of cell types. In avian species, somatic cells make up all of the internal organs, skin, feathers, bones, blood, and connective tissue. Somatic cells are diploid. According to the present invention any avian somatic cell may be used to induce pluripotent stem cells, but preferred somatic cells for use in the present invention include those cells which may be readily propagated, especially including fibroblast cells, including adult fibroblast cells. Embryonic fibroblast cells are preferred for use in the present invention although stomach cells, liver cells, keratinocytes, amniotic cells, blood cells, adipose cells, neural cells, melanocytes, among numerous others may also be used.

The term "avian induced pluripotent stem cells" (aiPSCs), of which "quail induced pluripotent stem cells" (qiPSCs), "chicken induced pluripotent stem cells" (ciPSCs), turkey induced pluripotent stem cells" (tiPSCs) and goose induced pluripotent stem cells (giPSCs) are a subset, are derived from avian somatic cells by reprogramming the somatic cells with at least three reprogramming genes including NANOG, LIN28 and c-MYC, and optionally one or more of Oct4 (POU5F1, PouV) SOX2 and KLF4 using transfection and/or transduction of the reprogramming genes in certain instances (when higher efficiency is desired) to preferably integrate the genes into the genome of the somatic cells. Avian induced pluripotent stem cells include both adult and embryonic induced pluripotent stem cells, often produced from adult fibroblast cells or embryonic fibroblast cells.

Included in the definition of avian induced pluripotent stem cells or (aiPSCs) are adult and embryonic cells from various types of somatic cells, preferably fibroblast cells. Any cells of avian origin that are capable of producing progeny that are derivatives of all three germinal

layers are included, regardless of whether they were derived from adult somatic cells, embryonic somatic tissue (e.g., embryonic fibroblasts), fetal, or other sources. The aiPSCs are preferably not derived from a malignant source.

aiPSCs according to the present invention exhibit a robust clonal rate of 20% (i.e. a single cell will produce a colony of cells 20% of the time). This unique feature of aiPSCs according to the present invention allows these cells to be particularly useful in studies that require multiple clonal isolation, such as complex genetic manipulation or virus screening, among others.

aiPSC cultures are described as "undifferentiated" when a substantial proportion of the stem cells and their derivatives in the population display morphological characteristics of undifferentiated cells, clearly distinguishing them from differentiated cells of embryo or adult origin. Undifferentiated aiPSCs are easily recognized by those skilled in the art, and typically appear in the two dimensions of a microscopic view in colonies of cells with high nuclear/cytoplasmic ratios and prominent nucleoli. It is understood that colonies of undifferentiated cells in the population may often be surrounded by neighboring cells that are differentiated. "Naïve" aiPSCs refer to aiPSCs that have not been exposed to a disease agent (such as Newcastle disease virus or avian flu virus). "Resistant" aiPSCs refer to aiPSCs that have been exposed to a disease agent and are viable after such exposure compared to non-resistant aiPSCs, which exhibit substantially reduced metabolic activity compared to resistant aiPSCs in the virus exposure cell viability assay as otherwise described herein.

aiPSCs according to the present invention generally display the following characteristics:

1. Form colonies with cells with a high nuclear to cytoplasmic ratio (ranging from about 4:1 to about 1:2 nuclear to cytoplasmic, often about 2.1);
2. Express alkaline phosphatase;
3. Are developmentally identical to avian embryonic stem cells (aESCs);

4. Are capable of *in vitro* differentiation to all three germ layer types (mesoderm, endoderm and ectoderm) as determined by gene expression;
5. Express the following genes and proteins: Nanog, PouV, Sox2;
6. Exhibit a normal karyotype (euploid with all avian chromosomes present and an absence of altered chromosomes);
7. Express high levels of telomerase (often 5-10 fold greater than somatic cells); and
8. Express Periodic Acid Schiff (PAS).

Of the above eight (8) characteristics, the first four (4) are always present; and any one or more (1, 2, 3 or 4, preferably all 4) of characteristics 5-8 are usually present.

aiPSCs according to the present invention often exhibit one or more (1, 2, 3, 4, 5 or 6) of the following characteristics:

9. Expression of at least one (i.e., one, two, three, four or five) of the cell surface markers SSEA1, SSEA3, SSEA4, TRA 1-61 and TRA 1-81;
10. Exhibit stable/robust pluripotency- can differentiate or contribute to chimeras even after more than 25 passages;
11. Fast cell cycle with high population doubling (often, at least about 1.2 or higher the rate or more of embryonic stem cells);
12. When injected into avian, preferably chick embryos, contribute to all germ cell Lineages during development;
13. Chimera generate protein and morphologically distinct cells *in vitro* such as neurons and muscle cells, among numerous others;
14. The cells are clonogenic- i.e., can give rise to a colony of aiPSCs from a single cell.

aiPSCs according to the present invention usually do not exhibit the following characteristics unless those characteristics are further induced in the cell population

13. Resistance to cell death induced by lethal viruses (Newcastle disease, avian flu, etc.)

aiPSCs according to the present invention are distinguishable over avian embryonic pluripotent stem cells (ESCs) in the following ways:

1. The embryonic stem cells (ESCs) and aiPSCs are produced in entirely different ways from different cellular origins (ESCs obtained from blastocyst stage embryo vs. somatic cells for aiPSCs).
2. The aiPSCs exhibit much more stable potency than ESCs. aiPSCs exhibit robust differentiation even after >25 passages, whereas ESCs exhibit differentiation slowdown after about 20-25 passages.
3. aiPSCs can contribute to chimeras even after >25 passages, whereas the ESCs lose the ability to contribute to chimeras after about 20-25 passages.
4. The aiPSCs exhibit a faster population doubling than ESCs (in some cases at least about 1.2 or higher the rate of population doubling of ESCs).
5. aiPSCs are often clonogenic (because of the fast cell cycle and stable pluripotency) and exhibit a clonal rate of about 10-20% or sometimes higher (i.e., where a single cell will produce a colony at least 10-20% of the time) whereas the ESCs are generally non-clonogenic.

A desirable phenotype of propagated aiPSCs is the high potential to differentiate into cells of all three germinal layers: endoderm, mesoderm, and ectoderm tissues. Pluripotency of pluripotent stem cells which may be confirmed, for example, by injecting cells into severe combined immunodeficient (SCID) mice, fixing the teratomas that form using 4% paraformaldehyde, and then examining them histologically for evidence of cell types from the three germ layers. Alternatively, pluripotency may be determined by the creation of embryoid bodies and assessing the embryoid bodies for the presence of markers associated with the three germinal layers, using RT-PCR as otherwise described herein.

Propagated pluripotent stem cell lines may be karyotyped using a standard G-banding technique and compared to published karyotypes of the corresponding primate species. It is

desirable to obtain cells that have a "normal karyotype," which means that the cells are euploid, wherein all chromosomes are present and not noticeably altered.

At any time the propagated aiPSCs may be re-exposed to the reprogramming vectors (DNA integrating or non-integrating transient expression) and selected again for pluripotent traits as provided above, especially including positive expression of the biomarker SSEA1.

The term "chimera" refers to cells and/or embryos in which aiPSCs according to the present invention have been introduced. Pursuant to the present invention, aiPSCs may be injected into avian embryos to instill those embryos with certain favorable characteristics which may introduced into the aiPSCs, including resistance to disease agents such as Newcastle disease virus, avian influenza virus or other traits. aiPSCs may be introduced into an embryo preferably at embryonic Stage X to XV (i.e., from the point that an egg is freshly laid (about 20 hours uterine age) to about 6-8 hours of that time, although embryos of greater maturity may also be manipulated to produce a chimeric offspring. See, for example, Kochav, et al., *Developmental Biology*, 79, 296-308, 1980 and Eyal-Giladi, et al., *Developmental Biology*, 49, 321-337, 1976. Alternatively, a germline chimera may be produced (introduction of the aiPSCs into germlines cells to produce germline chimeras) so that the germ cells may mature into gametes (ova or sperm) and any selected trait which is incorporated into the aiPSC may be transmitted to the offspring. Alternatively, aiPSCs may be injected directly into the testes of a chicken, such that in that environment

The term "avian embryonic stem cell" refers to embryonic pluripotent cells, of avian origin, which are isolated from the blastocyst stage embryo. Embryonic stem cell refers to a stem cell from an avian species and are used for comparison purposes to the avian induced pluripotent stem cells of the present invention. Many of the physical and functional characteristics of aESCs are identical to aiPSCs, although there are some difference of functional significance.

The term “reprogramming” refers to a process pursuant to the present invention whereby avian somatic cells are supplied, transfected or transduced with a population of reprogramming vectors comprising the reprogramming genes Nanog, LIN28 and c-MYC and optionally one or more of Oct4 (POU5F1 or PouV), SOX2 and KLF4, reprogrammed such that each of the genes which are transfected or transduced into the somatic cells is incorporated into cell and preferably integrated with the somatic cell genome, whereby the reprogrammed genes instill characteristics in the somatic cells to produce a population of avian induced pluripotent stem cells (aiPSCs). Accordingly, the term reprogramming is used generically herein to describe a process by which a somatic cell is induced to become a pluripotent cell pursuant to the present invention. In certain preferred aspects, the reprogramming genes integrate into the genome of the somatic cells, but integration is not required to induce the somatic cells to a pluripotent stem cell state, although such an approach is often efficient. In other preferred aspects of the invention, reprogramming genes are non-integrating and instead activate the endogenous pluripotency genes, resulting in alternative favorable characteristics (including the tendency to have much lower incidence of mutation and tumor formation).

The term “reprogramming genes” refers to the genes which are transfected or transduced into the avian somatic cells, are preferably integrated into the somatic cell genome and as a consequence of the expression of the induced polynucleotides reprogram or induce the somatic cells to a pluripotent stem cell state which the art refers to as an induced pluripotent stem cell or avian induced pluripotent stem cell (aiPSC). These genes include the following six genes, the first three of which are necessary and the last three of which are optionally used. These genes include Nanog, LIN28 and c-MYC as being required to produce avian induced pluripotent stem cells according to the present invention and additional genes which are optional, including one or more of POU5F1 (PouV), SOX2 and KLF4. It is noted that POU5F1 and PouV are the human and chicken homologues of Oct4 (octamer-binding transcription factor 4). It is an unexpected result that reprogramming of avian (preferably including chicken) somatic cells may be induced using only the three genes Nanog, LIN28 and c-MYC as described. It is also noted (unexpectedly) that the

reprogramming genes used to reprogram may be from any number of avian or mammalian species, including humans, but preferably these genes are from avian sources, preferably a source which exhibits species identity with that of the somatic cell which is to be reprogrammed. In general, the genes which are employed to reprogram the avian somatic cells pursuant to the present invention, have a sequence homology identity which is at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99% up to 100% of the genes from the species from which the somatic cells to be induced to pluripotent cells are obtained.

The term "reprogramming vector" is used to describe a transfection or transduction vector which may be used to reprogram a somatic cell to produce aiPSCs according to the present invention. Preferred reprogramming vectors for use in the present invention may be integrating or non-integrating and include viral vectors, especially integrating lentiviral vectors (see, Yu, et al., *Science*, 38, 1917-1920, 2007; Stadtfeld, et al., *Curr. Biol.*, 18, 890-894, 2008; Sommer, et al., *Stem Cells*, 27, 543-549, 2009; Anoyke, Danso, et al., *Cell Stem Cell*, 8, 376-388, 2011), retroviral vectors (see, Takahashi, et al., *Cell*, 126, 663-676, 2006; Takahashi, et al., *Cell*, 131, 861-872, 2007; Lowry, et al., *Proc. Natl. Acad. Sci. USA*, 105, 2883-2888, 2008; and Huangfu, et al., *Nature Biotechnol.*, 26, 1269-1275, 2008) and inducible retroviral vectors (see, Maherali, N., *Cell Stem Cell*, 3, 340-345, 2008 and Stadtfeld, et al., *Cell Stem Cell*, 2, 230-240, 2008). Alternative vectors which may be used include, for example, non-integrating vectors, which may be preferred in certain aspects of the invention, such as adenoviral vectors (Zhou, et al., *Stem Cells*, 27, 2667-2674, 2009 and Stadtfeld, et al., *Science*, 322, 949-953, 2008), plasmid vectors (Okita, et al., *Science*, 322, 949-953, 2008 and Si-Tayeb, et al., *BMC Dev. Biol.*, 1081, 2010), excisable vectors including transposon (Woltjen, et al., *Nature*, 458, 766-770, 2009) and loxP-lentiviral vectors (Somers, et al., *Stem Cells*, 28, 1728-1740, 2010) and DNA free vectors, including Sendai virus vectors (Fusaki, et al., *Proc. Jpn Acad.* 85, 348-362, 2009), protein vectors (Kim, et al., *Cell Stem Cell*, 4, 472-476, 2009 and Zhou, et al., *Cell Stem Cell*, 4, 381-384, 2009), modified mRNA vectors (Warren, et al., *Cell Stem Cell*, 7, 618-630, 2010) and MicroRNA vectors (Miyoshi, et al., *Cell Stem Cell*, 8, 633-638, 2011), as discussed generally in Robinton & Dailey, *Nature*, 481, 295-305, January 2012 and as generally known in the art. Vectors used in the present

invention are well known in the art and may be purchased, readily constructed and manipulated to incorporate reprogramming genes using standing genetic engineering methods.

In the present invention vectors are prepared using methods which are generally known in the art. A number of reprogramming vectors are available commercially, from establishments including the viPSTM Vector Kit (integrating), developed by ArunA Biomedical Inc., Athens, Georgia, and made available commercially by Thermo Fisher Scientific, Inc., Waltham Massachusetts, USA or Minicircle DNA[™] (non-integrating episomal DNA vectors) available from System Biosciences, LLC, Mountainview California, USA), the PiggyBac[™] Mouse 4-in-1 Transposon Vector (non-integrating), also available from System Biosciences, LLC. and Non-integrating modified mRNAs of the reprogramming genes (e.g., Stealth Express[™] exogenous gene expression system, available from Allele Biotechnology, San Diego, California, USA) expressing at least the reprogramming genes required for reprogramming (Nanog, LIN28, c-Myc).

The viPSTM Vector Kit for reprogramming fibroblasts (or other differentiated somatic cells) into induced pluripotent stem cells was developed by Thermo Fisher Scientific, Inc. in collaboration with ArunA Biomedical, Inc. Six transcription factors/genes (Lin28, c-Myc, Klf4, Nanog, Sox2 and Oct4 (Pou5f1)) have been cloned into a lentiviral vector system to create a resource for producing induced pluripotent stem (iPS) cells allowing for the generation of patient- and disease-specific cells. Ectopic expression of these factors has been shown to create pluripotent cells which resemble embryonic stem (ESCs) cells. The set of six factors is available in high-titer virus, ready for transduction. Separate vectors each of which expresses a single reprogramming gene are preferably used in the present invention. These vectors are commercially available from Thermo Fisher Scientific, Inc. or Systems Biosciences, LLC, as well as other sources and alternatively, may be readily prepared by inserting the relevant DNA sequence into the vector, the sequence of which genes are available online at Genebank.

The viPSTM vector kit lentiviral vectors express human Oct4 (POU5F1, Sox2, Nanog, Klf4, c-Myc, and Lin28 under control of the human elongation factor-1 alpha promoter (EF1 α) or alternatively a CMV, SV40, UBC, PGK and/or CAGG promoter. Vectors are generated by PCR amplifying each pluripotency factor open reading frame (ORF) followed by direct cloning downstream of the EF1 α promoter. Endonuclease restriction sites placed within the PCR primers are used to facilitate cloning. A Kozak consensus sequence may also be included to ensure efficient translation.

Minicircles are episomal DNA vectors that are produced as circular expression cassettes devoid of any bacterial plasmid DNA backbone. Their smaller molecular size enables more efficient transfections and offers sustained expression over a period of weeks as compared to standard plasmid vectors that only work for a few days. They are non-integrating. Non-integrating or footprint free transfection of reprogramming genes using mRNA (transposon vectors or cDNA expression vectors (minicircles) or even non-integrating virus such as Sendi virus or adenovirus, described above, may often be used to generate aiPSCs according to the present invention because these systems are less likely to induce a mutation (and possibly a tumor in the resulting chimeric bird) because the reprogramming genes do not integrate into the somatic cell genome using these methods of transfection/transduction. With non-integrating systems the gene cannot be turned on later (as with integrated genes) and induce tumors, which may be preferably in certain instances. However, the integrating vectors described above (lentivirus, retrovirus, inducible retrovirus and additionally, adenoassociate virus AAV vectors) are usually more efficient, with higher and more uptake and reprogramming to aiPSCs.

The cDNAs used for PCR are obtained from the Human Pluripotency Tool KitTM (Catalogue number PPK4919), which consists of: Oct4 (Pou5f1) (NM_002701, Incyte clone ID:LIFESEQ259583); Sox2 (NM_003106, IMAGE clone ID:2823424); Nanog (NM_024865, IMAGE clone ID: 40004920); Klf4 (NM_004235, IMAGE clone ID: 5111134); c-Myc (NM_002467, IMAGE clone ID: 2985844); and Lin28 (NM_024674, IMAGE clone ID: 841184), although a number of other cDNAs may also be used (see, for

example, below). In the case of alternative reprogramming genes from other mammalian and/or avian species, including chickens, these are obtained by nucleotide synthesis of the gene sequences for each of the identified reprogramming genes. Examples include the following genes listed in the table below:

Table 1A Pluripotent Reprogramming Genes

Species	Genes Name	Gene ID	mRNA reference
Chicken (<i>Gallus gallus</i>)	OCT4	427781	<u>NM 001110178.1</u>
	SOX2	396105	<u>NM 205188.1</u>
	NANOG	100272166	<u>NM 001146142.1</u>
	LIN28	428206	<u>NM 001031774.2</u>
	CMYC	420332	<u>NM 001030952.1</u>
	KLF4	770254	<u>XM 001233583.2</u>
Mouse (<i>Mus musculus</i>)	OCT4	18999	<u>NM 001252452.1</u>
	SOX2	20674	<u>NM 011443.3</u>
	NANOG	71950	<u>NM 028016.2</u>
	LIN28	83557	<u>NM 145833.1</u>
	CMYC	17869	<u>NM 001177352.1</u>
	KLF4	16600	<u>NM 010637.3</u>
Human (<i>Homo sapiens</i>)	OCT4	5460	<u>NM 001173531.1</u>
	SOX2	6657	<u>NM 003106.3</u>
	NANOG	79923	<u>NM 024865.2</u>
	LIN28	79727	<u>NM 024674.4</u>
	CMYC	4609	<u>NM 002467.4</u>
	KLF4	9314	<u>NM 004235.4</u>
Pig (<i>Sus scrofa</i>)	OCT4	100127461	<u>NM 001113060.1</u>
	SOX2	407739	<u>NM 001123197.1</u>
	NANOG	100170132	<u>NM 001129971.1</u>
	LIN28	100142662	<u>NM 001123133.1</u>
	CMYC	448810	<u>NM 001005154.1</u>
	KLF4	595111	<u>NM 001031782.2</u>
Cattle (<i>Bos Taurus</i>)	OCT4	282316	<u>NM 174580.2</u>
	SOX2	784383	<u>NM 001105463.1</u>
	NANOG	538951	<u>NM 001025344.1</u>
	LIN28	614997	<u>NM 001193057.1</u>
	CMYC	511077	<u>NM 001046074.2</u>
	KLF4	520842	<u>NM 001105385.1</u>

One of ordinary skill can identify the various reprogramming genes from a variety of

avian and/or mammalian species with the requisite homology, synthesize the cDNA's in each instance and incorporate the cDNA's into reprogramming vectors as otherwise described herein to transfect/transduce avian somatic cells and provide aiPSCs according to the present invention.

In the case of the pVIPS lentiviral vector, each of the pluripotency factor ORFs has been sequenced verified. For efficient expression, the preferred lentiviral vector used in the present invention also contains a woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) and the pput/cPPT (CTS, DNA flap) sequence. The pVIPS lentiviral vector is self-inactivating (SIN).

Features of the pVIPS lentiviral vector (see Figure 20) make it a versatile tool for generating induced pluripotent stem cells include:

- The ability to perform transductions using the replication incompetent lentivirus even on difficult to transduce cell lines
- Expression driven by a RNA Polymerase II EF1 α promoter
- Self inactivating (SIN) LTR to ensure minimal secondary recombination to form infectious particles

The following Table 2a provides a number of features of the pVIPS vector

Table 2A: Features of the pVIPS vector

Vector Element	Utility
5'LTR	5' long terminal repeat
RRE	Rev response element
CTS	Central Polypurine tract helps translocation into the nucleus of non-dividing cells
EF1 α Promoter	RNA Polymerase II promoter
WPRE	Enhances the stability and translation of transcripts
SIN-LTR	3' Self inactivating long terminal repeat (Shimada, <i>et al.</i>)
SV40 Ori	SV40 Ori Allows for episomal replication of plasmid in eukaryotic cells
pUC Ori	High copy replication and maintenance of plasmid in <i>E. coli</i>
Amp resistance	Ampicillin (carbenicillin) bacterial selectable marker

The terms “treat”, “treating”, and “treatment”, etc., as used herein, refer to any action providing or imparting a benefit to a subject at risk for or afflicted by a disease state, condition or deficiency which may be improved using cellular compositions according to the present invention. Treating a condition includes improving the condition through lessening or suppression of at least one symptom, delay in progression of the effects of the disease state or condition, including the prevention or delay in the onset of effects of the disease state or condition, etc.. Treatment, as used herein, encompasses both prophylactic and therapeutic treatment and especially includes instilling resistance to disease agents such as Newcastle disease virus or avian (bird) influenza.

