(71) Applicant (for all designated States except US): ADVANCED CELL TECHNOLOGY, INC. [US/US]; One Innovation Drive, Worcester, MA 01605 (US).

(72) Inventor; and
(75) Inventor/Applicant (for US only): WEST, Michael, D. [US/US]; 333 Commonwealth Avenue #7, Boston, MA 02115 (US).

(54) Title: PLURIPOTENT CELLS COMPRISING ALLOGENIC NUCLEUS AND MITOCHONDRIA

(57) Abstract: Allochimeric ES cells are provided having nuclei from one individual of a species and mitochondria from a different individual of the same species. The ES cells can be used for differentiation into differentiated cell types for therapy or for studying various developmental processes in forming embryos and differentiation.

# PLURIPOTENT CELLS COMPRISING ALLOGENIC NUCLEUS AND MITOCHONDRIA

Field of the Invention

The field of the invention is the manipulation of cells to provide non-human oocytes for nucler transfer procedures wherein such nuclear transfer units possess allogenic nucleus and mitochondria from two different cellular sources of the same species.

5

Background

The demonstration that nuclei can be transferred from a differentiated cell to an enucleated term cell resulting in dedifferentiated pluripotent cell provides extensive opportunities for biological manipulation, investigation and therapy. There is a growing

10     opportunity to correct biological defects or adverse medical conditions by using cells or organs which can substitute or complement such defect or condition. In the case of transplantation, until now reliance has been on organs obtained from donors, where the donor was dead or there were two copies of the organ and the donor gave up one of the copies. Despite the efforts to encourage people to provide that upon their death their

15     organs may be used, there remains a desperate shortage of available organs and a long waiting list. In those instances where failure to replace the defective organ is mortal, there is a limited period of time in which to find a replacement organ.

Not only are the organs in short supply, but the donor must be histocompatible with the recipient. Even with close histocompatibility matching between the donor and

20     recipient, the recipient normally must rely on immunosuppressing drugs to prevent rejection of the graft. These drugs frequently have serious adverse effects on the graft recipient, but are suffered because the loss of the graft would be mortal. As surgical capabilities have expanded, there is an increasing number of possibilities for cellular and organ grafts to correct an extensive number of indications.

25     Embryonic stem ("ES") cells offer opportunities to produce differentiated cells, organs and complete individuals upon demand, so that one may clone a particular genotype. In the case of livestock animals, e.g. ungulates, nuclei from like preimplantation

livestock embryos support the development of enucleated oocytes to term (Smith et al.,
 *Biol Reprod.* 40:1027-1035 (1989); Keefer, et al., *ibid* 50:935-939 (1994)). Because of the
interest and importance of ES cells, there have been a large number of papers reporting
various aspects of this technology. For example, Notarianni et al., *J. Reprod. Fert.*

5     *Suppl.* 43:255-260 (1991) report the establishment of stable, pluripotent cell lines from pig
and sheep blastocysts; Gerfen et al., *Anim. Biotech.* 6:1-14 (1995) report the isolation of
embryonic cell lines from porcine blastocysts, which cells differentiate into several
different cell types during culture; Cherny et al., *Theriogenology* 41:175 (1994) report
pluripotent bovine primordial germ cell-derived cell lines maintained in long-term culture

10    which formed embryoid bodies and spontaneously differentiated into at least two different
cell types; Campbell et al., *Nature* 380:64-68 (1996) reported the production of live lambs
following nuclear transfer of cultured embryonic disc ("ED") cells from day nine ovine
embryos cultured under conditions which promote the isolation of ES cell lines in the
mouse; Van Stekelenburg-Hamers et al., *Mol. Reprod. Dev.* 40:444-454 (1995) report the

15    isolation and characterization of permanent cell lines from inner cell mass ("ICM") cells of
bovine blastocysts; Smith et al., WO94/24274, published October 7, 1994, and Wheeler et
al., WO94/26889, published November 24, 1994 report the derivation of bovine and
porcine pluripotent ES cells useful for the production of transgenic animals; Collas et al.,
*Mol. Reprod. Dev.* 38:264-267 (1994) report nuclear transplantation of bovine ICMs by

20    mincroinjection of the lysed donor cells into enucleated mature oocytes (See also, Keefer et
al., *supra*); Sims et al., *Proc. Natl. Acad. Sci. USA* 90:6143-6147 (1993) report the
production of calves by transfer of nuclei from short-term *in vitro* cultured bovine ICM
cells into enucleated mature oocytes (See also, Stice et al., *Biol. Reprod.* 54:100-110
(1996). Finally, Robl et al., PCT/US97/12919 report ES cells resulting from cross-species

25    nuclear transplantation.

The extraordinary opportunities which the manipulation of ES cells provide warrant
the continued investigation and improvement in the character of the ES cells, the sources
from which they may be derived, the manner in which they are expanded and brought to
term and the like. Therefore, there is substantial interest in finding ways to allow for

30    expanded uses of the ES cells in the development of cells and organs or organ fragments

for transplantation, the husbandry of animals, and the investigation of developmental processes leading to differentiation of cells and formation of organoids, organs and portions thereof.