The term “differentiation” is used to describe a process wherein an unspecialized (“uncommitted”) or less specialized cell acquires the features of a more specialized cell such as, for example, a neural cell, a muscle cell, a cardiomyocyte or other somatic cell. The term “differentiated” includes the process wherein a pluripotent stem cell according to the present invention becomes a more specialized intermediate cell. A differentiated or differentiation-induced cell is one that has taken on a more specialized (“committed”) position within the lineage of a cell. The term “committed”, when applied to the process of differentiation, refers to a cell that has proceeded in the differentiation pathway to a point where, under normal

circumstances, it will continue to differentiate into a specific cell type or subset of cell types, and cannot, under normal circumstances, differentiate into a different cell type or revert to a less differentiated cell type. “De-differentiation” refers to the process by which a cell reverts to a less specialized (or committed) position within the lineage of a cell. As used herein, the lineage of a cell defines the heredity of the cell, i.e., which cells it came from and what cells it can give rise to. The lineage of a cell places the cell within a hereditary scheme of development and differentiation. A lineage-specific marker refers to a characteristic specifically associated with the phenotype of cells of a lineage of interest and can be used to assess the differentiation of an uncommitted cell to the lineage of interest. Pursuant to the present invention aiPSCs may be used to differentiate into a large number of somatic cells, in the same manner that ESCs may be differentiated.

As used herein, the terms “cell growth medium”, “cell propagating medium”, “cell transfecting medium”, “cell reprogramming medium” and “differentiation medium” are all used to describe a cellular growth medium in which (depending upon the additional components used) the somatic cells and/or aiPSCs are grown and/or propagated, transfected and/or reprogrammed or instilled with resistance to a disease agent (e.g. Newcastle disease virus and/or avian influenza). Specific examples of these are presented in the examples section which follows. The somatic cells are generally maintained and/or propagated in a minimum essential medium such as a basic cellular medium (basic salt solution) which includes one or more components such as ascorbic acid, glucose, non-essential amino acids, salts (including trace elements), glutamine, insulin (where indicated and not excluded), and other agents well known in the art and as otherwise described herein. A useful cell growth medium is Dulbecco’s modified Eagle’s medium (DMEM) with greater than 0.1% bovine serum albumen (Sigma), 5% CO₂ at 37°C. For propagation or expansion of cells (generally, more than a million cells), a minimum essential medium with additional components, such as the preferred medium Dulbecco’s modified Eagle’s medium (DMEM) high glucose (Hyclone) with 10% fetal bovine serum (Hyclone), 4mM L-glutamine (Gibco) and 50U/ml penicillin and 50 mg/mL streptomycin (Gibco), 4ng/ml of basic FGF in 5% CO₂ at 37° C may be used. Alternatively, cells can be expanded in 10% knockout DMEM conditioned by

exposure to buffalo rat liver cells for 24 hours, 7.5% fetal bovine serum, 2.5% chicken serum, 1% glutamax, 2% GS nucleoside supplement, 1% antibiotic, 2-mecaptoethanol, 4ng/ml of basic FGF and 6 ng/ml of stem cell factor. Alternative media may also be used but may not be as effective as the above described media.

Reprogramming of the somatic cells should be done in an enriched culture medium ("reprogramming medium"). A minimum medium is (Dulbecco's modified Eagle medium (DMEM)/F12 (Gibco) or other similar minimum essential medium. In the reprogramming medium, a protein source is required to be added to the medium to assist in the reprogramming of the somatic cells, but the amount of protein required can range from 0.1% to 20%. Additional components such as knockout serum replacer (KSR) may also be preferably included. In addition, preferably, bFGF at effective concentrations (about 5-50 ng/ml) is added as well. A preferred reprogramming medium is [DMEM/F12 (Gibco), supplemented with 20% knockout serum replacer (KSR; Gibco), 2mM L-glutamine (Gibco), 0.1mM nonessential amino acids (Gibco), 50U/mL penicillin/50 mg/mL streptomycin (Gibco), 0.1mM b-mercaptoethanol (Sigma-Aldrich), and 10 ng/mL basic fibroblast growth factor (bFGF; Sigma-Aldrich and R&D Systems)]. Other growth factor rich medium which can be used as a reprogramming medium includes a medium such as mTeSR1 medium (from Stemcell Technologies), but the reprogramming media, to be maximally effective must contain some protein source in effective amounts.

Induced pluripotent cells are generated and propagated in tissue culture plates. Cells can be in suspension or adherent with or without any additional extracellular matrix or feeder layer. Optimally a matrix like Matrigel or other is used and is adherent to the matrix. Most optimal is use of a mouse embryonic fibroblast feeder layer, although the cells may be grown feeder free.

Cells are propagated to the desired cell number and express Avian pluripotent markers as otherwise described herein. At a minimum, cells should display a high nucleus to cytoplasmic ratio and large nucleoli. Cells should also express alkaline phosphatase and be

positive for the periodic acid shift assay. At a higher level of pluripotency, cells should be positive for the pluripotency markers Sox2, Nanog and PouV, possess high levels of telomerase whereas somatic cells are largely negative or low expressers of these genes and markers. Optimally pluripotent cells expression cell surface marker SSEA1.

Differentiation media are well known in the art and comprise at least a minimum essential medium plus one or more optional components such as growth factors, including fibroblast growth factor (FGF), ascorbic acid, glucose, non-essential amino acids, salts (including trace elements), glutamine, insulin (where indicated and not excluded), Activin A, transferrin, beta mercaptoethanol, and other agents well known in the art and as otherwise described herein. Preferred media includes basal cell media which contains between 1% and 20% (preferably, about 2-10%) fetal calf serum, or for defined medium (preferred) an absence of fetal calf serum and KSR, and optionally including bovine serum albumin (about 1-5%, preferably about 2%). Preferred differentiation medium is defined and is serum free. In certain embodiments wherein MMCs are produced and Activin A inhibitor is used, the medium may eliminate or substantially reduce the amount of Activin A.

Other agents which optionally may be added to differentiation medium according to the present invention include, for example, nicotinamide, members of TGF- β family, including TGF- β 1, 2, and 3, Activin A, nodal, serum albumin, members of the fibroblast growth factor (FGF) family, platelet-derived growth factor-AA, and -BB, platelet rich plasma, insulin growth factor (IGF-I, II, LR-IGF), growth differentiation factor (GDF-5, -6, -8, -10, 11), glucagon like peptide-I and II (GLP-I and II), GLP-1 and GLP-2 mimetobody, Exendin-4, parathyroid hormone, insulin, progesterone, aprotinin, hydrocortisone, ethanolamine, epidermal growth factor (EGF), gastrin I and II, copper chelators such as, for example, triethylene pentamine, forskolin, Na-Butyrate, betacellulin, ITS, noggin, neurite growth factor, nodal, valporic acid, trichostatin A, sodium butyrate, hepatocyte growth factor (HGF), sphingosine-1, VEGF, MG132 (EMD, CA), N2 and B27 supplements (Gibco, CA), steroid alkaloid such as, for example, cyclopamine (EMD, CA), keratinocyte growth factor (KGF),

Dickkopf protein family, bovine pituitary extract, islet neogenesis-associated protein (INGAP), Indian hedgehog, sonic hedgehog, proteasome inhibitors, notch pathway inhibitors, sonic hedgehog inhibitors, heregulin, or combinations thereof, among a number of other components. Each of these components, when included, are included in effective amounts.

By way of further example, suitable media may be made from the following components, such as, for example, Dulbecco's modified Eagle's medium (DMEM) with high glucose (Hyclone) with 10% fetal bovine serum (Hyclone), 4mM L-glutamine (Gibco) and 50U/ml penicillin and 50 mg/ml streptomycin, DMEM/F12 (Gibco), supplemented with 20% knockout serum replacement KSR (Gibco), 2mM L-glutamine (Gibco), 0.1 mM nonessential amino acids (Gibco), 50U/ml penicillin/50mg/ml streptomycin (Gibco), 0.1 mM b-mercaptoethanol (Sigma-Aldrich) and 10 ng/mL basic fibroblast growth factor (bFGF; Sigma Aldrich and R&D systems) as well as the following five (5) media, which utilized Matrigel as the substrate:

1. **20% KSR medium:** DMEM/F12 (Gibco), supplemented with 20% knockout serum replacement (KSR; Gibco), 2mM L-glutamine (Gibco), 0.1mM nonessential amino acids (Gibco), 50U/mL penicillin/50 mg/mL streptomycin (Gibco), 0.1mM b mercaptoethanol (Sigma-Aldrich), and 10 ng/mL basic fibroblast growth factor (bFGF; Sigma-Aldrich and R&D Systems)];

2. **TGFβ1/LIF medium:** DMEM/F12, supplemented with 20% KSR, 2mM L-glutamine, 0.1mM nonessential amino acids, 50U/mL penicillin/50 mg/mL streptomycin, 0.1mM b mercaptoethanol, 0.12ng/ml TGFβ1 (Pepro Tech), 1000 unites/ml LIF(Millipore).

3. **LIF/ Wnt3a medium:** DMEM/F12, supplemented with 20% KSR, 2mM L-glutamine, 0.1mM nonessential amino acids, 50U/mL penicillin/50 mg/mL streptomycin, 0.1mM b mercaptoethanol, 100ng/ml Wnt3a (R&D Systems), 1000 unites/ml LIF.

4. **2i/LIF medium:** DMEM/F12 supplemented with N2 (Gibco) and mix 1:1 with Neurobasal medium (Gibco) supplemented with B27 (Gibco), 1mM L-glutamine, 0.8μM

PD0325901(Sigma), 3 μ M CHIR99021 (Selleckchem), 20ng/ml LIF.

5. TGF β 1/activin A/nodal medium: DMEM/F12, supplemented with 20% KSR, 2mM L-glutamine, 0.1mM nonessential amino acids, 50U/mL penicillin/50 mg/mL streptomycin, 0.1mM β mercaptoethanol, 0.12ng/ml TGF β 1 (Pepro Tech), 10ng/ml Activin A (R& D Systems), 50ng/ml mouse recombinant nodal (R & D systems).

An alternative medium growing/culturing aiPSCs and for differentiating aiPSCs in the present invention (depending upon the components which are used) is DMEM/F12 (50:50) which contains about 2% proalbumin (albumin; Millipore/Serologicals), 1x Pen/Strep, 1x NEAA, 1x Trace Elements A,B, C (Mediatech), Ascorbic Acid (10-100 ng/ml, about 25-65 ng/ml, about 50 ng/ml), about 0.1mM (0.025-0.5mM) β -Mercaptoethanol (Gibco), about 2-10 ng/ml, about 5-9 ng/ml, about 8 ng/ml bFGF (Sigma), with additional components added depending upon the cells to which the aiPSCs are to be differentiated.

Various media which are useful in the present invention include commercially available media available from and can be supplemented with commercially available components, available from Invitrogen Corp. (GIBCO), Cell Applications, Inc. and Biological Industries, Beth HaEmek, Israel, among numerous other commercial sources, including Calbiochem. In preferred embodiments at least one differentiation agent such as fibroblast growth factor (FGF), LR-IGF (an analogue of insulin-like growth factor), Heregulin and optionally, VEGF (preferably all three in effective amounts) is added to the cell media in which a PSC is cultured and differentiated into a mature differentiated cell line. One of ordinary skill in the art will be able to readily modify the cell media to produce any one or more of the target cells pursuant to the present invention. Cell differentiation medium is essentially synonymous with basal cell medium but is used within the context of a differentiation process and includes cell differentiation agents to differentiate cells into other cells. Growth/stabilizing medium is a basal cell medium which is used either before or after a reprogramming or differentiation step in order to stabilize a cell line for further use.

Growth/stabilizing media refers to media in which a pluripotent or other cell line is grown or cultured prior to differentiation. In general, as used herein, the various cell media which are used may include essentially similar components of a basal cell medium, but are used within different contexts and may include slightly different components in order to effect the intended result of the use of the medium. In the case of a reprogramming medium, the inclusion of a protein in an effective amount is highly important.

As discussed, pluripotent stem cells may be cultured (preferably) on a layer of feeder cells that support the pluripotent stem cells in various ways which are described in the art. Alternatively, pluripotent stem cells may also be cultured in a culture system that is essentially free of feeder cells, but nonetheless supports proliferation of pluripotent stem cells. The growth of pluripotent stem cells in feeder-free culture without differentiation is often supported using a medium conditioned by culturing previously with another cell type. Alternatively, the growth of pluripotent stem cells in feeder-free culture without differentiation may be supported using a chemically defined medium. These approaches are well known in the art. In preferred aspects of the present invention, the cells are grown in the presence of feeder cells.

Approaches for culturing cells on a layer of feeder cells are well known in the art. For example, Reubinoff et al. (*Nature Biotechnology* 18: 399-404 (2000)) and Thompson et al. (*Science* 6 Nov. 1998: Vol. 282. no. 5391, pp. 1145-1147) disclose the culture of pluripotent stem cell lines from human blastocysts using a mouse embryonic fibroblast feeder cell layer, the teachings of which may be adapted for use in the present invention for aiPSCs. Approaches for culturing PSCs in media, especially feeder-free media, are well known in the art. U.S. Pat. No. 6,642,048 discloses media that support the growth of pluripotent stem (pPS) cells in feeder-free culture, and cell lines useful for production of such media, which can be readily adapted for use in the present invention. In another example, US20070010011 discloses a chemically defined culture medium for the maintenance of pluripotent stem cells, also adaptable for use in the present invention.

As discussed above, the cells, especially including the aiPSCs may be grown on a cellular support or matrix, as adherent monolayers, rather than as embryoid bodies or in suspension. In the present invention, the use of Matrigel as a cellular support is preferred. Cellular supports preferably comprise at least one reprogramming or substrate protein. The term “reprogramming protein” or “substrate protein” is used to describe a protein, including a matrix protein, which is used to grow and/or propagate cells and/or to promote reprogramming of a somatic cell into a pluripotent stem cell. Reprogramming proteins which may be used in the present invention include, for example, an extracellular matrix protein, which is a protein found in the extracellular matrix, such as laminin, tenascin, thrombospondin, and mixtures thereof, which exhibit growth promoting and contain domains with homology to epidermal growth factor (EGF) and exhibit growth promoting and differentiation activity. Other reprogramming proteins which may be used in the present invention include for example, collagen, fibronectin, vitronectin, polylysine, polyornithine and mixtures thereof. In addition, gels and other materials such as methylcellulose or other gels which contain effective concentrations of one or more of these reprogramming proteins may also be used. Exemplary reprogramming proteins or materials which include proteins which may be used to reprogram cells include, for example, BD Cell-Tak™ Cell and Tissue Adhesive, BD™ FIBROGEN Human Recombinant Collagen I, BD™ FIBROGEN Human Recombinant Collagen III, BD Matrigel™ Basement Membrane Matrix, BD Matrigel™ Basement Membrane Matrix High Concentration (HC), Growth Factor Reduced (GFR) BD Matrigel™, BD™ PuraMatrix™ Peptide Hydrogel, Collagen I, Collagen I High Concentration (HC), Collagen II (Bovine), Collagen III, Collagen IV, Collagen V, and Collagen VI, among others. The preferred material for use in the present invention includes Matrigel™.

Another composition/material which contains one or more proteins for use in reprogramming is BD Matrigel™ Basement Membrane Matrix. This is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumor rich in ECM proteins. Its major component is laminin, followed by collagen IV, heparan sulfate, proteoglycans, entactin and nidogen.

The induced pluripotent stem cells are generated and propagated in tissue culture plates. Cells can be in suspension or adherent with or without any additional extracellular matrix (e.g. Matrigel or other matrix protein as described above) or feeder layer. Preferably, a matrix like Matrigel or other as described above is used and is adherent to the matrix. In preferred aspects, use is made (in all instances, but preferably during reprogramming and/or propagation) of a mouse embryonic fibroblast feeder layer. The pluripotent stem cells may be plated onto the substrate in a suitable distribution and in the presence of a medium that promotes cell survival, propagation, and retention of the desirable characteristics. All these characteristics benefit from careful attention to the seeding distribution and can readily be determined by one of skill in the art.

Cells are reprogrammed and propagated to the desired cell number and express avian pluripotent biomarkers. At a minimum, cells should display a high nucleus to cytoplasmic ratio and large nucleoli. Cells should also express alkaline phosphatase and be positive for the periodic acid shift assay. At a higher level of pluripotency, cells should be positive for the pluripotency markers Sox2, Nanog and PouV, possess high levels of telomerase whereas somatic cells are largely negative or low expressers of these genes and markers. Optimally pluripotent cells express the cell surface marker SSEA1.

aiPSCs which are produced according to the present invention can be selected for certain in vitro traits such as expression of an added exogenous gene (transgene) or loss of a endogenous trait (knockout) which may be considered for the production of transgenic animals or a new trait such as resistance to cell death when exposed to a virus such as Newcastle virus as described in detail herein. A method of instilling resistance to a disease agent such as Newcastle disease virus or avian influenza represent an additional embodiment according to the present invention.

At any time the propagated reprogrammed cells can be re-exposed (reprogrammed) to reprogramming gene expression (DNA integrating or non integrating transient expression)

and selected again for pluripotent traits as described above, including optimally SSEA1 expression.

As used herein, the term “activate” refers to an increase in expression or upregulation of a marker such as or an upregulation of the activity of a marker associated with aiPSC, a chimeric cell or a differentiated cell including a neuron, muscle cell or a related cell as otherwise described herein (e.g., nanog, SSEA1, etc.). The term “deactivate” generally refers to a decrease in expression or down regulation of the activity of a marker associated with a cell.

As used herein, the term “isolated” when referring to a cell, cell line, cell culture or population of cells, refers to being substantially separated from the natural source of the cells such that the cell, cell line, cell culture, or population of cells are capable of being cultured *in vitro*. In addition, the term “isolating” is used to refer to the physical selection of one or more cells out of a group of two or more cells, wherein the cells are selected based on cell morphology and/or the expression of various markers. In the present invention, in aspects related to isolating full reprogrammed cells from non-fully reprogrammed cells, magnetic and florescence cell sorting have been found to significantly improve upon the ability to isolate fully reprogrammed selectively. Isolated cells typically express higher levels of pluripotency markers and behave more like pluripotent stem cells with respect to expandability.

As used herein, the term “express” refers to the transcription of a polynucleotide or translation of a polypeptide (including a marker) in or on the surface of a cell, such that levels of the molecule are measurably higher in or on a cell that expresses the molecule than they are in a cell that does not express the molecule. Methods to measure the expression of a molecule are well known to those of ordinary skill in the art, and include without limitation, Northern blotting, RT-PCT, *in situ* hybridization, Western blotting, and immunostaining.

As used herein, the term “markers” or “biomarkers” describe nucleic acid or polypeptide molecules that are differentially expressed in a cell of interest. In this context,

differential expression means an increased level for a positive marker and a decreased level for a negative marker. The detectable level of the marker nucleic acid or polypeptide is sufficiently higher or lower in the cells of interest compared to other cells, such that the cell of interest can be identified and distinguished from other cells using any of a variety of methods known in the art.

As used herein, the term "contacting" or "exposing" (i.e., contacting or exposing a cell with a vector, compound or disease agent, among others) is intended to include incubating the compound and the cell together *in vitro* (e.g., adding the vector or compound to cells in culture). The step of contacting the cell with reprogramming or differentiation medium and one or more growth proteins or other components such as reprogramming vectors as otherwise described herein can be conducted in any suitable manner. For example, the cells may be treated in adherent culture as an adherent layer, as embryoid bodies or in suspension culture, although the use of adherent layers may be preferred because they provide an efficient process oftentimes providing reprogramming to a target cell population of high relative purity (e.g. at least 50%, 60%, 70-75%, 80% or more). It is understood that the cells contacted with the reprogramming or differentiation agent may be further treated with other cell differentiation environments to stabilize the cells, or to differentiate the cells further.

As used herein, the term "differentiation agent" refers to any compound or molecule that induces a cell such as an aiPSC or other cell to partially or terminally differentiate. The term "differentiation agent" as used herein includes within its scope a natural or synthetic molecule or molecules which exhibit(s) similar biological activity.

The term "effective" is used to describe an amount of a component, compound or composition which is used or is included in context in an amount and/or for a period of time (including sequential times) sufficient to produce an intended effect. By way of example, an effective amount of a differentiation agent is that amount which, in combination with other components, in a differentiation medium for an appropriate period of time (including

sequential times when different differentiation agents are exposed to cells to be differentiated) will produce the differentiated cells desired.

Relevant Uses of the Present Invention

Agriculture/Veterinary: The present invention is broadly applicable given that the avian species are a widely used developmental research and agricultural species, but gene targeting studies have been limited given an absence of robust pluripotent stem cells that can serve as a vector for these changes. Birds supply about 25-30% of the animal protein consumed in the world. Recent publications in Science and PLoS One have highlighted a number of gene based strategies (siRNA, DNA and recombinant protein) to prevent the spread of Avian Influenza (4-6). Genetically distinct disease resistant birds would in theory not require vaccination and offer the people of underdeveloped and developed countries alike animals that require reduced veterinary care and increased food source safety. However, these significant advances have several short comings that can be potentially overcome using avian induced pluripotent stem cells including:

1) the need to continually use DNA or recombinant protein vaccination of birds which is not economically or logistically possible in many of the poorest villages in southeast Asia or Africa, which makes up the majority of the farming population in these countries;

2) the ability to generate birds that carry resistant genes and transfer resistance to offspring is limited;

3) the restricted ability to generate animals with multiple genetic modifications to create disease resistance at multiple viral stages or to even more than one disease.

Utilizing avian stem cells that can be genetically manipulated, clonally isolated, easily expanded over numerous passages and can form chimeric animals, can overcome these major challenges and provide an economically advantageous approach to advantageous phenotypic

modification of the animal.