## SUMMARY OF THE INVENTION

Methods and compositions are provided concerning modified oocytes and ES cells capable of being expanded in culture and differentiating to different cell types, engineered ES cells, differentiated cells derived therefrom, organs or portions thereof and organisms derived thereby. The methods comprise employing an allogenic egg cell, where allogenic intends the ultimate species intended for the cell, which is enucleated and into which is introduced a xenogenic nucleus, to provide a chimeric cell having cytoplasm and mitochondria from one species and a nucleus from a different species. After expansion of the chimeric cell to provide a nuclear transfer (NT) unit from which ES cells may be derived, the ES cells may be used for further expansion and scientific investigation, or the NT unit may be transferred into an allogenic, syngenic or other compatible xenogenic host uterus (based on the original oocyte) for pregnancy and birth. Oocytes from this F1 animal may then be harvested, enucleated and an allogenic nucleus introduced to provide an egg cell which can provide cells having proteins of a single species, be immunologically competent and which can be accepted with a reduced risk of rejection when implanted in an allogenic host. For purposes of this application, these cells and their progeny will be referred to as "allochimeric." Of particular interest is the use of nuclei from a host to whom will be given differentiated allochimeric cells.

## DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Allochimeric cells are provided comprising mitochondria from a first individual of a first species and a nucleus from a second individual from the same first species. Broadly speaking, pluripotent cells capable of development into a fetus or into a plurality of different cell types will be produced comprising mitochondria from an allogenic species, where allogenic species intends the species of interest, and a xenogenic nucleus, where

xenogenic intends the species in which the cells will be implanted or a different species
compatible with implantation of the cells and the cells differentiating into a fetus. The
developed or matured fetus will then be used for harvesting oocytes, enucleating the
oocytes, and introducing a nucleus from the same species as the mitochondria. The
5       resulting allochimeric cells may be used *in vitro* and *in vivo* for studying differentiation, for
producing cells along different differentiation paths, e.g. ectoderm, endoderm or
mesoderm, and cells within these classifications, such as neuronal cells, neurons,
astrocytes, glial cells, ganglions, etc., hematopoietic cells, e.g. lymphocytes, macrophages,
NK cells, erythrocytes, megakaryocytes, etc., fibroblasts, myoblasts, etc.

10              The subject methodology involves the following primary stages: ·

1.      Harvesting and enucleating egg cells.


2.      Introducing nuclear mitochondrial genes from a species different from the egg cells
        into the nuclear genome of somatic cells of the different species.

15

3.      Introducing nuclei from a species different from the egg cells into the enucleated
        egg cells to provide chimeric cells.


4.      Expanding the chimeric cells to provide competent chimeric ES cells or incubation
20      to develop nuclear transfer (NT) units.


5.      Inseminating competent chimeric NT units into a compatible female host.


6.      Allowing the chimeric NT units to grow to a fetus or to term and provide a neonate.

25

7.      Growing the neonate to an age to provide chimeric oocytes.


8.      Harvesting chimeric oocytes from the offspring.

9.      Enucleating the chimeric oocytes and introducing nuclei allogenic to the
        mitochondria of the oocyte to provide allochimeric NT units.

10.     Culturing the allochimeric NT units to provide ES cells.

11.     Optionally genetically modifying the allochimeric ES cells.

12.     Using the allochimeric ES cells to produce other cell types.

        Each of these stages in the subject process will now be elaborated upon.

1.      Harvesting and enucleating egg cells
        Egg cells may be harvested from any convenient host of interest, for which one
wishes mitochondria from a species of interest. Thus, any mammalian species of interest
may serve as the source of the egg cells, particularly domestic animals, more particularly
large domestic animals, exemplified by equine, bovine, ovine, porcine, feline, canine,
lagomorpha, murine, etc., and primate, exemplified by human, monkey and ape. The
oocytes may be harvested in any convenient way, depending upon whether there are
sources of follicles from slaughtered animals or a surgical procedure is required. Of
particular interest are primate cells, more particularly human cells, which may serve for
production of differentiated cells. The cells may come from any member of the species, as
the nucleus which will be subsequently employed will determine the species and the
histocompatibility of the cells. The subject invention finds particular application with
human cells, although there will be instances with other species where allochimeric cells
can play a role, e.g. rare and valuable animals, animals for which *in vitro* fertilization is not
reasonably feasible, and the like.
        Human cells have been isolated and enucleated. In Zhang et al., *J. Assist. Reprod.
Genet.* 12:361-8 (1995), human fetal ova are isolated and cryopreserved, and upon
thawing are found to be capable of maturation to the formation of polar bodies. Follicular
aspiration is described in Messinis, et al., *Br. J. Obstet. Gynaecol.* 93:39-42 (1986);

Pellicer, et al., *Hum. Reprod.* 4:536-40 (1989); Wahlstrom et al., *Ann. N. Y. Acad. Sci.* 442:402-7 (1985); and Wood et al., *Br. J. Obstet. Gynaecol.* 88:756-60 (1981). Techniques for enucleation follow techniques used for other species and a particular mode is described in the Experimental section.

5          The egg cells may be matured *in vitro* in accordance with known techniques, using appropriate maturation medium. Oocytes having polar bodies (metaphase II oocytes) may be employed as the host cells. See, for example, Prather et al., *Differentiation* 48:1-8 (1991) and Seshagine et al., *Biol. Reprod.* 40:544-606 (1989))

Enucleation is achieved in accordance with conventional ways, conveniently using
10   a micropipette to remove polar bodies and surrounding cytoplasm. The oocytes are then screened to establish the successful enucleation.


2.       Introducing nuclear mitochondrial genes from a species different from the egg cells into the nuclear genome of somatic cells of the different species.