Research Biomedical Applications: Avian embryonic models have a long history of providing critical new insights into developmental biology including organ function (13, 14), disease progression (e.g. Pompe disease) (15), eye disorders (16, 17) and many others (18, 19). The advantage that avian species have is their relative size and ease of access to the embryo for manipulation. Cells and tissues including whole sections of the spinal column can be transplanted into the avian embryo and can be monitored in real time during development (20). This is not possible in mammalian species. Moreover, the quail-chicken chimera is an attractive and widely used model for developmental patterning and cell fate studies given that cells can be readily tracked in this model (21, 22). The quail also has a short generation interval (3-4 generation per year) (23), facilitating genetic selection studies and experiments requiring multiple generational observations (24). Coupling a robust and clonal feeder free iPSC lines and derived committed cell lines or tissues with these model systems offers new opportunities to manipulate and study developmental process both in vitro and in vivo.

Vaccines: For decades, vaccines have provided effective protection from influenza for Americans. While they have traditionally been produced in chicken eggs, a new technology-cell-based vaccine production-could save hundreds of thousands of lives in the event of an outbreak of pandemic influenza, or some other infectious disease.

A new approach would use avian iPSC to grow the influenza viruses. Cell-based vaccine production could more easily meet "surge capacity needs" because iPSCs could be frozen and stored in advance of an epidemic or developed rapidly in response to an epidemic. Cell-based vaccine production dramatically reduces the possibility for contamination and promises to be more reliable, flexible, and expandable than egg-based methods. In place of eggs, cell-based vaccine production utilizes avian iPSC cell lines that are capable of hosting a growing virus in bioprocessing cell culture vessels. The virus is introduced into the cells where it multiplies produce a large amount of virus per iPSC. Generally the cells' outer walls are removed, harvested, purified, and inactivated. A vaccine can be produced in a matter of

weeks. While other cell lines are capable of generating a vaccine. The avian iPSC will produce high concentrations and thus be more economical and faster than mammalian cell lines.

Examples/Results

Quail Pluripotent Stem Cells

qiPSCs Display Morphological Characteristics Consistent with a Pluripotent Cell Type

The generation of qiPSCs was initiated by testing the lentiviral transduction efficiency of isolated (QEF) (Fig. 1A) with an eGFP reporter construct using both GeneJammer and TransDux transduction reagents. A 20 MOI transduction with Transdux resulted in the highest efficiency with 40.5% (GFP) positive cells (S. Fig. 1). QEFs were then transduced with the six human pluripotency genes *hPOU5F1*, *hNANOG*, *hSOX2*, *hLIN28*, *hC-MYC* and *hKLF4* driven by the elongation factor 1-alpha (EF1- α) promoter with each construct in individual lentiviral vectors. After 24hrs, cells were replated on feeder cells in stem cell expansion medium.

Colonies were observed beginning 6 days after transduction with irregular shaped borders and fibroblast-like cell morphology (Fig. 1B). These initial colonies failed to proliferate and expand indicating that these colonies were not fully reprogrammed. Potential qiPSCs were observed around 17 days after transduction and grew as compact colonies (Fig. 1C). The compact colonies were mechanically picked and initially replated on feeder plates in stem cell expansion medium. However, replated cells failed to proliferate and appeared apoptotic. Additional colonies were collected and replated on matrigel coated plates in mTeSR1 stem cell medium. This system supported the growth and expansion of colonies and subsequent qiPSC expansion was performed using this system.

Morphologically, qiPSC colonies were highly refractive and at the single cell level

showed clear cell borders, high nuclear to cytoplasm ratio and prominent nucleoli (Fig. 1D, 1E). qiPSCs were strongly positive for alkaline phosphatase (AP) and periodic acid Schiff staining (PAS; Fig. 1F, 1G). PCR and RT-PCR using human specific primers revealed that 5 out of 6 pluripotent stem cell factors, *hPOU5F1*, *hSOX2*, *hNANOG*, *hLIN28* and *hC-MYC*, were integrated and expressed in qiPSCs, while *hKLF4* was not present (Fig. 1H).]

qiPSCs are Highly Proliferative, Express Pluripotent Marks and are Capable of Clonal Expansion After Genetic Manipulation

Rapid proliferation and high levels of telomerase activity are hallmarks of pluripotent stem cells. To determine the doubling time, plated cells were quantified every 12 hours for 48 hr. The population doubling time of qiPSCs was 16.6 hr, much faster than the QEF parent cell line (36.9 hr, $P < 0.01$) (Fig. 2A). qiPSCs are highly proliferative and are passaged every 4 days. Cells have been maintained for more than 50 passages without loss of the pluripotent phenotype. Telomerase activity revealed a significant ($P < 0.01$) increase of >11 fold from 8.4 total product generated (TPG) in QEFs to 95.3 TPG in qiPSCs (Fig. 2B). Telomerase activity of qiPSCs was comparable to that of positive control HeLa cell line (50.8 TPG, $P = 0.07$), which indicates the immortality of qiPSCs. Moreover, these cells were capable of clonal expansion after genetic manipulation. qiPSCs were transduced with the eGFP gene resulting in a 36.1% GFP+ population (Fig. 2C-E). Single GFP+ cells were FACS sorted into each well of a 96-well plate and colonies were found in 2 wells 9 days after sorting (Fig. 2F, 2G). Flow cytometry analysis of cells expanded from one colony showed that >96% of the cells still expressed GFP after serial subculture (Fig. 2H).

Immunocytochemistry revealed that POU5F1 and SOX2 proteins were absent in QEFs (Fig. 3A, 3B, 3C), but positive in qiPSCs (Fig. 3D, 3E, 3F). Immunocytochemistry of qiPSCs showed cells were negative for the pluripotency markers SSEA4, TRA-1-81 or TRA-1-60 (data not show).

Embryoid Body Differentiation of qiPSC Results in the Formation 3 Germ Layers

To derive embryoid bodies (EBs), qiPSCs were plated in AggreWell plates for 24 hrs and then transferred to suspension culture in mTeSR1 medium for differentiation. Six days of suspension culture resulted in round and compact EBs from qiPSCs (Fig. 4A). EBs were collected for RNA isolation and RT-PCR or replated for additional differentiation for 2 days in stem cell expansion medium without bFGF- the removal of which will enable differentiation (Fig. 4B). Results of RT-PCR showed expression of *TUJ1* (ectoderm), *PAX6* (ectoderm), *Vimentin* (endoderm) and *Brychury* (mesoderm) in EBs, but not in qiPSCs or QEF cells (Fig. 4C). Immunocytochemistry showed cells positive for TUJ1 (ectoderm, Fig. 4D), SOX17 (endoderm, Fig. 4E) and alpha smooth muscle actin (α SMA, mesoderm, Fig. 4F) in plated EBs. These results indicated that qiPSCs could differentiate into various cell types from all three germ layers.

qiPSC Differentiate In Vitro Into Neuronal, Astrocytes and Oligodendrocytes

To derive neural cells, qiPSCs were subjected to a 3 step neural differentiation process. Cells were initially cultured in neural derivation medium for 12 days, proliferation medium for 7 days and differentiation medium continuously. Immunostaining showed that these cells were positive for neural proteins Hu C/D+ and MAP2+ (Fig. 5A, 5B) after 10 days of differentiation. A significant number of neurite extensions were observed after differentiation. Differentiated qiPSCs were found to be positive for the astrocyte and oligodendrocyte associated proteins GFAP and O4 after 23 and 39 days of differentiation, respectively, in neural differentiation medium (Fig. 5C, 5D). These data demonstrated the neural competence of qiPSC and differentiation into all 3 neural lineages, which is not seen in any previous avian stem cell lines.

Incorporation of qiPSCs Into Chimeric Embryo and Live Offspring

To generate qiPSC-chicken chimeras, GFP+ qiPSCs at passage 26 were injected into the subgerminal cavity of stage X embryos (Fig. 10). Embryos were incubated for 14 or 19 days and were then dissected to determine GFP+ qiPSC incorporation into embryos. GFP+

qiPSCs were incorporated in brain (Fig. 6A), eye (Fig. 6B), trachea/lung (Fig. 6C), heart (Fig. 6D) and yolk sac (Fig. 6F) tissues. PCR was performed for the human *POU5F1* gene used to reprogram QEFs into iPSCs to further determine qiPSC contribution in chimeric animals. qiPSCs were present in tissues from the ectoderm (brain, eye and skin), endoderm (intestine, liver and lung), mesoderm (muscle and heart), extraembryonic tissue (yolk sac) and the gonad (Fig. 6E and 6G, Fig. 9). PCR products from qiPSCs from the yolk sac and skin were sequenced to validate that PCR primers were solely expanding the human *POU5F1* sequence. Blast of the sequenced DNA amplified from these tissues showed identity of 99% to 100% for human *POU5F1* genomic context sequence NC_000006.11, but only 72% to 74% maximum identity for chicken *PouV* (Oct4 homologue) mRNA NM_001110178.1 and no significant similarity to its genomic context sequence NW_001471503.1. These results indicate that qiPSCs incorporated and contributed to chicken embryonic tissues from all 3 germ layers, extraembryonic tissues and potentially the germline.

An additional two rounds of injections were performed to generate chimeric chicks using passage 7 and passage 45 qiPSCs. Early passage qiPSCs were injected into 30 eggs. Three embryos developed to term and hatched after 21 days of incubation, while 18 embryos failed to hatch. Hatched offspring were not chimeric, but two chicks at late stages of development showed feather chimerism (Fig. 7A, 7B). Passage 45 qiPSCs were injected into 102 eggs, resulting in 47 live offspring. Two chicks died soon after hatching and the rest of 45 chicks were healthy, but no feather chimerism was observed. Brain, muscle, liver and gonad tissues were collected from 15 chickens for PCR detection of qiPSC incorporation. Results showed that brain, liver and gonad samples from two individuals were positive for the human *POU5F1* reprogramming gene (Fig. 7C). The presence of feather chimerism with early passage cells and incorporation of qiPSCs into tissue with later passages indicate that these cells retain pluripotent characteristics following long term culture and are still capable of contributing to multiple lineages following prolonged culture.

Discussion

Induced pluripotent stem cells have been generated from numerous mammalian species (1, 2, 5-10), but never before in a non-mammalian species. Here we report the first non-mammalian iPSCs and paradoxically these avian iPSC were generated using human reprogramming factors. Also a constitutively expressed GFP marker was introduced into qiPSCs and subclones were selected and expanded based on expression of GFP in a feeder free culture system. Therefore these qiPSC are amenable to future gene targeting, and exhibit a proliferative potential never before reported for any avian pluripotent cell lines (11, 25-27). The qiPSCs show morphology consistent with previously established pluripotent stem cells at the single cell level. The qiPSCs are highly positive for the stem cell markers AP, PAS, POU5F1 and SOX2 that have been previously used to characterize avian ESCs and PGCs (26, 28-31). These iPSCs are highly proliferative with a doubling time of 16.6 hr, similar to iPSCs from mouse (1) and pig (32), and have undergone over 50 passages. qiPSCs generated all three germ layer cells after spontaneous EB differentiation and then using a mammalian neural directed in vitro differentiation process we generated astrocytes, oligodendrocytes and neuronal cells. In the chick/quail chimera model, qiPSC contributed to fetal tissues from all 3 germ layers and extraembryonic tissues and ultimately contributed to tissues in live offspring. Although avian ESC and PGC have generated chimeric offspring (29, 33), the qiPSC differ because for the first time an avian stem cell exhibits the robust in vitro proliferative and clonal attributes needed for future gene targeted birds.

Quail and quail-chick chimeras have long been used in understanding the development of the neural system (34-37). With the benefit of this avian system, numerous facets of neural development in the brain (13, 34) and neural crest (35) have already been deciphered. In the present study, qiPSCs were found to significantly contribute to the brain and eye tissue when injected into stage X chicken embryos in an undifferentiated state. Upon proper signaling in vitro, qiPSCs could differentiate into a neural progenitor (TUJ1+) and all 3 lineages of neural cells: neuronal (Hu C/D+ and MAP2+), astrocyte (GFAP+) and oligodendrocyte (O4+) in vitro. This level of neural competence in vitro and in vivo has not

been reported in previous avian pluripotent cell lines (11, 12), indicating their potential use in neural development and disorder research (38-40). Given that we have derived neuronal, astrocyte and oligodendrocytes using processes first developed in human pluripotent cells (41), the repertoire of “tools” and translational potential for embryonic graft studies is greatly enhanced. Temporal and spatial studies investigating the role and interaction among neural cell types during embryo development is now possible. Pluripotent derived neural cells vs tissues isolated from fetal and adult tissues can be compared faster and in a relevant but comparatively simple models in contrast to mammalian systems (20).

The successful use of human reprogramming factors to generate avian iPSC suggests that direct reprogramming mechanisms are widely conserved among species. In the chicken genes cPouV and cNanog were found as key factors in the maintenance of chicken pluripotency (42). However, homology between avian and human is not obvious for the reprogramming genes with homology being relatively low when comparing chicken, the only avian species with sequence data, and humans with homology ranging from approximately 53 to 81% for POU5F1, NANOG, LIN28, C-MYC and KLF4. SOX2 is the lone exception with homology of 94% between chicken and human genes. Based on the diverse number of species (mouse, human, pig) from which cells have been reprogrammed using human factors and now a non-mammalian species, we hypothesize that the reprogramming process is highly conserved. Therefore, direct reprogramming with transcriptional factors could be a universal strategy for generating iPSC lines in distantly related species. This would provide new species iPSCs for divergent species including species where embryonic stem cells are hard to isolate, maintain and expand, which arguably would include all species other than those of primates and some rodents.

The methods used here for qiPSCs overcome impediments inherent to avian ESC and PGC. Previously quail or chicken PGCs (31, 33) and ESCs (29) contributed to chimeras when injected into embryos immediately after collection from the donor embryo or after only a few passages. After serial subculture in this system, qiPSC still efficiently incorporated into tissues from all three germ layer in chimeric embryos at passages 26 and 45. This will further

enable complex genetic manipulations like homologous recombination, multiple gene introductions, drug selection and other strategies that may require extended culture. This significantly increases the value of these cells for future developmental studies. In addition, cultures beyond 50 passages that maintain a short doubling times and a pluripotency phenotype have not been previously reported. Beyond long term culture, qiPSCs demonstrated the capability of clonal expansion after isolating individual cells in a 96 well format providing the possibility of targeting genes of interest. Little is known about the maintenance of pluripotency and expansion of avian pluripotent cells therefore we used methods based on the significant body of knowledge for mammalian pluripotent cells (43-47). The feeder free culture system that supported qiPSC cultures for more than 50 passages without loss of the pluripotent phenotype was developed for human pluripotent cells and contains high levels of bFGF (48). In the future this system can be used to investigate individual factors and their role in maintaining pluripotency of avian stem cells. Using qiPSC gene targeted avian models will compliment important rodent models in disease and developmental gene function studies (49).

The present invention is directed to avian iPSCs which will greatly facilitate the insertion of genetic reporters and gene targeting. Future studies generating cells with gene specific and multiple promoters, inducers and conditional expression systems in avian iPSCs is likely feasible; thus enhancing the research communities' capabilities when it comes to investigating cell migration and contribution in developing embryos in ova (50). Since these qiPSC and ciPSC were capable of clonal expansion after genetic modification, targeting genes of interest is potentially possible, which would facilitate research on gene function and signaling pathways underlying the development process in chimeric embryo. Furthermore, avian iPSC derivatives such as neural cells should compliment mammalian cell transplant models for regenerative medicine (51). In total, this unique source of avian iPSC and derivative cells will provide biologist with multiple opportunities to enhance and expedite developmentally related discoveries.

Materials and Methods for Quail Pluripotent Stem Cells

Cell culture and Transduction

QEFs were isolated from day 11 embryos and cultured in fibroblast medium (DMEM high glucose (Hyclone) with 10% FBS (Hyclone), 4mM L-Glutamine (Gibco) and 50U/ml penicillin and 50 µg/ml streptomycin (Gibco)) in 5% CO₂ at 37°C. Cells were split using 0.05% trypsin (Gibco) upon confluency. For transduction, a total of 150,000 QEF cells were plated in one well of a 12-well plate. After 24hrs QEFs underwent lentiviral transduction utilizing the viPS kit (Thermo Scientific) with viruses containing the human stem cell genes *POU5F1*, *NANOG*, *SOX2*, *LIN28*, *KLF4* and *C-MYC* under the promoter of human elongation factor-1 alpha (EF1α). Transduction was performed in the presence of 1X TransDux (System Biosciences). QEFs were trypsinized 24 hrs after transduction and passaged onto inactivated feeder cells in embryonic stem cell expansion medium (Dulbecco's modified Eagle medium (DMEM)/F12 (Gibco), supplemented with 20% knockout serum replacement (KSR; Gibco), 2 mM L-glutamine (Gibco), 0.1 mM non-essential amino acids (Gibco), 50 U/ml penicillin/50 µg/ml streptomycin (Gibco), 0.1mM β mercaptoethanol (Sigma-Aldrich) and 10 ng/ml bFGF (Sigma-Aldrich and R&D Systems). qiPSC was manually harvested and plated on Matrigel (BD Biosciences; diluted 1:100 in DMEM/F12) coated dishes in mTeSR1 (Stemcell Technologies) medium. qiPSCs were mechanically dissociated using glass Pasteur pipette every 4 to 5 days. For clonal expansion, qiPSCs were transduced with GFP viral vector and single cells were FACS sorted into individual wells of a 96-well plate.

Alkaline Phosphatase and Periodic Schiff's Acid Staining

AP staining was carried out with VECTOR Red Alkaline Phosphatase Substrate Kit (Vector Laboratories) according to the manufacturer's instructions. PAS staining was performed by 4% fixation for 5 min. PAS (Sigma-Aldrich) was added to the plate and incubated at room temperature (RT) for 5 min, followed by PBS washes. Schiff's reagent (Sigma-Aldrich) was added and incubated at RT for 15 min, followed by PBS washes and then observation.

Immunocytochemistry

Protocol for immunostaining followed the methods previous reported (6). Primary antibodies used were POU5F1 (R&D Systems), SOX-2 (R&D Systems), Tuj1 (Neuromics), α SMA (Santa Cruz), Sox17 (Santa Cruz), SSEA4 (Developmental Studies Hybridoma Bank), TRA-1-60 (Chemicon), TRA-1-81 (Chemicon), Hu C/D+ (Invitrogen), MAP2+ (Millipore), GFAP (Chemicon) and O4 (Chemicon).

Proliferation and Telomerase Activity

Proliferation assay was performed by manual counts ($n=3$) at 12, 24, 36 and 48 hrs after plating. Population doubling time was determined using an exponential regression curve fitting (<http://www.doubling-time.com/compute.php>). Telomerase activity of QEFs, qiPSCs and HeLa cells (positive control) was determined using TRAPeze XL Telomerase Detection Kit (Millipore) following manufacturer's instructions. Statistics analysis was done utilizing ANOVA and Tukey pair-wise comparisons between each population with p -value < 0.05 being considered significant.

Embryoid Body Formation and Differentiation

Embryoid bodies (EBs) were formed by plating 2.0×10^6 qiPSCs in mTeSR1 medium and 0.1 mM Y-27632 ROCK inhibitor (Calbiochem) in AggreWell plate (Stemcell Technologies). After 24 hrs, aggregates were harvested and maintained in mTeSR1 medium for 7 days. Differentiation was assessed by RT-PCR using the primers in S. Table 2.

Neural Differentiation

To induce neural differentiation qiPSCs were sequentially cultured in neural derivation medium (DMEM/F12 supplemented with 200mM L-Glut, 4ng/ml bFGF and 1x N2) for 12 days, proliferation medium (AB Medium supplement with 200mM L-Glut, 1x ANS and 20ng/mL bFGF) for 7 days and then in differentiation (AB Medium supplement with 200mM L-Glut, 1x ANS and 10ng/mL LIF) mediums continuously.

Production of Chimera

Stage X white leghorn chicken embryos were used to produce chimeras. Egg shells were removed by Dremel rotary tool to make injection window (Figure 9A). qiPSCs were introduced into the subgerminal cavity using a glass micropipette (Figure 9B) with pressure controlled microinjector (Parker Automation). The window was sealed by hot glue (Figure 9C) after injection and eggs were incubated at 37°C.

RNA isolation, DNA isolation, PCR and Sequencing

RNA was isolated using RNeasy QIAprep Spin miniprep Kit (Qiagen) per manufacture's instructions. mRNA extractions were transcribed into cDNA using iScript cDNA Synthesis kit (Bio-Rad Laboratories). DNA was isolated using DNeasy kit (Qiagen) following the manufacturer's instruction. All DNA samples were subjected to RNase treatment to eliminate the RNA contamination. Primers used in PCR and RT-PCR are listed in attached Figure 8. Sequencing verification of hPOU5F1 was performed by extracting DNA from agarose gel after electrophoresis and sequenced. The product was compared by Blast in NCBI data base to both human and chicken genomes.