15         The somatic nuclei may be engineered by the introduction of genes important for mitochondrial function in the type of mitochondria present in the egg cell. The subject invention finds particular application in the introduction of human nuclear mitochondrial genes into animal, particularly bovine somatic cells to facilitate cellular function of an animal cell with human mitochondria.

20

3.       Introducing nuclei from a species different from the egg cells into the enucleated egg cells to provide chimeric cells.

A wide variety of mammalian hosts may be employed as the source of the nuclei,
where the issue will be primarily one of convenience. Besides the necessity for growing
25   the chimeric NT unit to term, other considerations in the selection of the host source will be ease of harvesting the oocytes from the host, ease of manipulation and growth in culture, maturity of the technology, prior success rate in achieving progeny, ease of growth of the host, viability of the resulting chimeric oocytes, number of available oocytes, compatibility of the mitochondria with the host nucleus, as well as other practical
30   considerations. While any mammalian host may be employed, of particular interest are

domestic animals, more particularly, large domestic animals or primates other than humans. Exemplary mammals include ungulates, bovine and ovine, porcine, feline, equine, lagomorpha, murine, canine, etc. The isolated oocytes are matured *in vitro* and selected for the presence of polar bodies.

5          The nuclei may come from any convenient source, germ or somatic cell, which can provide for the production of progeny. Therefore, the nuclei may come from fetal cells, neonate cells, mature cells, cells produced in a culture medium, or the like. Generally, neoplastic cell nuclei will not be used, but may have application depending on the nature of the neoplasm. The source may ne differentiated cells, which may be quiescent, $G_0$ or in an

10         active state e.g. $G_1$ or $G_2$. Cell types include one cell embryos (zygotes) or pronuclei, egg cells, blastomeres, epithelial cells, endothelial cells, muscle cells, keratinocytes, skin cells, alveolar cells, hepatocytes, renal cells, neuronal cells, hematopoietic cells, fibroblasts, endothelial cells, parenchymal cells, adipose cells, glial cells, follicular cells, etc., where the choice may be determined by a number of factors associated with the end purpose.

15         Various techniques exist for introducing the nucleus into the enucleated egg cell. Fusion and injection has been shown to be efficient, where the cell, which is the source of the nucleus, is placed into the perivitelline space of the enucleated oocyte and fused in a fusion chamber by means of an electrical pulse or using a fine glass needle, the donor nucleus or pronuclei from a fertilized oocyte can be isolated and delivered into the

20         cytoplasm of the recipient egg. These pronuclei may be from animals whose genomes have been modified to include nuclear mitochondrial genes of the same species as the recipient oocyte. (See, for example, Prather et al., U.S. Patent No. 4,997,384.) Fusion can also be accomplished by using electrical pulses or by using various fusogenic reagents, e.g. Sendai virus. (See, for example, Graham, *Wister Inot. Symp. Monogr.* 9:19 (1969)

25         and Collas and Barnes, *Mol. Reprod. Dev.* 38:264-267 (1994).)


4.    Expanding the chimeric cells to provide competent chimeric ES cells or incubation
      to develop nuclear transfer (NT) units.

      After fusion, the resultant fused nuclear transfer ("NT") units are placed in a

30         suitable medium until activation, e.g. CRIaa medium. Typically, activation will be

effected shortly thereafter, usually within 24h, more usually 4 - 9h later. Activation may

be accomplished by methods known for mammalian cells, such as culturing the NT unit at

sub-physiological temperature, e.g. room temperature, penetration of oocytes by sperm,

electrical and chemical shock, etc. See, for example, Susko-Parrish et al., U.S. Patent No.

5, 496, 720. Other methods include effecting simultaneous or sequential increasing of

levels of divalent cations in the oocyte, e.g. calcium, magnesium, barium or strontium,

conveniently in the form of an ionophore, by using electrical shock, treatment with ethanol

or treatment with caged chelators; or reducing phosphorylation of cellular proteins in the

oocyte, employing kinase inhibitors, e.g 6-dimethylaminopurine, staurosporine,

roscovitine, butyrolactone, 2-aminopurine, and sphingosine, or introducing one or more

phosphatases into the oocyte, e.g. phosphatase 2A or 2B. A technique of interest for

activation is: activation of NT units within 18 to 30 hours after the start of culture in

maturation medium. The NT units are then removed from the culture media and placed in

activation media (2mL TL Hepes with 2 mg of bovine serum albumin, 5mM of Ionomycin

and 2mM of 6-DMAP for 4 minutes on a slide warmer. At the end of 4 minutes, the NT

units are rinsed in TL Hepes and placed in culture media with 2mM of 6-DMAP at 38.5°

and 5% $CO_2$ for 2 to 5 hours. At the end of this period, NT units are rinsed 4 times in TL

Hepes and placed in culture. When the desired developmental stage is reached, ES cells

will be derived or NT units will be transferred to recipient females.