Table 1 Incorporation of qiPSCs in chicken chimeric embryonic tissues

Tissue		No. of Samples Subjected to PCR	No. of Samples Positive For <i>hPOU5F1</i>
Ectoderm	Brain	18	9
	Eye	18	7
	Skin	17	5
Endoderm	Intestine	18	4
	Liver	16	5
	Lung	18	7
	Trachea	6	3
Mesoderm	Muscle	18	4
	Heart	18	3
	Gonad	10	5
	Kidney	7	4
	Gizzard	8	1
Extra Embryonic	Yolk sac	1	1

Table 2 Sequence of primers used in the study

	Primer	Sequence	Product (bp)
hPOU5F1	Forward	GAGAAGGAGAAGCTGGAGCA	453
	Reverse	TCGGACCACATCCTTCTCG	
hSOX2	Forward	CCCCTGTGGTTACCTCTTCCTCC	177
	Reverse	TGCCGTTAATGGCCGTGCC	
hNANOG	Forward	CTATGCCTGTGATTTGTGGG	160
	Reverse	GGTTGTTTGCCTTTGGGAC	
hLIN28	Forward	GGCTCCGTGTCCAACCA	281
	Reverse	AACTCCACTGCCTCACCCCT	
hC-MYC	Forward	GTTTCATCTGCGACCCG	480
	Reverse	CAGGAGCCTGCCTCTTTT	
hKLF4	Forward	GGCTGATGGGCAAGTTCG	395
	Reverse	CTGATCGGGCAGGAAGGAT	
qGAPDH	Forward	TGCCCAGAACATCATCCCA	295
	Reverse	GCCAGCACCCGCATCAAAG	
hGAPDH	Forward	GAGTCAACGGATTTGGTCGT	283
	Reverse	TTGATTTTGGAGGGATCTCA	
Vimentin	Forward	GTCTGGATACTCGCAGTTAGG	175
	Reverse	GGTGTAGGGATTGGGGTAG	
Brachyury	Forward	TCTGGATACTCGCAGTTAGGT	365
	Reverse	ATGGTGCTGTTACTCACGGAC	
PAX6	Forward	CAGAAGATCGTGGAACCTCGC	280
	Reverse	CACTGGGTATGTTATCGTTGGTA	
TUJ1	Forward	CAGCGATGAGCATGGCATAGAC	576
	Reverse	CGGAAGCAGATGTCGTACAGG	

Chicken Pluripotent Stem Cells

The generation of integrated virus induced pluripotent cells using chicken cells was unique and unexpected because previously used key reprogramming factors was not required

In previous reports, iPS cell lines were derived by using all 6 factors of OCT4, SOX2, NANOG, LIN28, KLF4 and CMYC. In the present invention, as set forth herein, we find that exogenous expression of NANOG, LIN28 and CMYC were sufficient to reprogram the chicken fibroblast cell into pluripotent status, the first time demonstrating OCT4 is dispensable in iPSC derivation. Moreover, these ciPSC could be cultured on feeder free

system, on plates coated with Matrigel or directly on plate without any matrix. This feature would reduce the cost of cell culture and facilitate the cell screening or vaccine production where large amount of culture are required.

Materials and Methods

Chicken embryonic fibroblast culture

Black australorp chickens embryonic fibroblast cells (CEFs) were isolated from a day 11 embryo. CEFs were then cultured in DMEM media (Hyclone) supplemented with 10% FBS (Hyclone), 4mM L-Glutamine (Gibco) and 50U/ml penicillin and 50 µg/ml streptomycin (Gibco) and incubated in 5% CO₂ and at 37°C, with media changes every other day. Cells were subcultured using 0.05% trypsin (Gibco) upon confluency.

ciPSC derivation

A total of 150,000 CEF cells were plated in one well in a 12-well plate. After 24hrs CEFs underwent lentiviral transduction utilizing the viPS kit (Thermo Scientific) with viruses containing the human stem cell genes POU5F1, NANOG, SOX2, LIN28, KLF4 and C-MYC under the promoter of human elongation factor-1 alpha (EF1α). Transduction was carried out at a multiplicity of infection (MOI) of 10 in the presence of 1X TransDux (System Biosciences). The transduced CEFs were replated onto inactivated mouse embryonic fibroblast (MEF), fed with conditioned embryonic stem cell (ESC) expansion media (Dulbecco's modified Eagle medium (DMEM)/F12 (Gibco), supplemented with 20% knockout serum replacement (KSR; Gibco), 2 mM L-glutamine (Gibco), 0.1 mM non-essential amino acids (Gibco), 50 U/ml penicillin/50 µg/ml streptomycin (Gibco), 0.1mM β mercaptoethanol (Sigma-Aldrich) and 10 ng/ml FGF-2 (Sigma-Aldrich and R&D Systems). Conditioning of the medium was done by incubating the medium on MEF for 24 hours. Upon 50% confluent, the transduced CEFs were dissociated using 0.05% trypsin and replated on fresh MEF plates. Attrition of incorrectly reprogrammed cells was performed by serial trypsin passages until the majority of the colonies were iPSC-like cells. At the end of the

attrition, the ciPSCs were manually passaged onto tissue culture plate coated with Matrigel in ESC medium. The stable ciPSC cells were culture on plate coated with Matrigel or without any matrix and passaged every 3-4 days using accutase.

Alkaline Phosphatase and Periodic Schiff's Acid Staining

Following the protocol used in derivation of quail iPSC as described for the quail experiments described above.

Immunocytochemistry

Follow the same protocol as in quail iPSC, described above..

The primary antibody used in this experiment were SSEA1 (1:20; Developmental Studies Hybridoma Bank), DDX4 (1:500, Abcam). Secondary antibodies were Alexa Fluor (Invitrogen) diluted at 1:1000.

DNA, RNA isolation and PCR

DNA was isolated from CEFs and ciPSCs using DNeasy kit (Qiagen) following the manufacturer's instruction. Human specific primers were use to analyze the incorporation of transgenes in ciPSCs.

RNA was isolated from CEFs and ciPSC using RNeasy QIAprep Spin miniprep Kit (Qiagen) following manufacturer's instructions and then transcribed into cDNA using iScript cDNA Synthesis kit (Bio-Rad Laboratories). Quantitative PCR was performed to analyze the chicken endogenous pluripotent genes expression using validated primers from Applied Biosystem.

Embryoid Body Formation and Differentiation

Embryoid bodies (EBs) were formed follow the same protocol as in quail iPSC as

described in the experiments described above.. The EBs were harvested on day 7 and RNA was isolated to for RT-PCR to check the expression of genes from different lineages.

Results

Derivation of ciPSC

CEFs were cultured in modified DMEM (Fig. 10A) were transduced with 6 human stem cell factors (hOCT4, hSOX2, hNANOG, hLIN28, hKLF4 and hCMYC) constructed in lentiviral vectors. The trasnduced CEFs were replated onto inactivated MEF plate 24hours after transduction and feed with conditioned ESC medium. Obvious colonies were observed 5 days after transduction (Fig. 10B). The ciPSC-like cells demonstrated typical stem cell characteristics as large nuclear and prominent nucleoli (Fig. 10C). A pure population of ciPSCs were derived by serial passages 50% confluence (Fig. 10D) using 0.05% trypsin and finally repated on Matrgel coated plate when the majority of the colonies demonstrating typical stem cell characteristics. The stable ciPSC were cultured in on feeder free system, on culture dish coated with Matrigel or without any matrix. The ciPSCs maintained in feeder free system are highly positive in AP (Fig. 10F) and PAS (Fig. 10G) staining. And some of the cells are positive for SSEA1 (Fig. 10H while all are positive for DDX4 (Fig. 10I).

ciPSCs express pluripotent genes

PCR using human specific primers revealed that, of the 6 human transcription factors that we used to derive the ciPSC, only hNANOG, hLIN28 and hC-MYC were incorporated in ciPSC BA3 and BA4 cell lines (Fig.11A). The quantitative PCR demonstrated that the endogenous OCT4, SOX2 were highly up regulated in ciPSCs comparing to the CEFs (Fig. 11-C), indicating the activation of the endogenous stem cell regulatory network. However, we found that the chicken C-MYC was expressed at a similar level in CEFs and ciPSC. The over expression of the exogenous C-MYC might be responsible for the maintenance of the pluripotent thus endogenous expression is dispensible. The telomerase reverse transcriptase (cTERT) gene was significantly up regulated in ciPSC (Fig. 11E), confirming that these cells

regained the immortality after reprogramming. And the chicken vasa homologue (cVH), a germ cell specified gene, was also expressed in high level in ciPSC (Fig. 11E). The expression of cVH was consistent with results of immunostaining, indicating these cells have the germ cell potential.

Differentiation of ciPSCs through embryoid body

Differentiation of ciPSCs was performed through embryoid body formation using AggreWell system. Around 2.0×10^6 cells were plated in one well in the AggreWell plate thus yielded 1,000 cells per aggregate. The aggregates were cultured in ESC medium for 24 hours and then transfer to a 100 mm plate for suspension culture in ESC medium without bFGF for 7 days. Compact EBs were formed at the end of the culture (Fig. 12A) and RNA was isolated from the EBs. The results of RT-PCR showed the gene expression of ectoderm (NESTIN, TUJ1), mesoderm (GLUT1, LPL, PPAR γ) and endoderm (HNF1 α , HNF4 α) was found in the EBs derived cells.

ciPSCs derived in this study are unique because conducting a 2nd round of reprogramming can yield more pluripotent cells (SSEA1) after selection for NDV resistant

Generation of disease resistant animals has been a major goal of the breeding programs using state-of-art biotechnologies and chimeric animal production using disease resistant pluripotent stem cells is a promising strategy for this purpose. However, the serial cell screening or genetic manipulation *in vitro usually* results in reduction of the pluripotency and thus fails to produce chimeric animals. The ciPSC pursuant to the present invention is easy to maintain in feeder free system and were used in serial screening to establish Newcastle Disease Virus screening (NDV) cell line. Following a 2nd round of reprogramming, the resistant ciPSCs demonstrated a more pluripotent state as indicating by

higher percentage of cells are positive for SSEA1 and other markers, and these markers were previously reported to be required in pluripotent cells that would produce chimeric chicken.

Materials and Methods

NDV screening

CEF and ciPSCs were plated in Matrigel coated 6-well plate at a density of 1×10^6 cells per well. NDV infection was performed 24 hours after plating at an MOI of 50. First, the medium from the culture is removed and washed with PBS once before infection. Second, the virus in ESC medium containing 1 % KSR for ciPSCs or in DMEM medium containing 1% FBS for CEFs. Add the infection solution to cells and incubate at 39°C in 5% CO₂ for 1 hour. Finally, remove the infection solution at the end of the incubation and add fresh ESC medium and return the cells to incubator. Cell viability was assayed 48 hours after the NDV infection. The survived cells were subjected to serial rounds of virus challenging with or without passaging.

2nd round reprogramming

The transduction protocol used in the 2nd round reprogramming is similar to the 1st round. ciPSCs are plated on Matrigel coated plate in ESC medium. Cells were transduced with 6 h human transcription factors (POU5F1, NANOG, SOX2, LIN28, KLF4 and C-MYC) constructed in lentiviral vectors at an MOI of 10 in the presence of 1X TransDux (System Biosciences). Transduction medium was removed 24 hours after transduction and fresh ESC medium was added and changed every other day. The reprogrammed ciPSCs were maintained and expanded in feeder free system and purification of the more-pluripotent ciPSCs were performed by MACS sorting using magnetic beads conjugated with anti-SSEA1 antibody (Miltenyi Biotec). The purified cells were cultured on MEF feeder for better maintaining of the pluripotency.

Results

Derivation of NDV resistant ciPSCs

NDV used in this study was genetically modified and labeled with GFP thus the infection could be tracked by fluorescence from the infected cells. Result of flow cytometry showed an infection efficiency of >99% in either CEFs (Fig. 13E) or ciPSCs (M). There was significantly cell death in CEFs and ciPSCs 48 hours after the first round of NDV infection (Fig. 13C, 13G and 13K, 13O). The survived ciPSCs recovered from the infection and formed obvious colonies around 9 days after the infection, with significant number of cells negative for GFP (Fig. 13L, 13P), while the CEFs remained infected and slowly proliferate or even stopped growing (Fig. 13D, 13H).

A total of 12 rounds of infection were carried to achieve a stable ciPSC line resistant to NDV. The established ciPSC lines showed significantly higher resistance to NDV upon infection comparing to CEFs or original ciPSCs never subjected to NDV infection (Fig. 14).

More pluripotent ciPSCs was derived by a 2nd round of reprogramming

The ciPSCs resistant to NDV were subjected to 2nd of reprogramming by transduction of human stem cell factors constructed in lentiviral vectors and transduced were expanded on feeder free culture system. Cells positive for OCT4, SOX2, NANOG and SSEA1 were found 7 days after transduction (Fig. 6A), this pluripotent state was not seen in the 1st round of reprogramming. We then enriched the SSEA1⁺ cells by MACS sorting and yielded a purity >82% SSEA1⁺ cells. The stable SSEA1⁺ ciPSCs were finally plated on MEF layer for a better maintaining of the pluripotency.

EXAMPLE

Chicken induced pluripotent stem cells using a non integrating system.

The benefits of non integrating systems are the following: The molecular basis of reprogramming has been revealed by exogenous expression of combinations of transcription factors described in prior examples for quail and chicken, described above. Induction of reprogramming was carried by these factors is mostly carried out by co-infection with retroviral or lentiviral vectors. The main problems of the retrovirus-based method are oncogenicity and mutagenesis. Chimeric mice derived from iPSCs as well as their offspring developed tumors, probably because of reactivation of the proviral *cMyc* oncogene. Even though three-factor (*Oct4*, *Sox2*, and *Klf4*) iPSC-derived animals did not develop tumors, ectopic expression of any one of these genes may have deleterious consequences. For instance, ectopic expression of *Oct4* in skin and intestine causes tumor development. Overexpression of *Klf4* induces dysplasia in skin.

A non integrating system potentially avoids this pitfall and we have used a commercially available system Minicircle DNA (System Biosciences) to generate these types of cells in chickens.

Materials and Methods

Cell culture and transfection

Chicken embryonic fibroblast (CEFs) from black australorp chickens were isolated from day-11 embryos and cultured in fibroblast medium [Dulbecco's modified Eagle's medium (DMEM) high glucose (Hyclone) with 10% fetal bovine serum (Hyclone), 4mM Lglutamine(Gibco), and 50U/mL penicillin and 50 mg/ml streptomycin (Gibco) in 5% CO₂ at 37°C. Cells were trypsinized and passaged using 0.05% trypsin (Gibco) upon reaching confluence. For transfection, a total of 1x10⁶ CEF cells were plated in a 35 dish. After 24 hrs, CEF cells were transfected with Minicircle DNA (System Biosciences) containing the four reprogramming factors *POU5F1*, *SOX2*, *LIN28*, *NANOG* and the green fluorescent protein (GFP) reporter gene all driven by

the cytomegalovirus promoter using four types of transfection reagents including lipofectamine (Invitrogen), Xfect (Clontech), purefection (System Biosciences) and genejammer (Agilent Technologies). 5 µg of minicircle DNA was diluted in 250ul DMEM/F12 and mixed with transfection reagent per transfection reagent manufacturer instructions. The mixture was incubated at room temperature for 20 min. The mixture was added to the CEF drop wise. After 24 hrs, the transfection reagent mixture was removed and replaced with fresh medium. The CEF cells were transfected a total of 3 times in this manner every other day over a 5 day period in. At day7, the CEFs were trypsinized and plated onto inactivated mouse embryonic fibroblast feeder cells in 20%KSR stem cell medium [DMEM/F12 (Gibco), supplemented with 20% knockout serum replacement (KSR; Gibco), 2mM L-glutamine (Gibco), 0.1mM nonessential amino acids (Gibco), 50U/mL penicillin/50 mg/mL streptomycin (Gibco), 0.1mM b mercaptoethanol (Sigma-Aldrich), and 10 ng/mL basic fibroblast growth factor (bFGF; Sigma-Aldrich and R&D Systems)]. To assess transfection efficiency, a subset of cells before plating in stem cell conditions from each transfection reagent treatment at day 6 was analyzed by flow cytometry for GFP expression.

ciPSCs were maintained on feeders and were mechanically dissociated using a glass pasteur pipette or passaged using 0.05% trypsin every 4–5 days. After 10 passages on feeder, the colonies were picked up and dissociated in 0.05% trypsin into single cells, and ciPSCs were directly passaged into feeder free conditions on Matrigel (BD Biosciences; diluted 1:100 in DMEM/F12) coated dishes in 20% KSR plus 10ng/ml bFGF.

Flow Cytometry

Cells were fixed in 4% paraformaldehyde (PFA) for 15 min at room temperature. Cells were washed 3 times in phosphate-buffered saline (PBS; Hyclone) without calcium and without magnesium (-/-) and were blocked in 6% fetal bovine serum (FBS) 94% PBS blocking solution for 45min. SSEA-1 (1:200; Developmental Studies Hybridoma Bank) primary antibody was added and cells were incubated for 1 hour. Cells were washed 3 times with blocking solution. Primary antibody was detected using fluorescently conjugated

secondary antibody Alexa Fluor 488 (1:1000; Invitrogen). Cells were analyzed using a DakoCytomation Cyan (DakoCytomation) and FlowJo Cytometry analysis software (Tree Star).

Alkaline phosphatase and periodic acid Schiff's staining

Following the protocol used in derivation of quail iPSC as described for the quail experiments described above.

Immunocytochemistry

Cells were passaged onto Matrigel coated slides. Cells were washed with PBS+/+ and fixed with 4% PFA at room temperature for 15 min. Fixed cells were blocked in 6% FBS 94% PBS+/+ blocking solution for 45 min. Cells were incubated with primary antibodies for 1 hour. Primary antibodies used were POU5F1 (1:500; Santa Cruz), SOX2 (1:200; R & D Systems), NANOG (1:200; Millipore), β III-Tubulin (1:200; Neuromics), Brachyury (1:200; Santa Cruz), Vimentin (1:100; BD Pharmingen), SSEA-1 (1:200; Developmental Studies Hybridoma Bank), SSEA-4 (1:200; Developmental Studies Hybridoma Bank), TRA-1-60 (1:200; Chemicon), TRA-1-81 (1:200; Chemicon). Primary antibodies were detected using a fluorescently conjugated secondary antibody, Alexa Fluor 488 (Molecular Probes, 1:500) and 594 (Molecular Probes, 1:500). Cells were observed and images were captured on the Ix81 microscope with Disc-Spinning Unit (Olympus) using Slide Book Software (Intelligent Imaging Innovations).

Proliferation and Telomerase Activity

ciPS cells were plated on 6-well plates at day 1 with 1×10^5 cells per well. Proliferation assay was performed by manual counts ($n=3$) at 12, 24, 36 and 48 hours after plating. Population doubling time was determined using an exponential regression curve fitting (website at doubling-time.com/compute.php).

Telomerase activity of CEFs, ciPSCs, WA09 human embryonic stem cells (hESCs) and Hela cells (positive control) were determined using TRAPeze XL Telomerase Detection Kit (Millipore) following the manufacturer's instructions. Statistical analysis was done utilizing ANOVA and Tukey pair-wise comparisons between each population with p-values < 0.05 being considered significant.

Embryoid Body Formation and Differentiation

Embryoid bodies (EBs) were formed by plating 3.6×10^6 ciPSCs in 20% KSR medium and 0.1 mM Y-27623 ROCK inhibitor (Calbiochem) in an AggreWell plate (Stemcell Technologies). After 24 hours, cell aggregates were harvested and cultured in differentiation medium [DMEM/F12 (Gibco), supplemented with 20% fetal bovine serum (FBS; Hyclone), 2mM L-glutamine (Gibco), 0.1mM nonessential amino acids (Gibco), 50U/mL penicillin/50 mg/mL streptomycin (Gibco), 0.1mM β -mercaptoethanol (Sigma-Aldrich)] for 10 days. Differentiation was assessed by RT-PCR using the primers in Table 1 to assess the differentiation by immunostaining. EBs were replated in 4 well chamber slides and differentiate further for 4 additional days in differentiation medium.

Re-transfection and medium comparison

To compare several established stem cell culture system in maintaining ciPSCs pluripotency, 1×10^6 cells were plated on matrigel coated 35mm dish and re-transfected with Minicircle DNA as before and were passaged directly into 1 of 5 feeder free culture systems. The medium components of the five culture system were as follows:

Group 1 20% KSR medium: DMEM/F12 (Gibco), supplemented with 20% knockout serum replacement (KSR; Gibco), 2mM L-glutamine (Gibco), 0.1mM nonessential amino acids (Gibco), 50U/mL penicillin/50 mg/mL streptomycin (Gibco), 0.1mM β -mercaptoethanol (Sigma-Aldrich), and 10 ng/mL basic fibroblast growth factor (bFGF; Sigma-Aldrich and R&D Systems)];

Group 2 TGF β 1/LIF medium: DMEM/F12, supplemented with 20% KSR, 2mM L-glutamine, 0.1mM nonessential amino acids, 50U/mL penicillin/50 mg/mL streptomycin,

0.1mM β-mercaptoethanol, 0.12ng/ml TGFβ1 (Pepro Tech), 1000 unites/ml LIF(Millipore).

Group 3 LIF/ Wnt3a medium: DMEM/F12, supplemented with 20% KSR, 2mM L-glutamine, 0.1mM nonessential amino acids, 50U/mL penicillin/50 mg/mL streptomycin, 0.1mM β-mercaptoethanol, 100ng/ml Wnt3a (R&D Systems), 1000 unites/ml LIF.

Group 4 2i/LIF medium: DMEM/F12 supplemented with N2 (Gibco) and mix 1:1 with Neurobasal medium (Gibco) supplemented with B27 (Gibco), 1mM L-glutamine, 0.8μM PD0325901(Sigma), 3μM CHIR99021 (Selleckchem), 20ng/ml LIF.

Group 5 TGFβ1/activin A/nodal medium: DMEM/F12, supplemented with 20% KSR, 2mM L-glutamine, 0.1mM nonessential amino acids, 50U/mL penicillin/50 mg/mL streptomycin, 0.1mM β-mercaptoethanol, 0.12ng/ml TGFβ1 (Pepro Tech), 10ng/ml Activin A (R& D Systems), 50ng/ml mouse recombinant nodal (R & D systems).

All systems used Matrigel as the substrate.

RNA isolation, DNA isolation, PCR

RNA was isolated using RNeasy QIAprep Spin miniprep Kit (Qiagen) according to manufacturer's instructions. Genomic DNA was removed using gDNA eliminator columns (Qiagen). The RNA quality and quantity was determined using the NanoDrop 8000 (Thermo Scientific). Total mRNA (500ng) extractions were reverse transcribed into cDNA using iScript cDNA Synthesis Kit (Bio-Rad Laboratories). PCR amplification was performed using GoTaq Green master mix (Promega). Primers used in RT-PCR are listed in Table 2A. PCR reactions were performed by initially denaturing cDNA at 95°C for 3 min followed by 30 cycles of denaturing at 95°C for 30 sec, annealing at 60°C for 30 sec, polymerization at 72°C for 30 sec and a final 10-min extension at 72°C. PCR products were loaded into 2% agarose gels containing 0.6 μg/mL ethidium bromide and run in Tris-acetate-ethylenediaminetetraacetic acid buffer for 45 min. The Alpha Innotech gel documentation station was used to observe PCR products.

Chimera Production

Stage-X White Leghorn chicken embryos were used to produce chimeras. Small injection windows were drilled into injection egg shells using a Dremel rotary tool. ciPSCs from black australorp chickens were transduced with Turbo-GFP Lentiviral Vector (Thermo Scientific Open Biosystems) before injection according to the manufacturer's instructions. ciPSCs were injected into the subgerminal cavity using a glass micropipette with pressure controlled microinjector (Parker Automation). Each embryo was injected with 10,000 cells. The window was sealed by using a hot glue gun after injection and eggs were incubated at 37.8 °C.

Eggs were opened and dissected at day 5 and day 10 to determine if GFP positive ciPSCs successfully incorporated into chick embryos or allowed to hatch day 22. Hatched chicks were checked for feather chimerism (ciPSCs were derived from black australorp chickens, chicks from this breed have black down feathering at hatch enabling tracing of these ciPSC derived from this breed if transplanted into breeds that produce yellow down chicks such as White Leghorn).

DNA isolation , PCR, and sequencing

Chicks were sacrificed and brain, liver, muscle, heart and gonad tissues were collected for PCR analysis for the integrated GFP gene.

DNA was isolated from five different organs using DNeasy kit (Qiagen) following the manufacturer's instructions. PCR reactions were performed by initially denaturing cDNA at 95°C for 3 min followed by 30 cycles of denaturing at 95°C for 30 sec, annealing at 58°C for 30 sec, polymerization at 72°C for 30 sec and a final 10-min extension at 72°C. GFP primer used in PCR are listed in Table 2, above.

Sequencing verification of GFP gene was performed by extracting DNA from agarose gels after electrophoresis. DNA was extracted from the agarose gels using the QIAquick Gel Extraction Kit (Qiagen) per manufacturer's instructions. Purified DNA was sent to the

Georgia Genomic Facilities for sequencing. The resulted sequence was compared by Blast in the NCBI database.

Results

ciPSCs generated by Minicircle transfection express pluripotent markers

Chicken embryonic fibroblasts (CEF; Fig. 16A) isolated from day-11 black australorp chicken embryos were transduced with the minicircle vector containing the *POU5F1*, *SOX2*, *NANOG* and *LIN28* reprogramming genes and the GFP reporter using four different transfection reagents: lipofectamine, Xfect, purefection and genejammer. The cells were transfected a total of 3 times every other day over a 5 day period. The GFP expression reached a peak at 72 hours post-transfection (Fig. 16B). The lipofectamine transfected group showed the highest transfection efficiency with 16.4% population being GFP+, while transfection with genejammer, Xfect and purefection resulted in 2.3%, 2.1%, and 15.1% GFP+ cells respectively (Fig 16. C, D). At day7, the cells were trypsinized and plated onto inactivated feeder cells in 20% KSR stem cell medium.

Compact colonies began to emerge on day 20 after transfection with defined borders (Fig. 17A). To determine the effect of transfection reagents on colony formation, the number of colonies was manually counted. Cells transduced with lipofectemine resulted in significantly (p -value < 0.05) higher levels of colony formation (21.7 ± 1.0) relative to cells transduce with genejammer (11 ± 2.1) and Xfect (10.7 ± 1.8). Transfection with purefection resulted in similar levels of colony formation (17.3 ± 1.4) (Fig. 2C). The compact colonies were mechanically isolated and initially replated on feeder plates in 20% KSR medium. After passaging, ciPSC displayed morphological characteristics consistent with iPSCs including a colonial growth pattern with colonies forming highly refractive colonies with well defined borders (Fig. 17B). At the single cell level, ciPSCs had a high nucleus to cytoplasm ratio and possessed large nucleoli indicative of a stem cell fate.

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Claims:

1. A population of avian induced pluripotent stem cells reprogrammed from avian somatic cells, said somatic cells being reprogrammed with at least the three reprogramming genes Nanog, LIN28 and c-MYC and optionally, at least one reprogramming gene selected from the group consisting of OCT4, SOX2 and KLF4.
2. The population of cells according to claim 1 wherein said cells have been reprogrammed with Nanog, LIN28, c-MYC, Oct4 and optionally SOX 2, KLF4 or a mixture of SOX 2 and KLF4.
3. The population of cells according to claim 1 wherein said cells have been reprogrammed with Nanog, LIN28, c-MYC, SOX2 and optionally Oct4 and/or KLF4.
4. The population of cells according to claim 3 wherein said cells have been reprogrammed with Nanog, LIN28, c-MYC and SOX2.
5. The population of cells according to claim 1 wherein said cells have been reprogrammed with Nanog, LIN28, c-MYC, KLF4 and optionally, SOX2 and/or Oct4.
6. The population of cells according to claim 1 wherein said cells have been reprogrammed with Nanog, LIN28, c-MYC, Oct4, SOX2 and KLF4.
7. The population of cells according to claim 1 wherein said cells have been reprogrammed with Nanog, LIN28 and c-MYC in the absence of Oct4, SOX2 and KLF4.
8. The population of cells according to any of claims 1-7 wherein said somatic cells are embryonic somatic cells.
9. The population of cells according to any of claims 1-7 wherein said somatic cells are embryonic fibroblast cells.
10. The population of cells according to any of claims 1-7 wherein said somatic cells are adult somatic cells.
11. The population of cells according to any of claims 1-7 wherein said somatic cells are adult fibroblast cells.
12. The population according to any of claims 1-11 wherein said somatic cells are reprogrammed by transfecting or transducing said cells with at least one vector comprising said reprogramming genes.
13. The population according to any of claims 1-12 wherein said reprogramming

genes are avian or mammalian genes each of which has a sequence homology of at least about 50% of the identical gene of the avian species from which the somatic cells are reprogrammed.

14. The population according to any of claims 1-13 wherein said reprogramming genes are chicken, mouse, human, pig or cow genes.

15. The population according to any of claims 1-14 wherein said cells are transfected or transduced with vectors wherein each of said vectors comprises a single reprogramming gene.

16. The population according to any of claims 1-12 wherein said avian induced pluripotent cell is a quail, chicken, turkey, duck or goose pluripotent cell.

17. The population according to claim 15 wherein said avian induced pluripotent cell is a chicken pluripotent cell.

18. The population according to claim 15 wherein said avian induced pluripotent cell is a quail pluripotent cell.

19. The population according to claim 15 wherein said avian induced pluripotent cell is a turkey pluripotent cell.

20. A population of induced avian pluripotent stem cells according to any of claims 1-19 exhibiting the following four characteristics:

- form colonies with cells with a high nuclear to cytoplasmic ratio ranging from about 4:1 to about 1:2;
- express alkaline phosphatase;
- are developmentally identical to avian embryonic stem cells (aESCs); and
- are capable of *in vitro* differentiation to all three germ layer types (mesoderm, endoderm and ectoderm) as determined by gene expression.

21. The population of pluripotent stem cells according to claim 20 wherein said cells exhibit one or more of the following characteristics:

- express the following genes and proteins: Nanog, PouV, Sox2;
- exhibit a normal karyotype;
- express high levels of telomerase; and

- express Periodic Acid Schiff (PAS).

22. The population of pluripotent stem cells according to claim 20 or 21 wherein said cells exhibit one or more of the following characteristics:

- express at least one of the cell surface markers SSEA1, SSEA3, SSEA4, TRA 1-61 and TRA 1-81;
- exhibit stable/robust pluripotency and can differentiate or contribute to chimeras even after more than 25 passages;
- fast cell cycle with high population doubling;
- when injected into avian embryos, contribute to all germ cell lineages during development;
- chimera generate protein and morphologically distinct cells *in vitro* such as neurons and muscle cells, among numerous others; and
- the cells are clonogenic.

23. The population of pluripotent stem cells according to any of claims 1-22 which have been instilled with disease resistance from a disease agent.

24. The population of pluripotent stem cells according to claim 23 wherein said disease agent is Newcastle disease virus or avian influenza virus.

25. The population of pluripotent stem cells according to any of claims 1-24 wherein said cells are reprogrammed after an initial reprogramming.

26. The population of cells according to claim 25 which cells express SSEA1.

27. A chimeric bird produced from pluripotent stem cells according to any of claims 1-26.

28. The chimeric bird of claim 27 which is a quail, chicken, turkey, duck or goose.

29. The chimeric bird of claim 28 which is a chicken or turkey.
30. The chimeric bird of claim 29 which is a chicken.
31. The chimeric bird according to any of claims 27-31 which exhibit resistance to a disease agent.
32. The chimeric bird according to claim 31 wherein said disease agent is Newcastle disease virus or avian influenza virus.
33. A method comprising reprogramming an avian embryonic or adult somatic cell to an avian induced pluripotent stem cell, said method comprising transfecting or transducing said somatic cell with the three reprogramming genes Nanog, Lin28 and c-Myc and optionally, at least one reprogramming gene selected from the group consisting of Oct 4, SOX2 and KLF4, allowing said genes to reprogram said somatic cells into pluripotent stem cells and optionally, isolating and/or expanding said stem cells, wherein each of said reprogramming genes is an avian or mammalian gene which exhibits at least about 50% sequence homology with the identical gene from the species from which the somatic are reprogrammed.
34. The method according to claim 33 wherein said avian somatic cell is reprogrammed with the reprogramming genes Nanog, Lin28, c-Myc, POU5F1, SOX2 and KLF4 to produce avian induced pluripotent stem cells.
35. The method according to claim 33 or 34 wherein said reprogramming genes are human, chicken, mouse, pig or cow genes.
36. The method according to any of claims 33-35 wherein said reprogramming genes are transduced into said somatic cells via a viral vector and integrated into the genome

of said somatic cell.

37. The method according to any of claims 33-35 wherein said reprogramming genes are transfected into said somatic cells via a non-integrating reprogramming vector.

38. The method according to any of claims 33-37 wherein said cells have been reprogrammed with Nanog, LIN28, c-MYC, Oct4 and optionally SOX 2, KLF4 or a mixture of SOX 2 and KLF4.

39. The method according to any of claims 33-37 wherein said cells have been reprogrammed with Nanog, LIN28, c-MYC, SOX2 and optionally Oct4 and/or KLF4.

40. The method according to any of claims 33-37 wherein said cells have been reprogrammed with Nanog, LIN28, c-MYC and SOX2.

41. The method according to any of claims 33-37 wherein said cells have been reprogrammed with Nanog, LIN28, c-MYC, KLF4 and optionally, SOX2 and/or Oct4.

42. The method according to any of claims 33-37 wherein said cells have been reprogrammed with Nanog, LIN28, c-MYC, Oct4, SOX2 and KLF4.

43. The method according to any of claims 33-37 wherein said cells have been reprogrammed with Nanog, LIN28 and c-MYC in the absence of Oct4, SOX2 and KLF4.

44. The method according to any of claims 33-43 wherein said somatic cells are embryonic somatic cells.

45. The method according to any of claims 33-44 wherein said somatic cells are embryonic fibroblast cells.

46. The method according to any of claims 33-43 wherein said somatic cells are adult somatic cells.

47. The method according to any of claims 33-43 or 46 wherein said somatic cells are adult fibroblast cells.

48. The method according to any of claims 33-47 wherein said somatic cells are reprogrammed by transfecting or transducing said cells with at least one vector comprising said reprogramming genes.

49. The method according to claim 48 wherein said vector is a viral vector and said reprogramming genes integrate into the genome of said somatic cell to produce said induced pluripotent stem cell.

50. The method according to claim 48 wherein said vector is a non-viral vector said reprogramming genes are non-integrating with the genome of said somatic cells to produce said induced pluripotent stem cell.

51. The method according to any of claims 33-50 wherein said reprogramming genes are chicken, mouse, human, pig or cow genes.

52. The method according to any of claims 33-51 wherein said cells are transfected or transduced with vectors wherein each of said vectors comprises a single reprogramming gene.

53. The method according to any of claims 33-52 wherein said avian induced pluripotent cell is a quail, chicken, turkey, duck or goose pluripotent cell.

54. The method according to claim 53 wherein said avian induced pluripotent cell is a chicken pluripotent cell.

55. The method according to any of claims 33-54 wherein said pluripotent stem cells once produced are reprogrammed at least one further time.

56. The method according to claim 55 wherein said pluripotent stems cells which are further reprogrammed express SSEA1.

57. A method of genetically manipulating avian species, especially chickens, comprising selecting and introducing genes into somatic cells to be reprogrammed into avian iPSCs or directly into avian iPSCs to produce genetically manipulated iPSCs, introducing the genetically manipulated avian iPSCs cells into avian embryos to form chimeric embryos; and generating offspring with said chimeric embryos, wherein said offspring exhibit a phenotype consistent with said introduced genes.

58. The method according to claim 57 wherein said genes are introduced directly into said iPSCs.

59. The method according to claim 58 wherein said genes are introduced into said somatic cells before being reprogrammed to iPSCs.

60. The method according to any of claims 57-59 wherein said genetically manipulated iPSCs are introduced into a subgerminal cavity of said embryos and said embryos are allowed to mature.

61. The method according to claim 60 wherein said embryo is a chicken, turkey or duck embryo.

62. The method according to claim 60 wherein said embryo is a chicken embryo.

63. A method of instilling disease resistance in an avian induced pluripotent stem

cell, said method comprising producing an avian induced pluripotent stem cell from somatic cells according to the method of any of claims 33-56, exposing said pluripotent cells to a disease agent for a period of time sufficient to produce resistance to said disease agent in said cells so that more than 99% of the population of said pluripotent stem cells succumb to said disease agent, isolating the remaining viable cells, optionally testing said cells for resistance to said disease agent and optionally, propagating said resistant cells to provide a population of avian induced pluripotent stem cells exhibiting resistance to said disease agent. .

64. A method of instilling disease resistance into a population of avian induced pluripotent stem cell from disease resistant somatic cells, said method comprising producing disease resistant somatic cells by exposing somatic cells to a disease agent for a period of time sufficient to produce resistance to said disease agent in said cells, more than 99% of the population of which succumb to said disease agent, isolating said resistant somatic cells, propagating said cells to provide a population of resistant somatic and producing a population of disease resistant avian induced pluripotent stem cell from said disease resistant somatic cells according to the method of any of claims 33-56.

65. The method according to claim 63 or 64 wherein said resistant aiPSCs are injected into an avian embryo to produce a chimeric bird, said bird exhibiting resistance to said disease agent.

66. The method according to claim 65 wherein said bird is a chicken and said disease agent is Newcastle disease virus or avian influenza virus.

67. The method according to claim 66 wherein said disease agent is Newcastle disease virus.

68. A method of producing a chimeric avian gamete comprising administering into a germline cell an avian induced pluripotent cell according to any of claims 1-16 to produce a chimeric germline cell, introducing said chimeric germline cell into the testes or ovaries of an

adult bird and isolated said chimeric gamete after a period sufficient to produce said gamete from said germlinje in said adult bird.

69. The method according to claim 68 wherein said chimeric gamete is an ovum.

70. The method according to claim 68 wherein said chimeric gamete is a sperm cell.

71. The population according to claim 23 wherein said disease agent is an agent against which a vaccine is to be made.

72. A method of producing immunogenic material for a vaccine comprising providing a population of aiPSCs resistant to a disease agent according to claim 71, growing said disease agent in said population of aiPSCs, removing said disease agent from said population of aiPSCs after said growing step, and optionally inactivating said disease agent after said removing step.

73. The method according to claim 72 wherein said disease agent is influenza virus.

74. The method according to claim 72 wherein said disease state is Newcastle disease virus avian influenza virus.

75. The method according to any of claims 72-74 wherein said disease agent is inactivated after said removing step.

76. The method according to any of claims 72-74 wherein said disease agent is inactivated before being grown in said aiPSCs.