The activated NT units of chimeric cells may then be cultured in a suitable *in vitro*

or *in vivo* culture medium until the generation of ES cells and cell colonies developing

embryos, such as blastocysts. Culture media suitable for culturing and maturation of ES

cells and embryos are well known in the art. The chimeric cells may be treated as cells

from the species of the nucleus, having the surface membrane proteins, cytoplasmic and

nuclear proteins defined by the nuclear genes. Examples of known media include Ham's

F-10 + 10% fetal calf serum ("FCS"), Tissue culture medium-199 ("TCM-199") + 10%

fetal calf serum, TyrodespAlbumin-Lactate-Pyruvate ("TALP"), Dulbecco's phosphate

buffered saline ("PBS"), Eagle's and Whitten's media. A common media is TCM-199 or

DMEM and 5 to 20% fetal calf serum or an equivalent source of factors to support growth,

such as newborn serum, estrual cow serum, lamb serum or steer serum. An exemplary

media is TCM-199 with Earl salts, 10% fetal calf serum, 0.2mM Na pyruvate and 50μg/ml
gentamycin. These media may be used with cell lines to provide for conditioned media,
using granulosa cells, oviduct cells, BRL cells, uterine cells and STO cells. Alternatively,
the media described by Rosenkrans, jr., U.S. Patent No. 5, 096, 822 may be used. This

5    medium referred to as CR1 contains hemicalcium L-lactate in from about 1 to 10mM,
usually 1 to 5mM, sodium chloride, potassium chloride, sodium bicarbonate, and a minor
amount of fatty-acid free bovine serum albumin, and when essential and non-essential
amino acids are added, the medium is referred to as CR1aa. An exemplary CR1 media
comprises 114.7mM NaCl, 3.1 mM KCl, 26.2mM $Na_2CO_3$, 5mM hemicalcium L-lactate

10   and 3mg/ml of fatty acid-free bovine serum albumin.

Conveniently, the activated NT unit chimeric ES cells are placed in CR1aa media
containing 1.9mM DMAP for about 4h, followed by a wash with HECM and then cultured
in CR1aa containing BSA, at about 38.5°C, 5% $CO_2$, for about 4 - 5 h. The cultured NT
units are usually washed and placed in a suitable conditioned media for growth. An

15   effective media is CR1aa media containing 10% FCS and 6 mg/ml BSA. Suitable feeder
layers include fibroblasts and epithelial cells, particularly uterine epithelial cells, form
sources such as ungulates, chicken, murine, STO, SI-m220 and BRL cells. Mouse
embryonic fibroblasts have been found to be particularly useful. After sufficient time,
which will vary with the species of the nucleus, chimeric ES cells are obtained which may

20   now serve for insemination in an appropriate host.


5.     Inseminating competent chimeric NT units into a compatible female host.


25   6.     Allowing the chimeric NT units to grow to a fetus or to term and provide a neonate.
These stages are well established in the literature for a number of species, including
murine and ungulates. For insemination, NT units are transferred to the uterus. After
insemination, the host is monitored to ensure that the NT units have been successfully
implanted. Depending on the nature of the host, the host may be restrained to prevent a

spontaneous abortion. The delivery of the fetus or neonate may be spontaneous or induced abortion, natural delivery or cesarean section.

7.      Growing the neonate to an age to provide chimeric oocytes.

5

8.      Harvesting chimeric oocytes from the offspring.

The above stages will depend on the nature of the host. Normal maintenance of the host is employed to permit the neonate to grow as a healthy host. The harvesting of the

10      oocytes may be performed as described above.

9.      Enucleating the chimeric oocytes and introducing nuclei allogenic to the
        mitochondria of the oocyte to provide allochimeric NT units.

The manner of enucleation and introduction of nuclei has already been discussed.

15      The selection of the species for supplying the nucleus will depend on the purpose for which the cells are to be used. The cells may be used for cloning a particular species, particularly where oocytes are only difficultly grown in the species, such as humans or rare species, or as a source of differentiated cells, where the cells have both a nucleus and mitochondria from the same species. Other than human, other species which find application with the

20      subject invention include the cloning of rare and endangered animals.

10.     Culturing the allochimeric NT units to provide ES cells.

The NT units are cultured on the feeder layer until the NT units reach a size suitable for the isolation of ES cells, generally requiring at least 4 cells, preferably at least 50 cells,

25      and usually not exceeding 400 cells. Culturing will usually employ conditions of 38.5°C and 5% $CO_2$, with the culture medium changed about every 1 - 5 days.

The final stage involves mechanically removing the NT units from the culture, isolating cells from the inner portion of the NT unit, although cells from other portions of. the NT unit may find application, washing the cells from the NT unit and plating the cells

30      on a feeder layer, e.g. irradiated fibroblast cells, from the same or different species from the

nucleus. The cells are maintained on the feeder layer in a suitable growth media, e.g alpha-MEM supplemented with 10% FCS, 0.1mM β-mercaptoethanol and L-glutamine. The growth media is conveniently changed about every 1 - 3 days.

In the case of a human allochimeric ES cell, the individual cells are not well defined and the perimeter of the colony is refractive and smooth in appearance. The colony does not possess an epithelial-like appearance.

One may use the NT unit cells to provide inner cell mass (ICM) cells of blastocysts. The ICM cells can be grown in culture using feeder layers, e.g STO (mouse fibroblasts) with charcoal stripped serum

11.     Optionally genetically modifying the allochimeric ES cells.

The ES cells may be modified with DNA in accordance with conventional techniques. Bare DNA, recA coated DNA, viruses, plasmids, YACs, extrachromosomal DNA, chromosomal fragments, cDNA or other source of the desired gene, regulating sequence or the like may be introduced into the ES cells, using appropriate media, liposomes, transporting sequences, permeabilization, electrofusion or the like. See, for example, Schneike et al., Science 278:2130-3 (1997).

The modifications can provide for the constitutive or inducible expression of a particular product, e.g. insulin angiogenesis factors, cytokines, e.g. interleukins, interferons and colony stimulating factors, blood factors, e.g. serum albumin, thrombin, fibrinogen, thrombopoietin, erythropoietin, tissue plasminogen activator,, etc. hormones, e.g. growth hormones, growth factors, e.g. epidermal growth factor, basic fibroblast growth factor, glial derived neurotrophic growth factor, etc., enzymes, enzyme inhibitors, e.g. α-antitrypsin, telomere associated proteins, e.g. Sir, telomerase, etc., neuronal proteins, e.g. neurotrophin-3,4/5, ciliary neurotrophic factor, etc., pancreatic proteins, basal proteins, taste bud proteins, eye proteins, hemoglobin, transcription factors, lipoproteins, immunoglobulins, surface membrane receptors, e.g. insulin receptor, oncogene repressors, L-dopamine, and angiogenesis inhibitors. Alternatively, to correct a defect or undesirable phenotype, one may use homologous recombination in the nucleus which was employed to produce the allochimeric ES cells. For example, nuclei from subjects suffering from sickle

cell anemia, may be genetically modified to provide cells which provide a native

hemoglobin; subjects suffering from hemophilia may have the mutant gene for the blood

factor corrected, e.g. Factor VIIIc or VIIIvw, Christmas factor; subjects susceptible to

diseases because of genetic factors, may have their nuclei modified to provide alleles less

5      susceptible to such diseases, e.g. AIDS, juvenile onset diabetes, muscular dystrophy,

Alzheimer's disease, Parkinson's disease, and so on.

Instead of introducing a genetic capability, in some situations one may wish to turn

off a genetic capability or make the gene inducible. Illustrative genes which may be

knocked out include oncogenes, histocompatibility proteins, blood type proteins and other

10     genes whose dominant expression leads to pathology. One may use homologous

recombination or screen for cells in which the desired knock-out has occurred.


12.      Using the allochimeric ES cells to produce other cell types.

Pedersen, *Reprod. Fertil. Dev.* 6:543-52 (1994) reviews a number of articles

15     concerned with differentiation of ES cells. The author reports the production of

differentiated cells expressing the CD markers specific for the cell type and stage of

differentiation or products produced by the cells, which products are not normally

produced by cells at the level of differentiation of ES cells. Development of ES cells into

specific cell types may be found in Bain et al., *Dev. Biol.* 168:342-357 (1995) (neural

20     cells); Palacios et al., *Proc. Natl. Acad. Sci. USA* 92:7530-7537 (1995) (hematopoietic

cells); and Rathjen et al., *Reprod. Fertil. Dev.* 10:31-47 (1998).

Cells of interest include hematopoietic cells, neuronal cells, skeletal and cardiac

muscle cells, skin cells, epithelial cells, endothelial cells, structural cells, osteoclasts and

osteoblasts, follicle cells, eye cells, cells associated with sensing, such as taste buds inner

25     ear cells, bone cells, renal cells, hepatocytes, pancreatic cells, e.g. β-islet cells, fibroblasts,

and chondrocytes.

The differentiated ES cells can find application in a wide variety of venues. The

cells having allogenic mitochondria and nucleus provide cells which represent normal cells

comprised of DNA from a single species. In this way, issues concerning incompatibility

30     between mitochondria and the nucleus or obviated, the proteins which are present in the

cytoplasm are all of the same species, the interaction between the nucleus and the
mitochondria, e.g. transport of proteins is natural, and the immunodominant sequences
present on the surface of the cell will all be from the same species. Furthermore, cellular
processes which depend on the interaction between the mitochondria and other intracellular

5　　compartments would also be natural.

The ES cells and differentiated cells offer many opportunities for dissection of
cellular processes, response to exogenous agents, e.g. genes, drugs, factors, etc., being used
for screening the effect of these exogenous agents in the development of drugs, identifying
specific alleles or mutations associates with the response to these outside agents, and

10　　investigating variations in transcription and expression in response to various agents.

In addition, because the ES cells may be maintained in culture and expanded, one
can repetitively produce the differentiated cells, where the cells will be based on the same
genotype. The subject invention also offers the capability to use the nucleus from an
individual for investigation and diagnosis. Thus, one may determine the susceptibility of a

15　　particular individual to outside agents, such as carcinogens, allergens, toxins, and mutagens
where there is a complete cell of a single species having the native metabolism of such
species. One can also investigate the cellular response to a particular regimen, e.g. drug
regimen, to determine the effect on the normal cells of a particular patient. For chronic
administration of a regimen, there is sufficient time to use the stored enucleated ova of the

20　　species of interest as a receptacle for introduction of nuclei from differentiated cells of a
particular individual of such species. One then has ES cells which can be directed to
produce various differentiated cells at different stages of differentiation, which can be
subjected to the regimen and changes in the character of the cells determined.

There will be situations where the subject invention allows for studying processes

25　　of mitochondria in conjunction with a particular nucleus. For example, where an
individual may have a nucleus which is defective in providing proteins used by the
mitochondria for its processes, the subject methodology allows for cloning of such cells
and seeing the effect of differentiation on the mitochondrial processes and the effect on
growth patterns, phenotype, etc.

13

In those instances where the ES cells or NT units are used for the maturation of a fetus and production of a neonate, the use of the native mitochondria avoids incompatibility when mating the resulting individual with other individuals of the same species. Thus, there is less likely to be rejection of the fetus by the mother and less likely to be immune responses to host cells, as a result of foreign mitochondrial proteins being present.