77. A vaccine comprising immunogenic material produced according to the methods of any of claims 72-76.

FIGURE 1

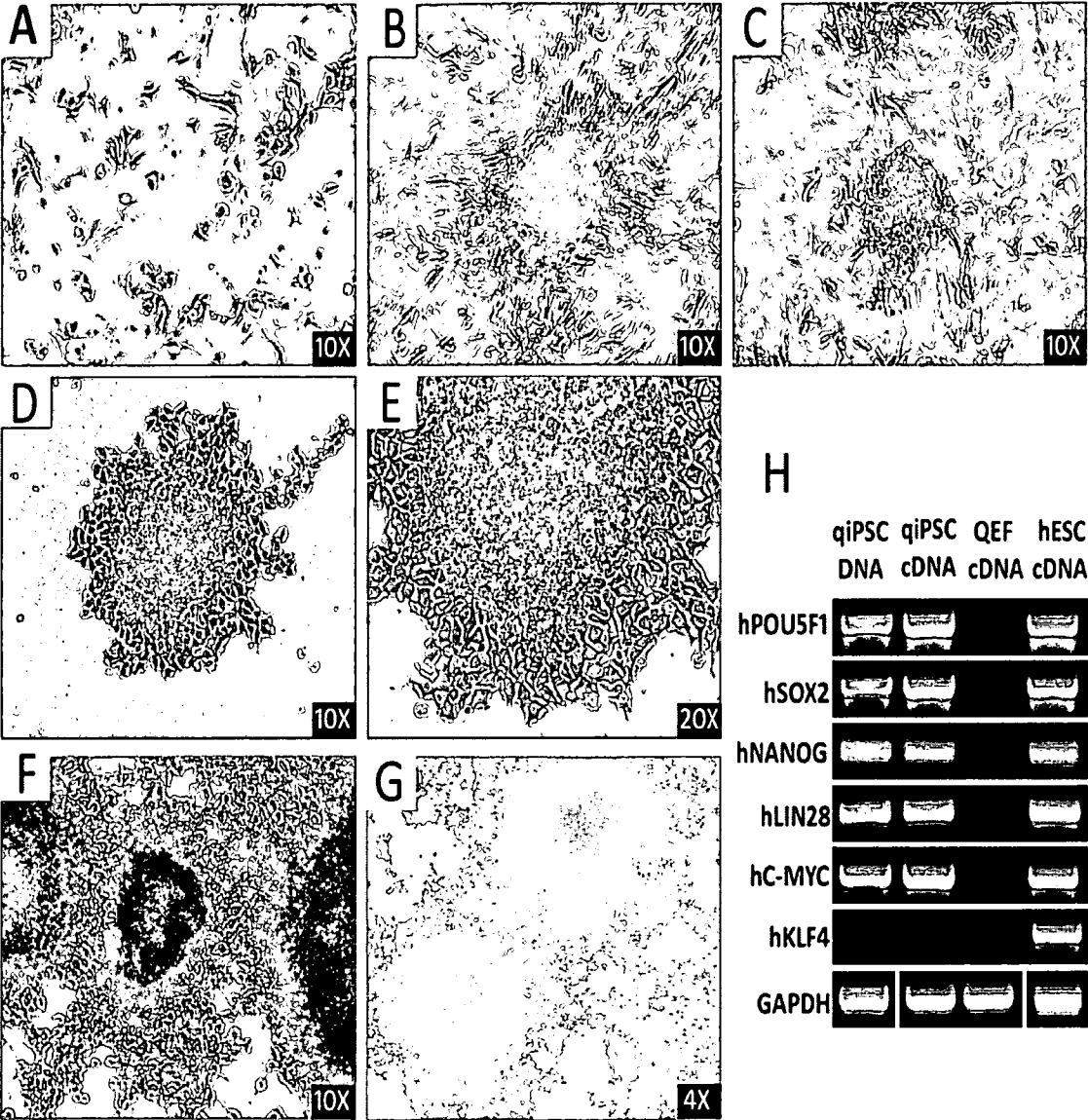


FIGURE 2

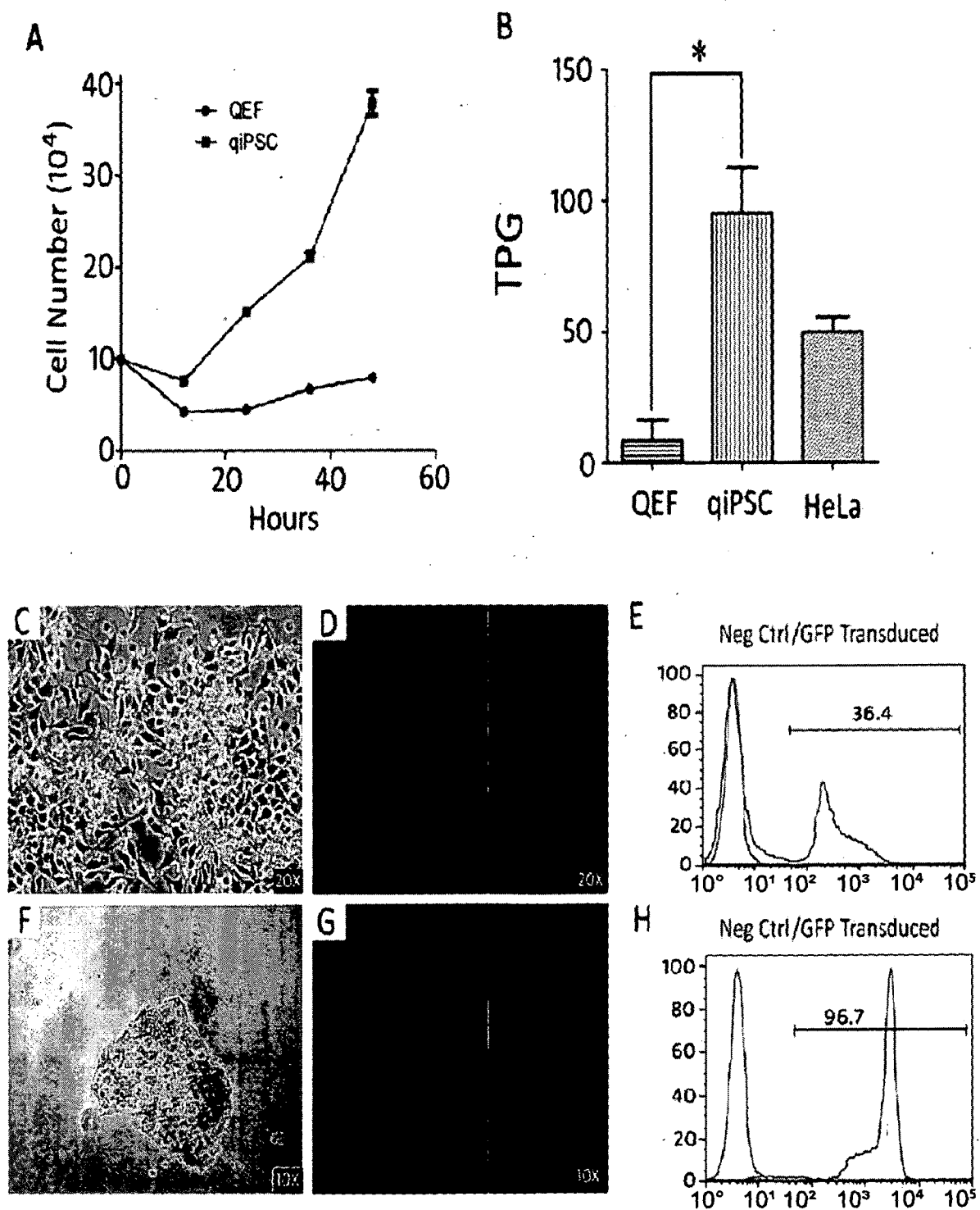


FIGURE 3

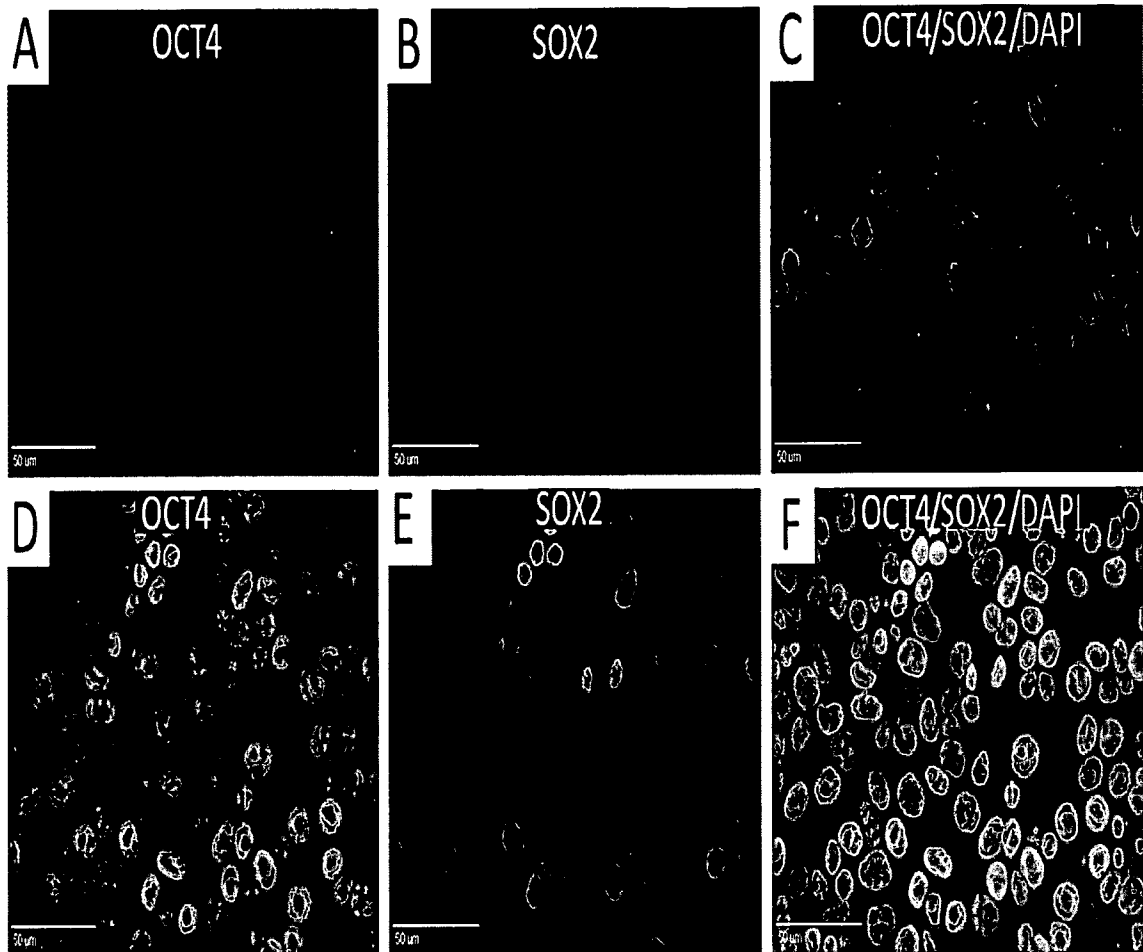


FIGURE 4

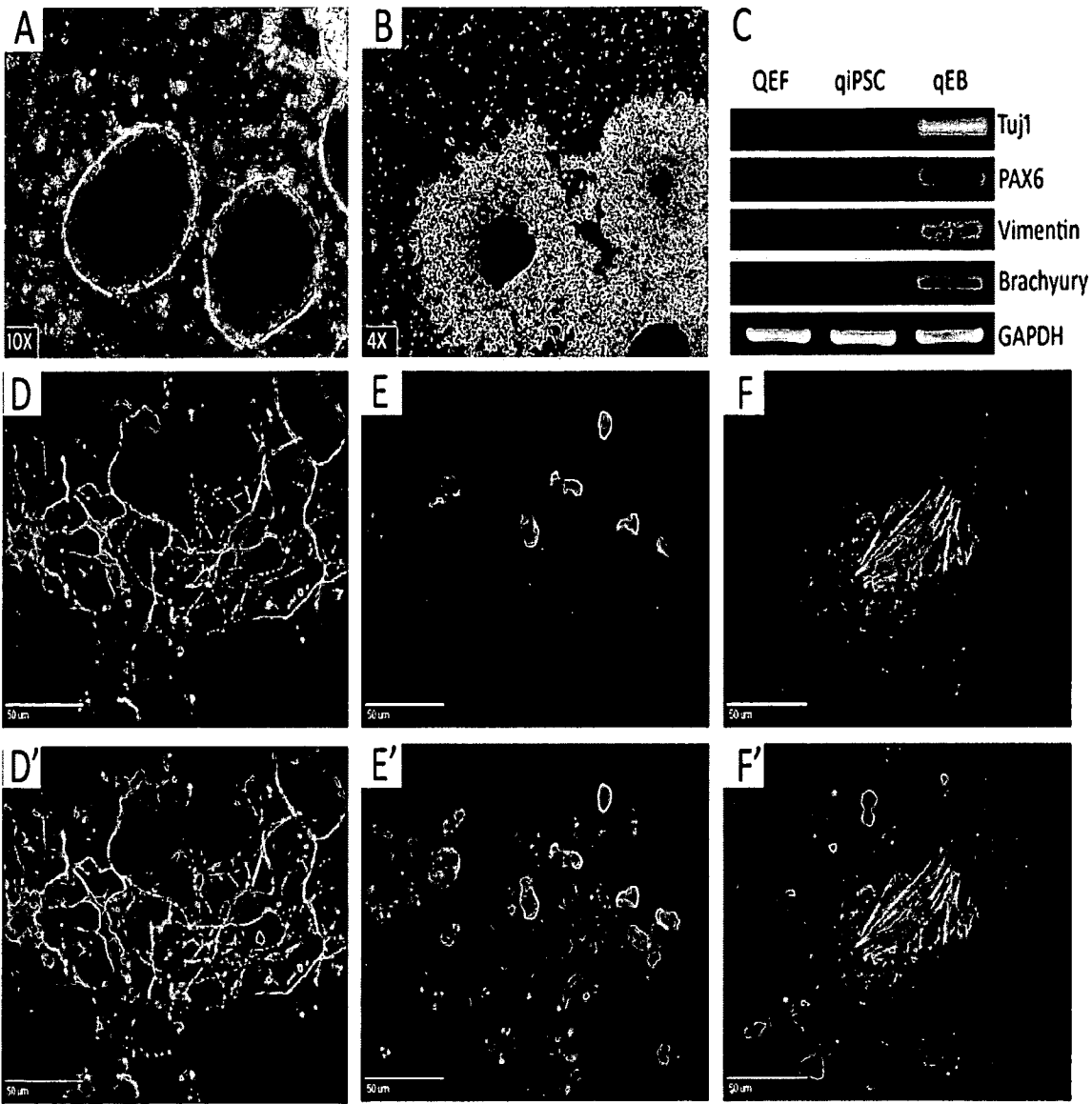


FIGURE 5

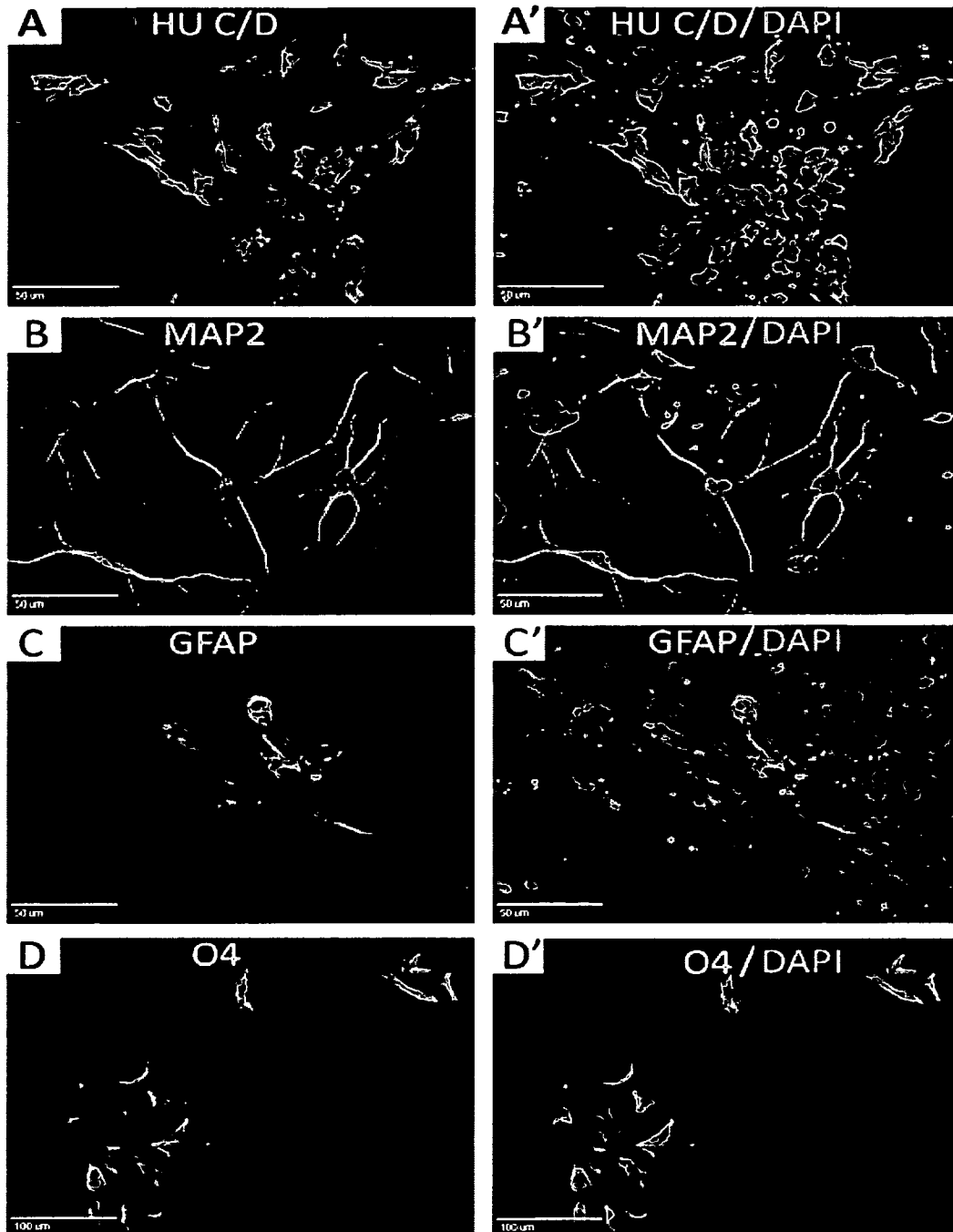


FIGURE 6

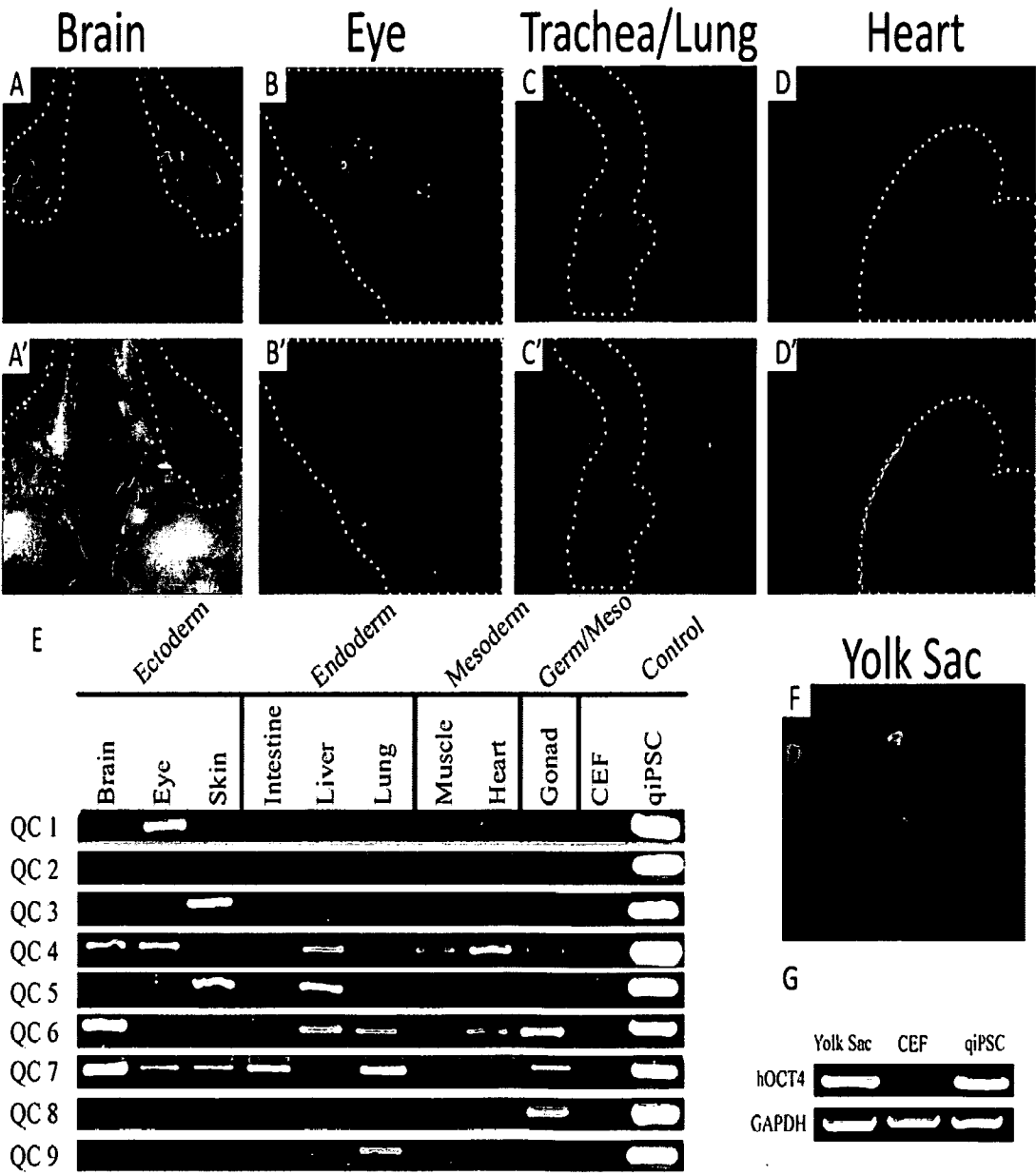


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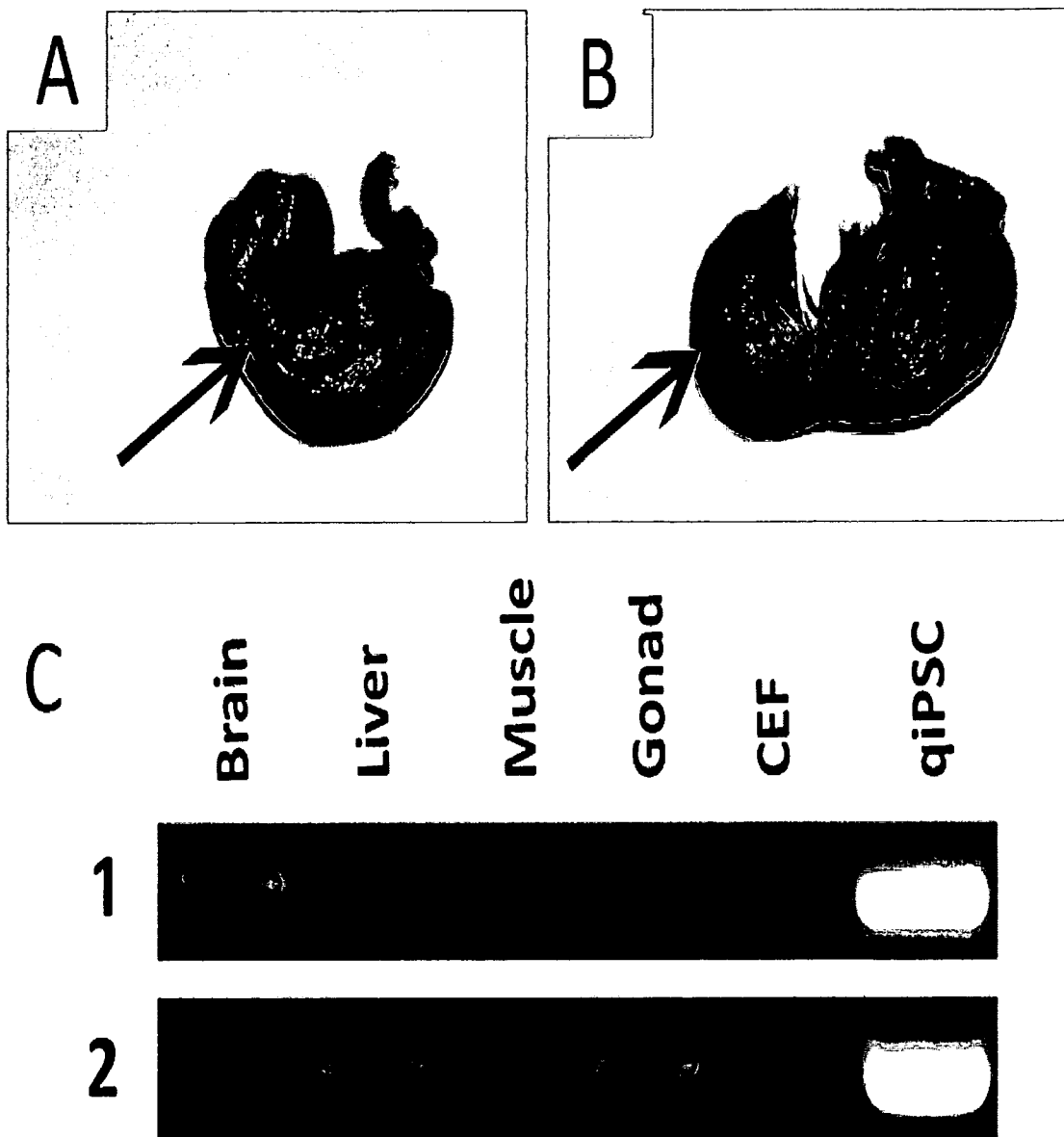