Compositions which find use in the subject invention are oocytes comprising mitochondria from one species and a nucleus from a different species, isolated and in culture. For the most part, the nucleus and mitochondria will be from species which substantially differ, generally being from a different genus and even different families. Conveniently, the nucleus will be chosen to provide for a useful source of the enucleated ovum, such as domestic animals (ungulates), e.g. bovine, ovine and porcine, and laboratory animals, e.g. murine. Also included in the subject invention are ova comprising mitochondria from one individual and a nucleus from a different individual of the same species, ES cells derived therefrom, cultures comprising such ES cells, differentiated cells derived therefrom, and cultures comprising such differentiated cells.

The compositions include allochimeric oocytes and ES cells in growth cultures for expansion of the cells. The compositions also include mixtures of allochimeric cells and differentiated cells of inner cell masses or concomitant growth or maintenance of ES cells and differentiated cells in culture media for cell differentiation.

For hematopoietic cells, a culture medium comprising monolayers of mitomycin C-treated (5-10$\mu$g/ml at 37°C for 3- 4h) or irradiated (2 - 4 x $10^3$ rads of $\gamma$rays; 1 rad = 0.01 G$\gamma$) RP.0.10 bone marrow stromal cells in Costar six-well plates, in the presence of recombinant interleukin-3 (rIL-3)(100 - 300 units/ml), rIL-6 and F (final concentration 10%v/v) supernatants from 2-day cultures of confluent FLS4.1 fetal liver stromal cells. FLS4.1 supernatant contains FLT3 ligand, steel factor and a new factor that supports growth of hematopoietic stem cells in 2 - 2.5 ml of culture medium [Iscove's Dulbecco's modified medium/50$\mu$M 2-mercaptoethanol/2mM L-glutamine/gentamycin at 50 $\mu$g/ml /7.5% v/v FCS] at 37°C in a 7.5%$CO_2$/92.5% air atmosphere. Every 5 - 7 days the cells are harvested and subcultured in new Costar 6-well plates containing mitomycin C-treated

14

RP.0.10 stromal cells and freshly prepared cytokine-supplemented culture medium. (Palacios, et al., *Proc. Natl. Acad. Sci. USA* 92:7530 (1995)). It is understood that other cell lines providing analogous conditioned media may be used, as well as other constituents which provide analogous activities.

5          For neuronal cells, the ES cells are subject to an 8-day induction procedure which consists of 4 days of culture as aggregates without retinoic acid (RA) followed by 4 days of culture in the presence of RA. The medium is DMEM (high glucose with L-glutamine, without pyruvate; GIBCO 11965-043), 10% fetal bovine serum, 10% newborn calf serum and nucleosides stock.

10         Other media may be employed for directing differentiation into other cell types, using factors which exist naturally and affect the differentiation *in vivo*, such as hematopoietic factors, e.g. G-CSF, M-CSF, GM-CSF, interleukins, interferons and other cytokines and growth factors.

The enucleated oocytes, all having the same mitochondria, may be stored frozen in
15     a suitable medium for extended periods and then carefully thawed before use.

The differentiated cells, whether genetically modified or not, find use in therapy, where healthy cells can be introduced into the appropriate compartment to perform a desired function. For example, myocytes may be used for grafting to myocardial tissue or other muscle tissue, where natural or genetically modified cells may provide an advantage,
20     e.g. correction of a mutant protein, as in the case of muscular dystrophy. See, for example, U.S. Patent No. 5,602,301. Hematopoietic cells may be infused into a patient, such as lymphocytes, natural killer cells, megakaryocytes, eosinophils, basophils, mononuclear cells, or the precursors thereof, where the precursors may be dedicated to a specific cell type or may be multipotent and will differentiate to a number of different type cells. β-islet
25     cells may be grafted onto the pancreas or provided in a protected container which may be innervated by a blood supply for treatment of diabetes. Neuronal cells may be introduced into the brain cavity to provide therapies for Parkinson's disease, Alzheimer disease, cerebral palsy, and the like, by providing a source of L-dopamine, NGE or other factors or other composition which can affect brain function. Other indications for use of the subject
30     ES cells or their differentiated progeny include spinal cord injuries, multiple sclerosis, liver

diseases, vascular diseases, burns cartilage replacement, heart diseases, kidney diseases, urinary tract diseases, prostate diseases, and diseases resulting from aging.

　　　　The subject cells may be used to provide for enhanced supplies of particular factors, constitutively or inducibly. Pruschy et al., *Chem. Biol.* 1: 163-72 (1997) provide

5　　modifications which allow for inducing production of a product by orally taking a pill, which activates a transcription factor. Thus, one can induce the production of tissue plasminogen activator in the case of a heart attack, production of insulin for diabetics, activation of lymphocytes in the case of infection, through the introduction of non-human receptors such as theecdysone receptor, and the like.

10　　　　The following examples are by way of illustration and not by way of limitation.


## EXPERIMENTAL


## Materials and Methods

15

Recipient human oocytes:

　　　　Oocytes from human fetuses: Fetal ovaries are obtained from human fetuses of 16-20 weeks gestation following elective abortion. Ovarian tissues are minced into ~1mm size and cultured in Waymouth media supplemented with 15%(v/v) fetal bovine serum,

20　　0.03 IU/ml FSH and 35 ng/ml insulin. The tissue is cultured at 37°C in 5%$CO_2$ air for 5 - 25 days in Falcon dishes and 3 - 40 days in Costar Transwell-COL membranes prior to induction of final maturation in the presence of LH and human follicular fluid. Patches of monolayer consisting of fibroblasts form within 2 - 3 days of the culture of the fresh tissues. After 1 week of culture, follicles separate out from the ovarian tissue, but remain

25　　attached to the monolayer. The maximal number of follicles separating out from the tissue appear about 1 week after initiating culture. After 40 days of culture in Costar dishes, a significant fraction of the ova reach a diameter of more than 80$\mu$, of which about one-third are surrounded by the zona pellucida. Following induction of final maturation, extrusion of the first polar body is noted in ova grown in Costar dishes for 40 days. The mature ova

30　　may be collected and used directly for enucleation.

Oocytes from mature human females. Human ova are obtained using the technique of Trotnow et al., *Arch. Gynecol.* 236: 211-7 (1985). A steering attachment is employed with the transducer of the Diasonics DS 1 sector scanner employing an aspiration needle gliding through a trocar, with continuous sonographic imaging of the aspiration needle.

5     The patient is given epidural anaesthesia.

Other techniques may be employed, including administering follicle stimulating hormone in accordance with the regimen described by Mercan et al., *Hum. Reprod.* 12: 1886-9 (1997).

The oocytes may then be matured *in vitro* as follows. The immature oocytes are

10    washed in TL-HEPES buffered medium containing 3 mg/ml bovine serum albumin (Fraction V). The oocyte cumulus complexes are placed in TCM-199 containing 10% fetal calf serum, LH and/or FSH and estradiol at 39°C. After about 20h in maturation medium, the oocytes are removed and placed in TL-HEPES with 1 mg/ml hyaluronidase and the cumulus cells are removed by repeated pipetting through a fine-bore pipet. The stripped

15    oocytes are screened for polar bodies and those containing polar bodies (metaphase II oocytes) are selected for further use.


Bovine donor cells:

Heifers are superovulated and artificially inseminated . Embryos are recovered

20    from the reproductive tract on day 5 or 6 after standing estrus. The embryos are flushed from the reproductive tract with PBS and collected. *In vitro* matured, fertilized and cultured embryos are used as donor embryos on day 5 after fertilization. The fertilization process is described in Keefer et al., *Mol. Reprod. Dev.* 36: 469-74 (1993).


25    Enucleation

Enucleation is performed using a beveled micropipette at approximately 18h post-initiation of maturation (hpm). Enucleation is confirmed in TL-HEPES medium plus bis-benzimide (Hoechst 33342, 3μg/ml).


30

Nuclear transfer

Individual donor cells are placed into the perivitelline space of the recipient enucleated oocyte. The human oocyte cytoplasm and the bovine donor nucleus (NT unit) are fused together using electrofusion: one fusion pulse of 90V for 15μsec at 24 h post-
5      initiation of maturation of the oocytes. The resulting NT units are placed in CR1aa media until 28h post initiation of maturation, at which time they are activated according to the following process. NT units are exposed for 4 min to ionomycin-6-DMAP (2mM) followed by 4 hrs in DMAP only (2mM) in TL-HEPES supplemented with 1mg/ml serum albumin and then washed for 5min in TL-HEPES supplemented with 30mg/ml serum
10     albumin. The NT units are then transferred into a microdrop of CR1aa culture media plus 10% FCS and 6mg/ml serum albumin in 4 well plates containing a confluent feeder layer of mouse embryonic fibroblasts. The NT units are cultured for 3 more days at 38.5°C and 5% $CO_2$. The culture medium is changed every 3 days until day 12 after the initiation of activation. At this time about 50 cells should be formed, which cells are mechanically
15     removed from the zona and used to produce embryonic cell lines.


Mouse embryonic fibroblast feeder layer:

Primary cultures of mouse embryonic fibroblasts are obtained from 14 - 16 day old murine fetuses. After the head, liver, heart and alimentary tract are aseptically removed,
20     the embryos are minced and incubated for 30min at 37°C in prewarmed trypsin EDTA solution (0.05% trypsin/0.02% EDTA; GIBCO, Grand Island, NY). Fibroblast cells are plated in in tisssue culture flasks and cultured in alpha-MEM medium (BioWhittaker, Walkersville, MD) supplemented with 10% FCS, penicillin (100IU/ml) and streptomycin (50μl/ml). Three to 4 days after passage, embryonic fibroblasts in 35 x 10 Nunc culture
25     dishes (Baxter Scientific, McGaw Park, IL) are irradiated. The irradiated fibroblasts are grown and maintained in a humidified atmosphere with 5% $CO_2$ in air at 37°C. Culture plates having a uniform monolayer of cells are used to culture embryonic cell lines.

Production of embryonic cell lines:

NT unit cells obtained as described above were washed and plated directly onto irradiated feeder fibroblast cells (see above). These cells included those of the inner portion of the NT unit. The cells are maintained in a growth medium consisting of alpha-

5     MEM supplemented with 10% FCS and 0.1mM β-mercaptoethanol. Growth medium is exchanged every 2 to 3 days. The initial colony is observed by the second or third day of culture. The colony is propagated and exhibits a similar morphology to previously disclosed mouse and bovine embryonic stem (ES) cells. Individual cells within the colony are not well defined and the perimeter of the colony is refractile and smooth in appearance.

10    The cells have an epithelial appearance.


Production of chimeric bovine NT units:

Chimeric NT units are selected and implanted in cows according to the following procedure: 1-5 NT units are implaned in the uterus. The resulting calves are grown to the

15    appropriate stage for oocyte development, at which time the ova are aspirated and isolated. Alternatively, oocytes are obtained from adult females by superovulation and ultrasound-guided oocyte retrieval or from slaughtered cow ovaries.