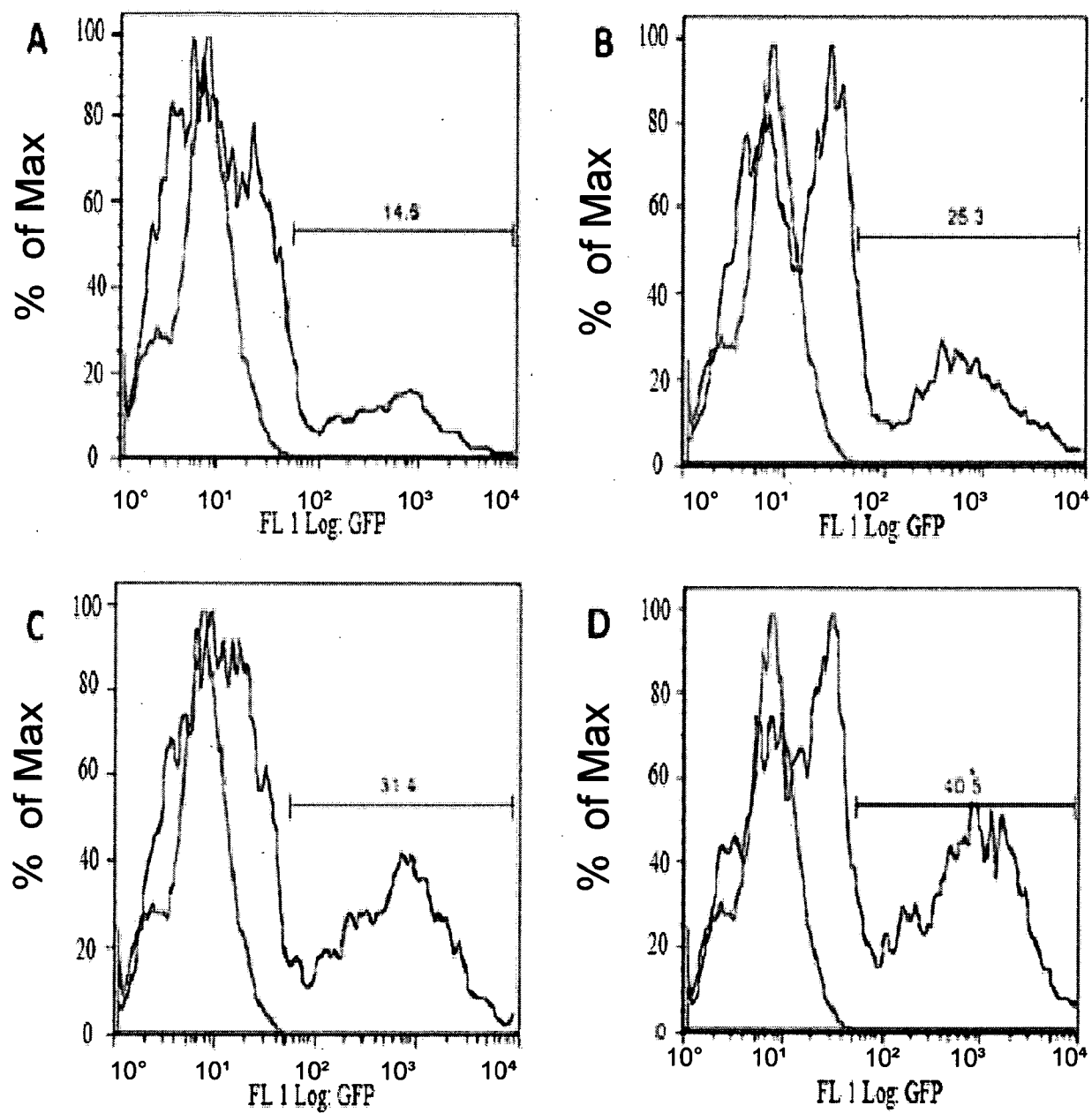
FIGURE 8

FIGURE 9

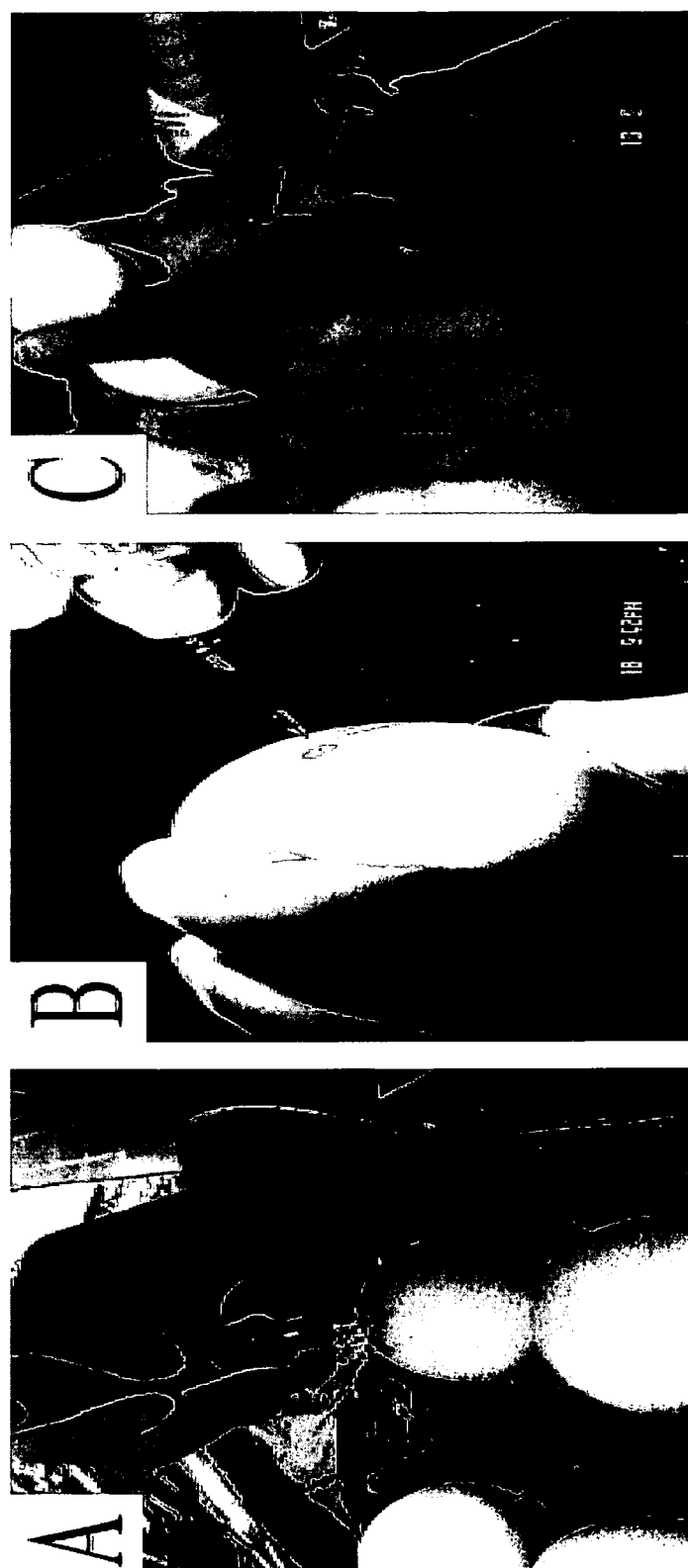


FIGURE 10

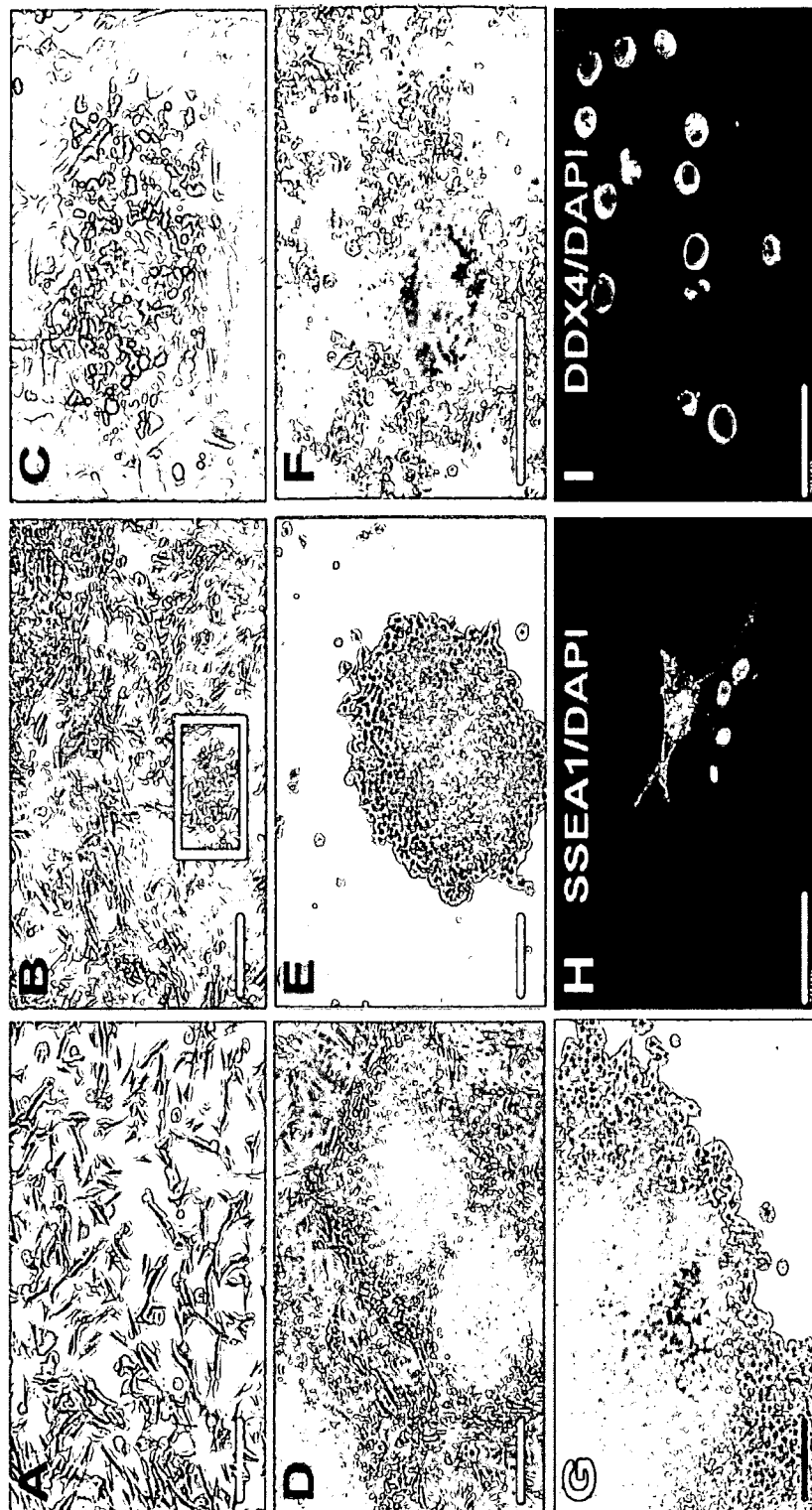


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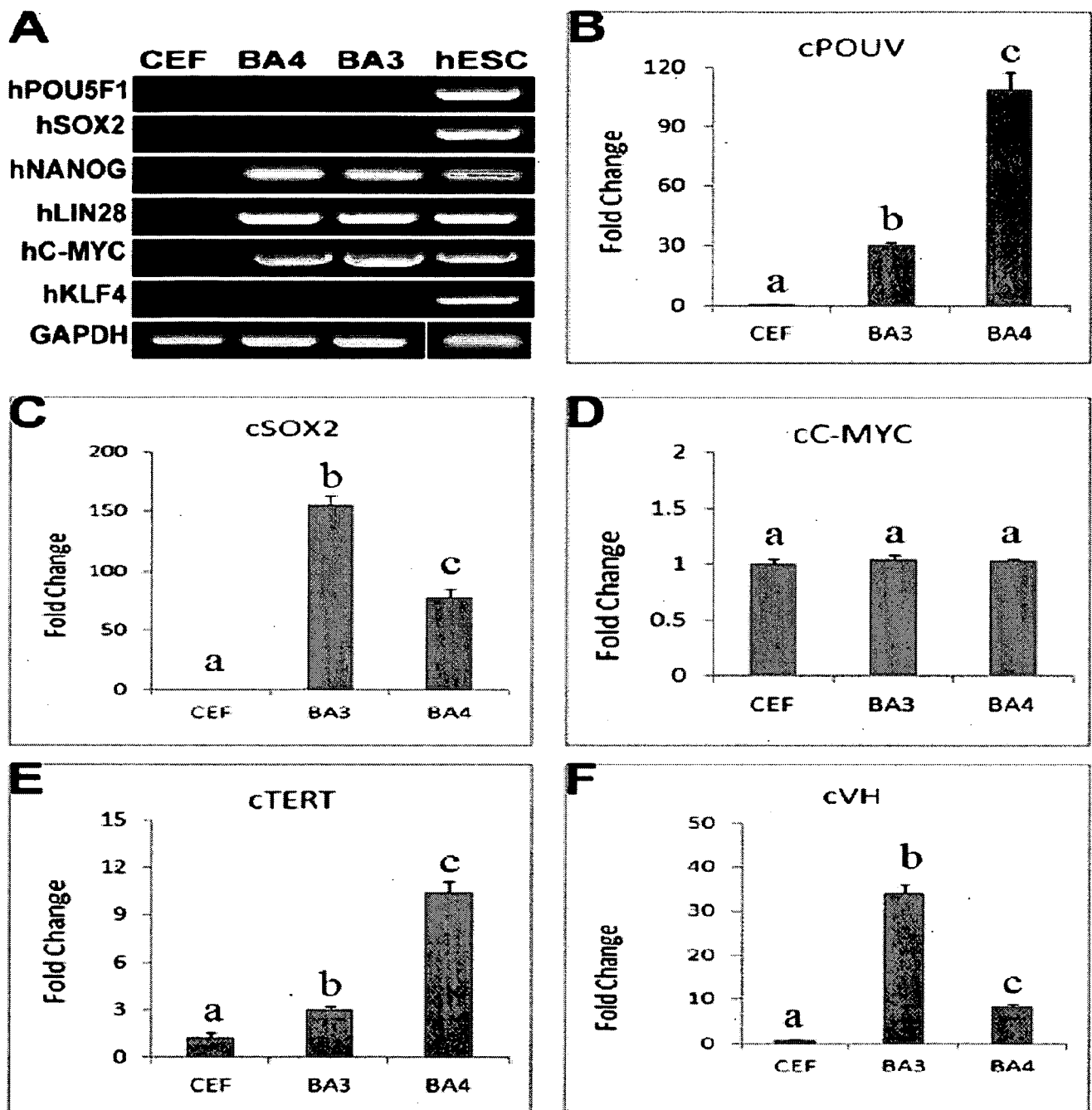


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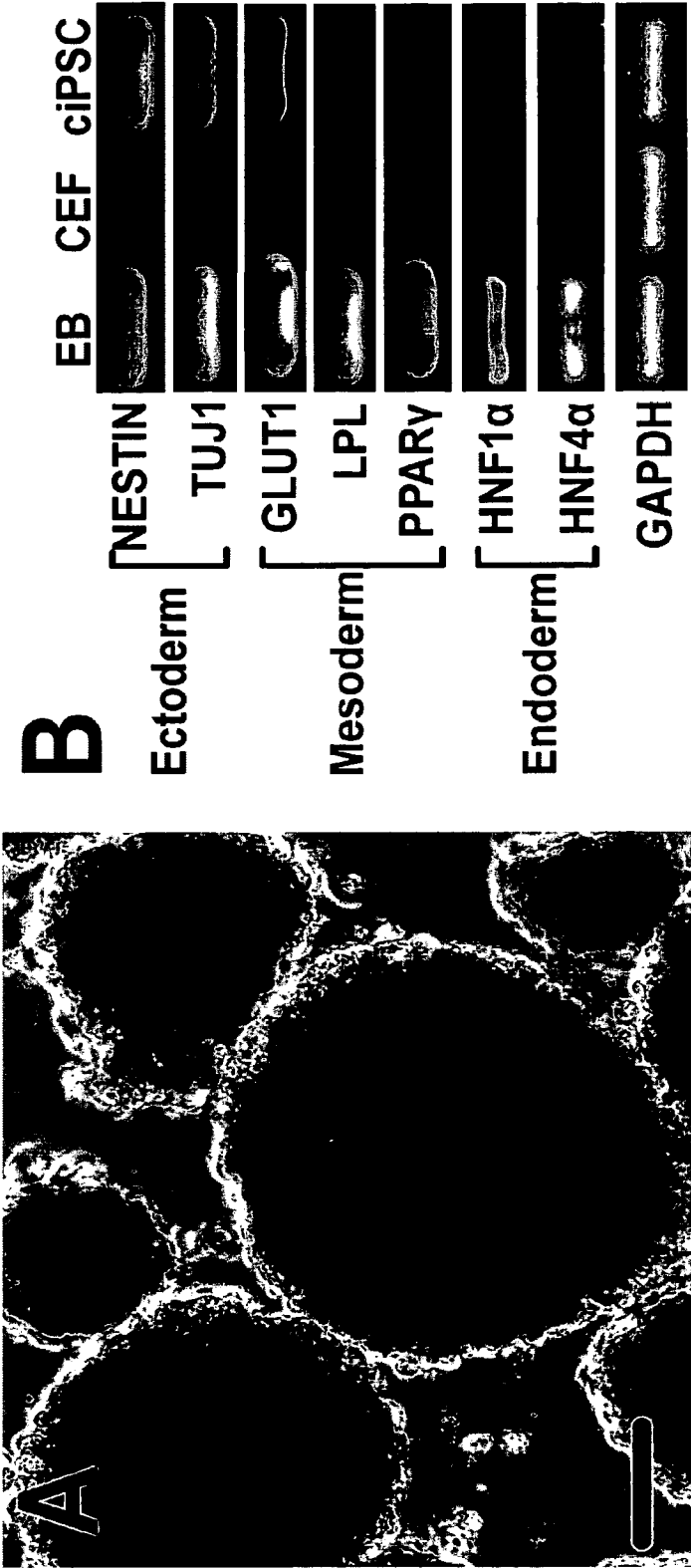


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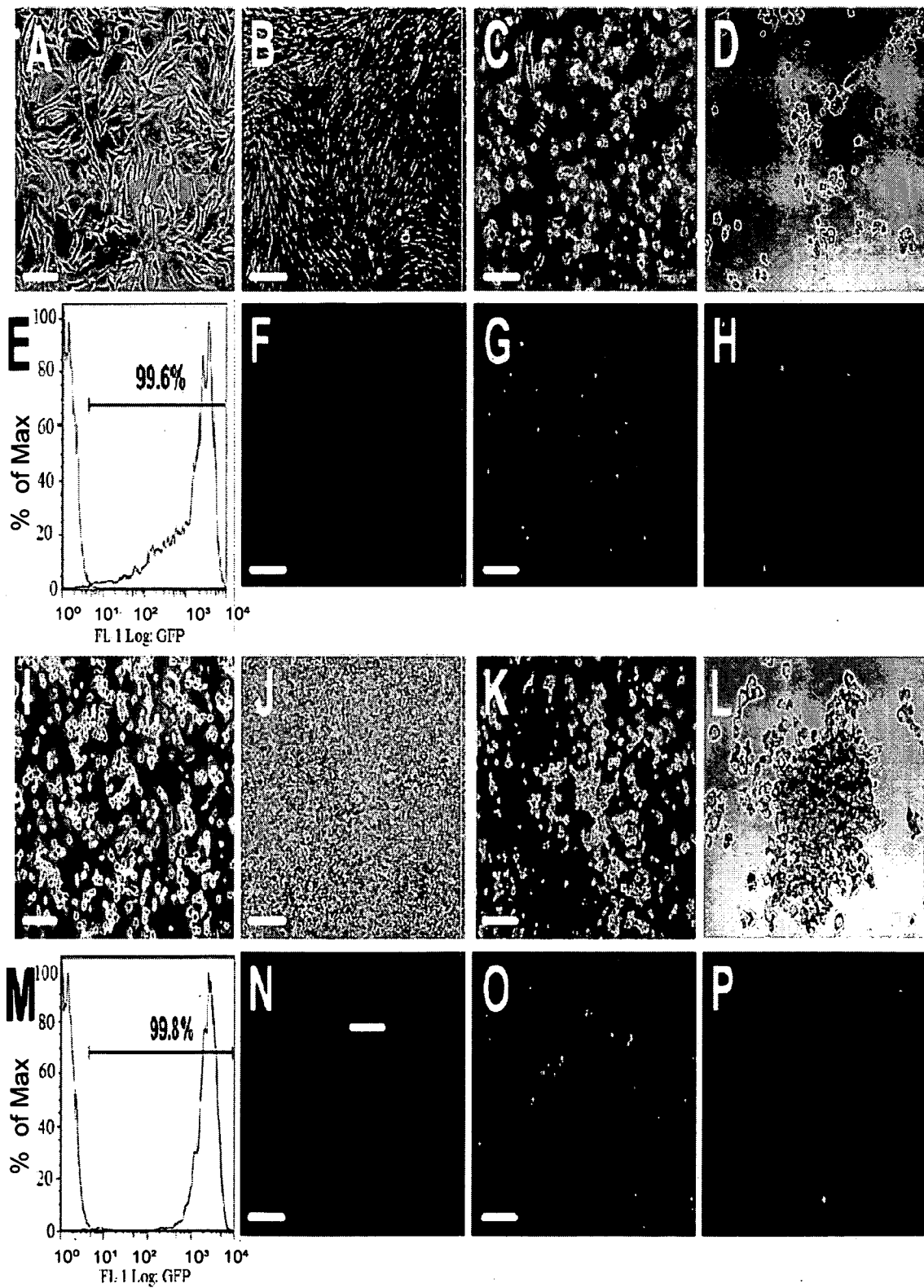