Production of allochimeric human cells:

20    The chimeric ova obtained as described above are treated as described above for growth and enucleation, and are then ready to be used for receipt of a human nucleus. Human epithelial cells are lightly scraped from the inside of the mouth from a consenting human adult with a standard glass slide. The cells are washed off the slide into a Petri dish containing PBS with Ca or Mg. The cells are pipetted through a small-bore pipette to

25    break up cell clumps into a single cell suspension. The cells are then transferred into a microdrop of TL-HEPES media containing 10% FCS under oil for nuclear transfer to enucleated chimeric bovine oocytes comprising human mitochondria using the procedure described above.

The allochimeric cells may employ nuclei from substantially any convenient human

30    cell source, such a fibroblasts, epithelial cells, keratinocytes, blood lymphocyte or bladder

epithelial cell. The allochimeric cells are grown in culture and directed into various differentiation patterns, as described by Stice et al., (1998), *supra*, including preparation of myocytes.

In accordance with the subject invention allochimeric human cells are provided having a human nucleus and human mitochondria from different individuals. These cells find extensive use in that they provide a simulated natural human cell for studying cellular processes in the development of embryos and differentiation to differentiated cell types. The cells can also be used for producing differentiated cells for cell therapy or production of human factors. The subject cells provide cells which produce only human proteins, so as to be less likely to induce an immune response, permit genetic manipulation of the nucleus, to provide human cells which may be introduced into an individual who is the source of the nucleus, but where the nucleus is modified for therapeutic or other purposes, and provide a source of genotypically identical cells, which may be used for cloning of a particular genotype.

The references cited in this application are incorporated herein by reference as if they had been completely set forth in the specification. All procedures and methods are included as if set forth and are to be made part of the procedures of this application, as applied to the subject matter of this application in accordance with those of skill in the art.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

**WHAT IS CLAIMED IS:**

1.      A method for producing allochimeric cells comprising mitochondria from a first human and a nucleus from a second and different human, said method comprising:

5          enucleating an oocyte from a first human;

introducing a nucleus from a species other than human into said enucleated oocyte to provide a first chimeric oocyte;

expanding said first chimeric cell in culture to provide NT units;

inseminating said ES cell into a compatible female host and allowing said NT units

10    to grow to at least a fetus comprising second chimeric oocytes:

harvesting and isolating at least one of said second chimeric oocytes;

enucleating at least one of said second chimeric oocytes and introducing a human nucleus into said second chimeric oocyte to provide an allochimeric NT unit; and

culturing said allochimeric NT unit in culture to provide allochimeric ES cells.

15

2.      A method according to Claim 1, wherein said allochimeric ES cells are grown in culture under conditions resulting in differentiated cells.

3.      A method according to Claim 2, wherein said differentiated cells are

20    neuronal, muscle or hematopoietic cells.

4.      A method according to Claim 2, wherein said species is ungulate and said female host is ungulate.

25        5.      A method according to Claim 1, wherein said human nucleus is from a differentiated human cell.

6.      A method according to Claim 1, wherein said nucleus from a species other than human is introduced by means of electrofusion.

30

21

7.      A method according to Claim 1, comprising the additional step of genetically modifying said allochimeric ES cells.

8.      A method for producing allochimeric cells comprising mitochondria from a
5   first human and a nucleus from a second and different human, said method comprising:

enucleating an oocyte from a first human;

introducing a nucleus from an ungulate into said enucleated oocyte to provide a first chimeric oocyte;

expanding said first chimeric cell in culture to provide at least 4 ES cells;

10      inseminating said ES cell into an ungulate which is the same as the source of said nucleus and allowing said ES cell to grow to at least a fetus comprising second chimeric oocytes:

harvesting and isolating at least one of said second chimeric oocytes;

enucleating at least one of said second chimeric oocytes and introducing a human
15  nucleus from a differentiated human cell into said second chimeric oocyte to provide an allochimeric oocyte; and

expanding said allochimeric oocyte in culture to provide allochimeric ES cells.

9.      A method according to Claim 8, wherein said allochimeric ES cells are
20  grown in culture under conditions resulting in differentiated cells in the neuronal, muscle or hematopoietic pathways.

10.     A method according to Claim 8, comprising the additional step of genetically modifying said allochimeric ES cells.

25

11.     A method for treating a human host for an indication susceptible to treatment with allochimeric cells, said method comprising:

producing differentiated allochimeric cells according to Claim 2; and

introducing said allochimeric cells into said human host at a site for treating said
30  indication.

12.    A method according to Claim 11, wherein said allochimeric ES cells are genetically modified prior to differentiation.

13.    A method according to Claim 11, wherein said human nucleus is from said human host.

14.    A composition comprising a plurality of allochimeric human ES cells.

15.    A composition according to Claim 14, wherein said allochimeric human ES cells are in culture.

16.    A composition comprising a plurality of differentiated allochimeric cells in other than a human host.

17.    A composition according to Claim 16, wherein said differentiated allochimeric cells are in culture.

18.    A composition according to Claim 16, wherein said differentiated allochimeric cells are in the neuronal, muscle or hematopoietic pathways.

19.    A composition comprising a mixture of allochimeric ES cells and allochimeric differentiated cells in culture.

20.    A composition comprising a plurality of genetically modified allochimeric ES cells as a result of *in vitro* introduction of exogenous DNA into at least one allochimeric ES cell.