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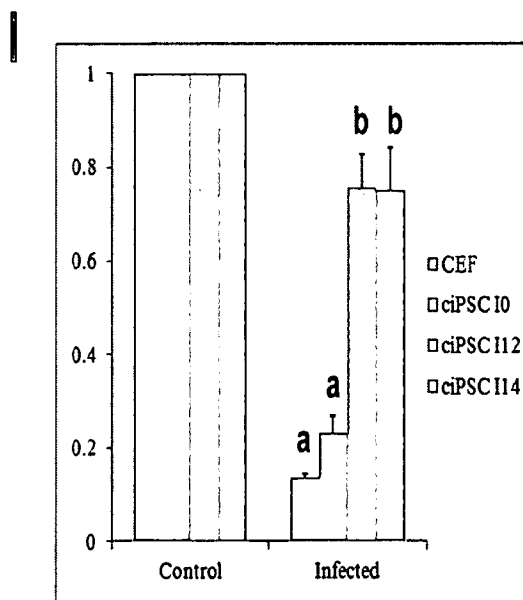
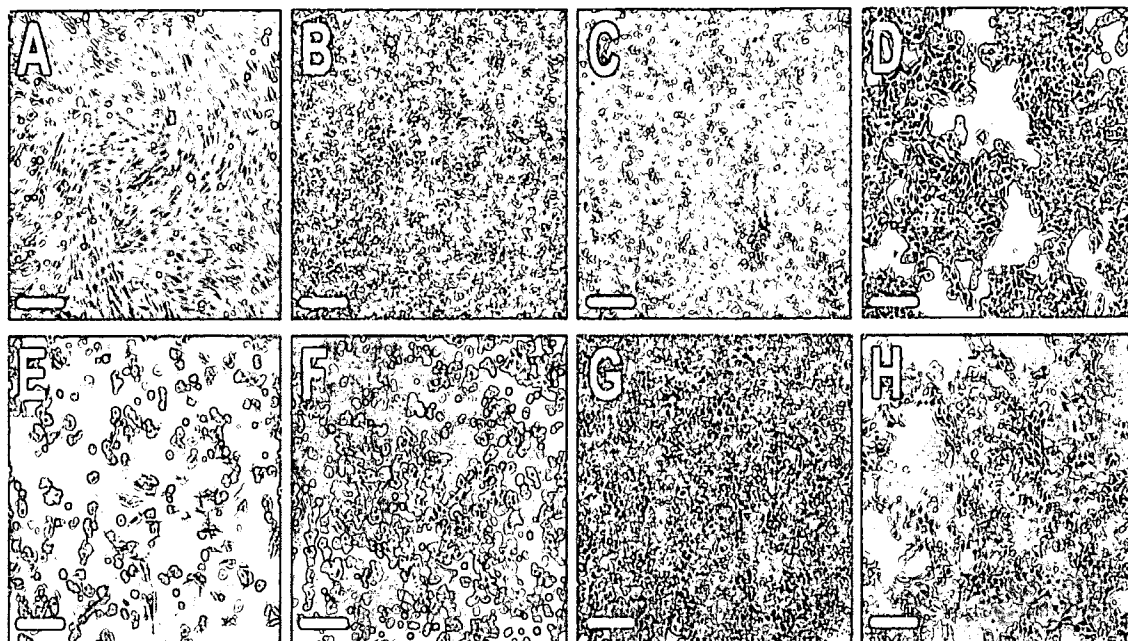


FIGURE 15

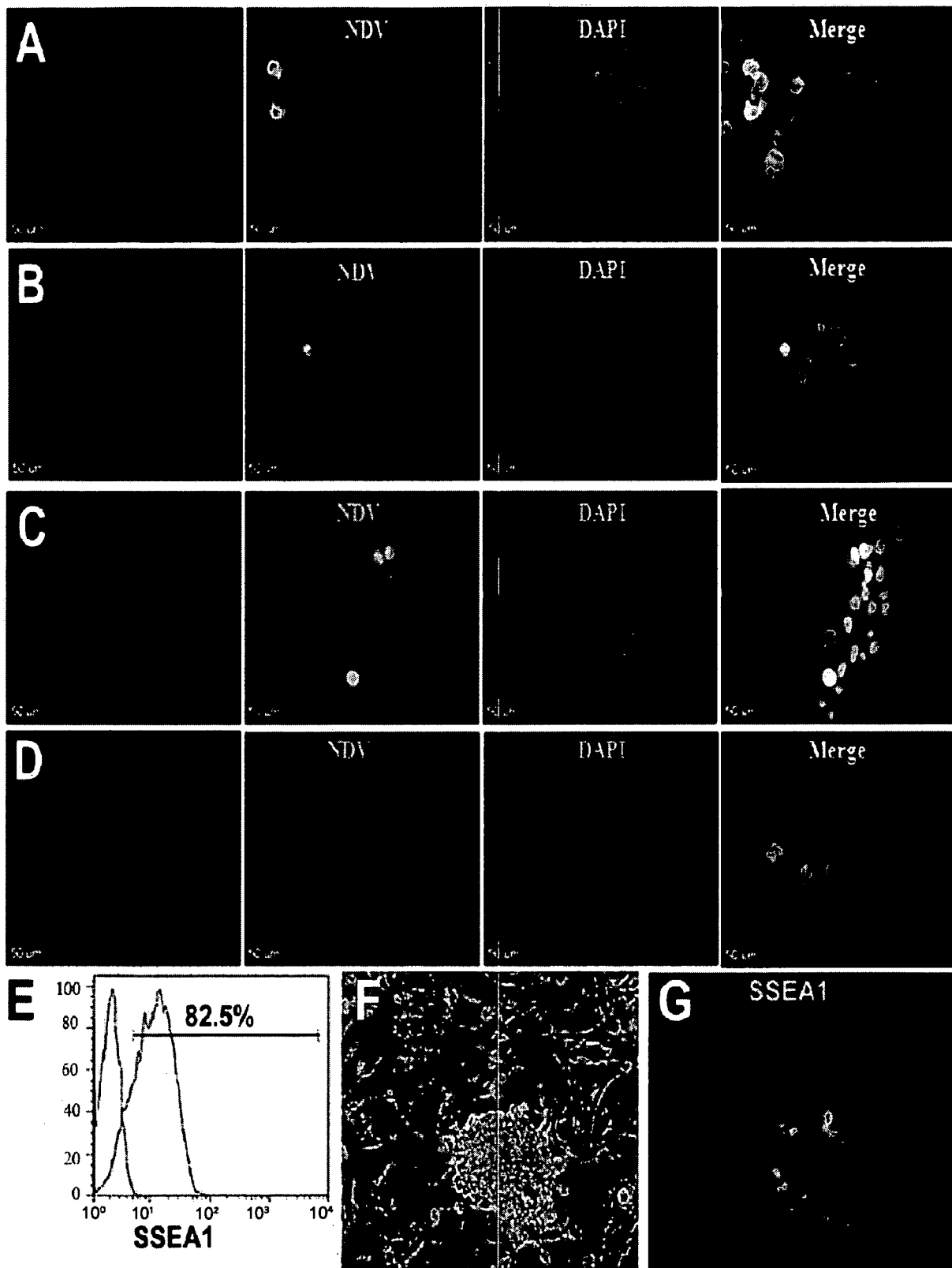
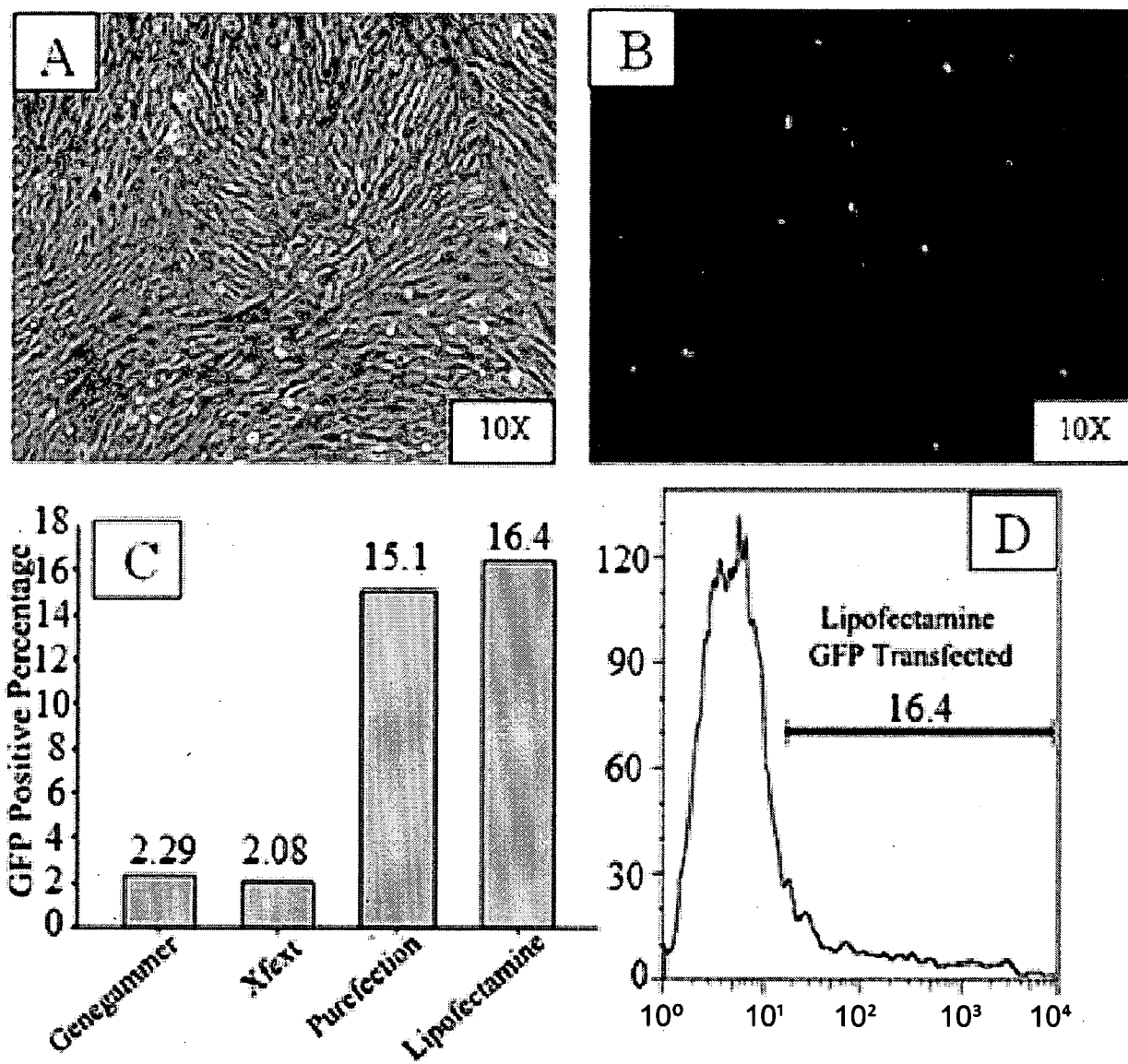


FIGURE 16



EXAMPLE 17

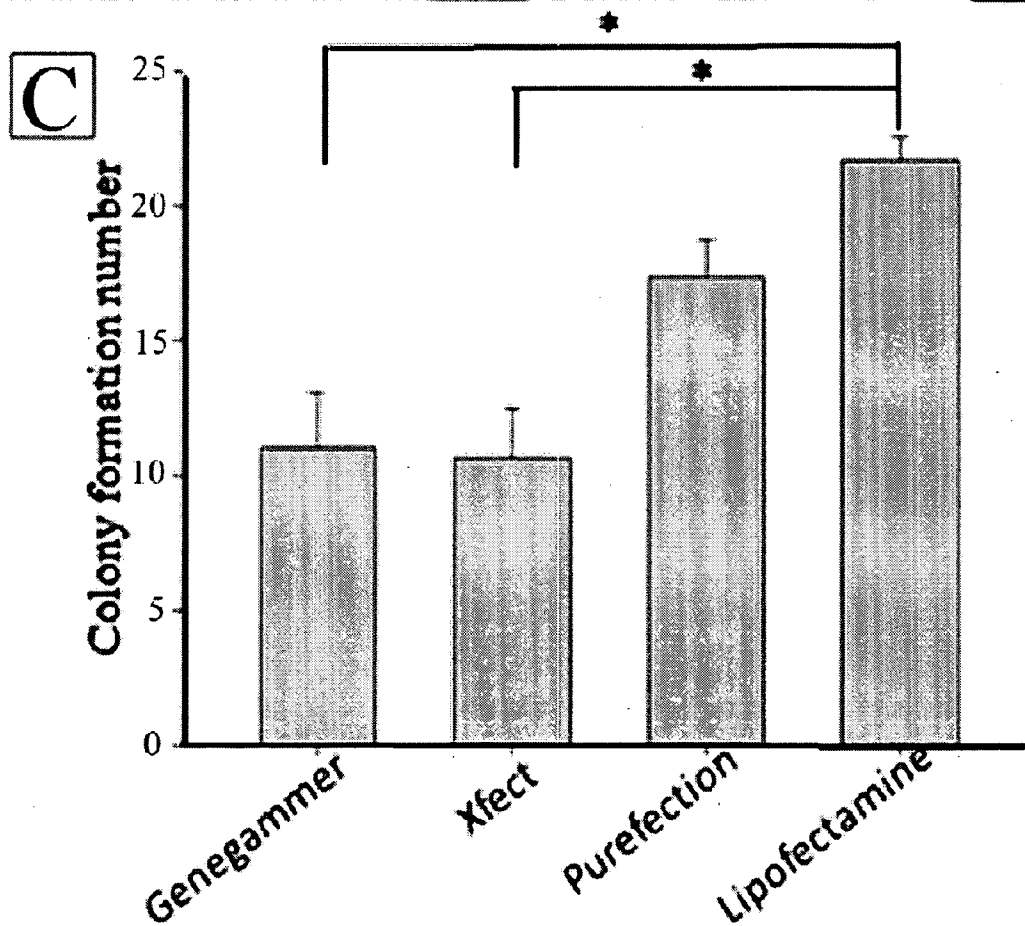
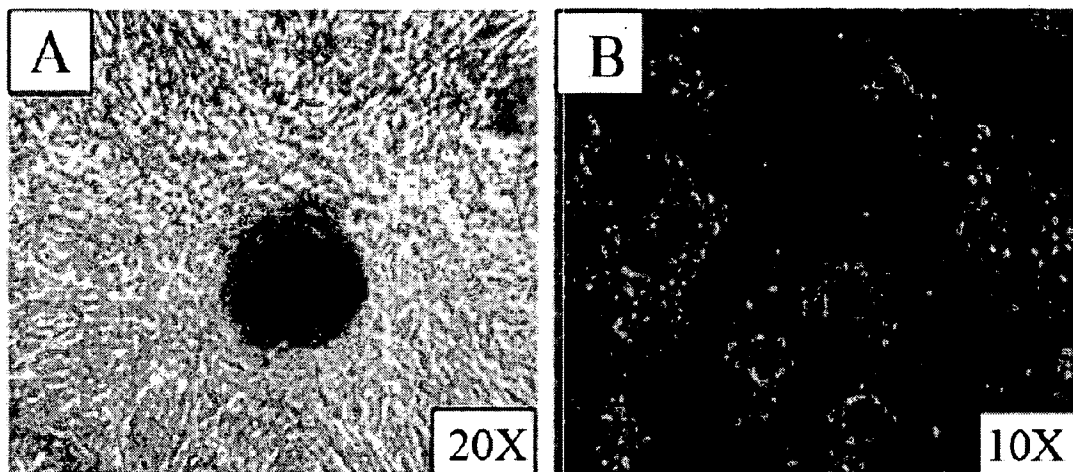


FIGURE 18

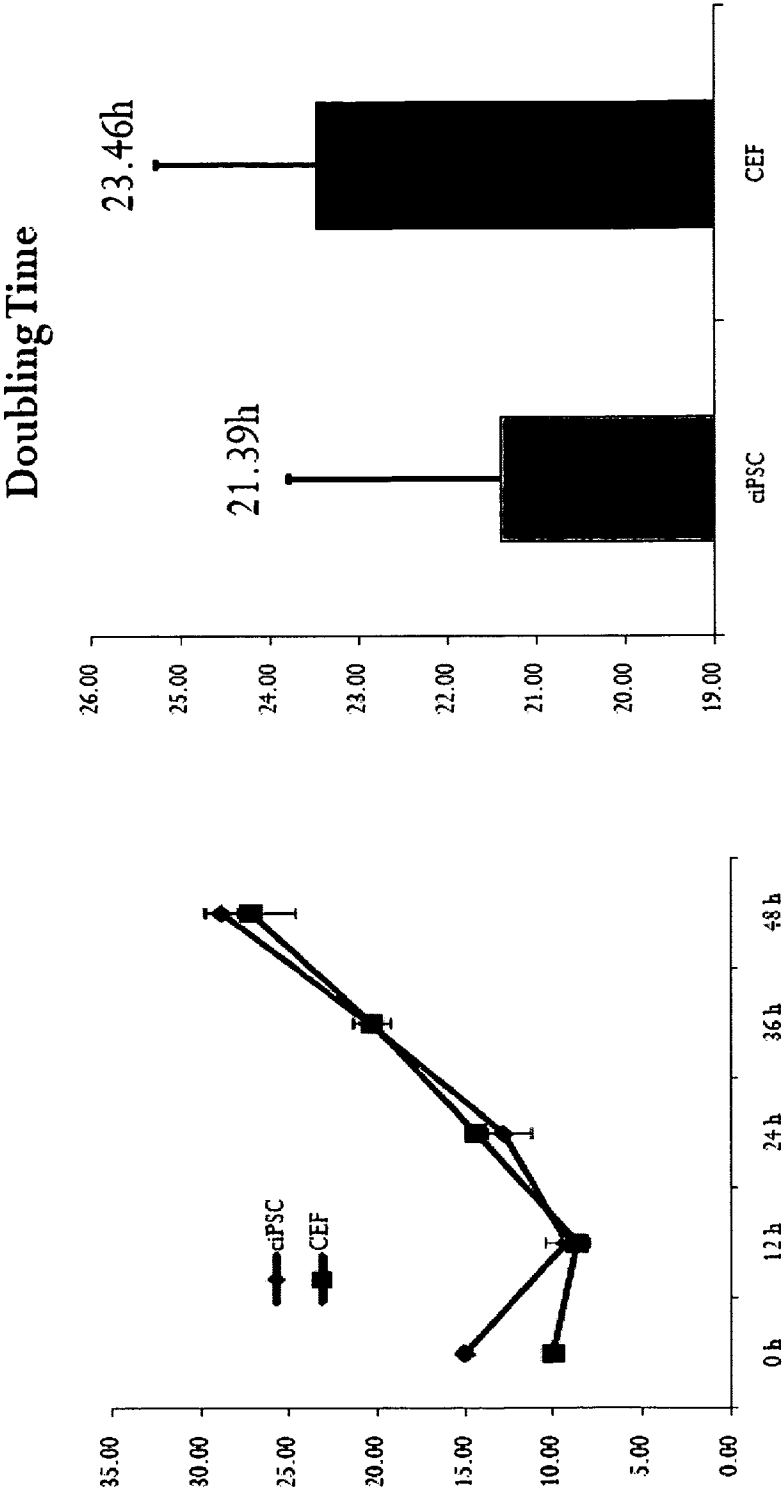


FIGURE 19

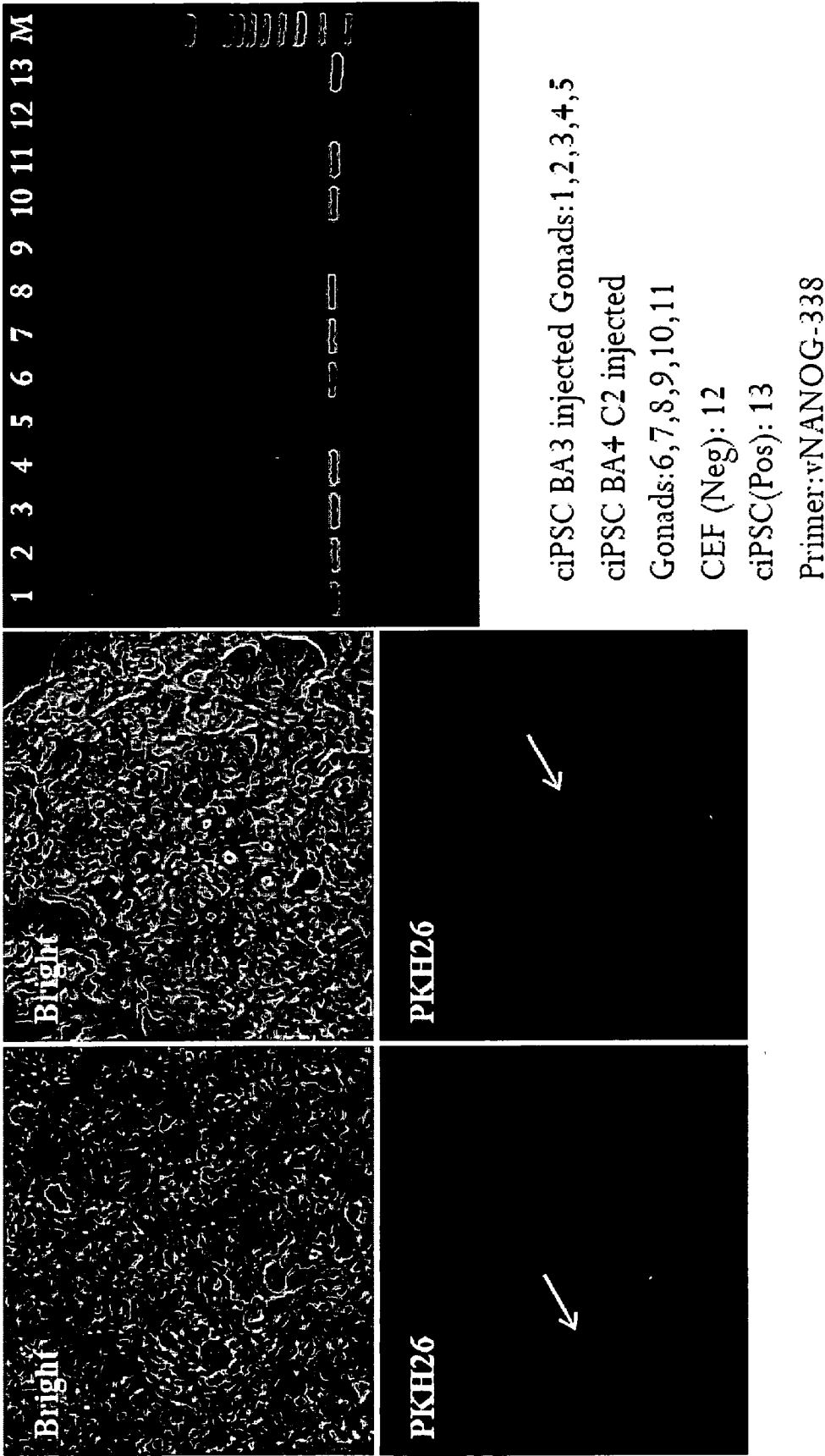


FIGURE 20

