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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 06-039628- -00004-0000

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Tra. 11 PATENTEIYII Eve: 379 FASENACIONALII
Act. 538 PETICION Folios: 2

Trámite : 011
Evento : 379
Actuación : 538

Referencia : Expediente No. 06.039.628
Asunto : Solicitud de Patente para **UN PROCESO PARA PRODUCIR PROTEINA EXOGENA EN LA LECHE DE MAMIFEROS TRANSGENICOS Y UN PROCESO PARA PURIFICAR PROTEINAS DE ESTA MANERA**
Solicitante : **STERRENBELD BIOTECHNOLOGIE NORTH AMERICA INC.**
Clase : A61K 067/027, A61P 005/006, C12N 015/000
Publicada en : La Gaceta No. 584 Del 31 de diciembre de 2007
Fecha de Solicitud : 26 de abril de 2006

1. Yo, Emilio FERRERO, identificado como aparece al pie de mi firma, actuando como apoderado en el asunto de la referencia, atentamente, solicito se efectúe el examen de patentabilidad de la solicitud en referencia presentada de acuerdo con el Capítulo II del PCT y según lo establecido en el artículo 44 de la Decisión 486 de la Comisión de la Comunidad Andina. Para tal efecto presento junto con este escrito el recibo por valor de \$210.000.00, que corresponde a la tasa fijada según lo dispuesto en la resolución No. 44372 de 26 de diciembre de 2007.

2. Ruego atentamente a la Señora Jefe se sirva ordenar que se anexe este recibo para que se efectúe dicho examen y se continúe con el trámite de la solicitud.

Señora Jefe,

EMILIO FERRERO
T.P.A. No. 31.929

COLO-15292-841-001-7
ASGAMGVD-129442WPC15292
No. M-2008-129442VSG

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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***** CONSIGNACION *****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PGO	Vr. PAGO
CAVELIER ABOGADOS	CONSIGNACION	BANCO DE BOGOTA	062754387	2678328	09/06/2008	89,445,000.00

***** CONCEPTO *****

CANT.	RENTISTICO	CONCEPTO	TOTAL CONCEPTO
1	50005-01-01	SOLICITUDES	
		114 PTC CAP-II EXAMEN DE PATENTIBILID	210,000.00
		TOTAL :	\$ 210,000.00

SON: DOSCIENTOS DIEZ MIL PESOS

RESPONSABLE :

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DIVISIÓN DE NUEVAS CREACIONES

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EL EXTRACTO DE LA PRESENTE SOLICITUD DE PATENTE DE INVENCION

FUE PUBLICADO EN LA GACETA DE PROPIEDAD INDUSTRIAL No 584 DE

FECHA 31 DE DICIEMBRE DE 2007 EL TÉRMINO HÁBIL SEÑALADO POR LA

LEY EXPIRA EL 31 DE MARZO DE 2008

NOTA: Dentro del plazo de los seis (6) meses siguientes a partir de la fecha de publicación en la gaceta de Propiedad Industrial, deberá solicitar y cancelar el examen de patentabilidad so pena de que la solicitud caiga en abandono, de acuerdo con lo establecido en el Artículo 44 de la Decisión 486/2000.

SE PRESENTARON OPOSICIONES POR PARTE DE TERCEROS.

SI _____

NO X



US006395958B1

(12) United States Patent
Strelchenko et al.**(10) Patent No.: US 6,395,958 B1**
(45) Date of Patent: May 28, 2002**(54) METHOD OF PRODUCING A POLYPEPTIDE**
IN AN UNGULATE**(75) Inventors:** Nikolai S. Strelchenko, DeForest;
Jeffrey M. Betthausen, Windsor; Gail
L. Jurgella, Madison; Marvin M.
Pace, DeForest; Michael D. Bishop,
Rio, all of WI (US)**(73) Assignee:** Infigen, Inc., DeForest, WI (US)**(*) Notice:** Subject to any disclaimer, the term of this
patent is extended or adjusted under 35
U.S.C. 154(b) by 0 days.**(21) Appl. No.: 09/354,276****(22) Filed: Jul. 15, 1999****Related U.S. Application Data****(63)** Continuation of application No. 09/239,922, filed on Jan. 28,
1999, now Pat. No. 6,011,197, and a continuation-in-part of
application No. PCT/US98/04345, filed on Mar. 5, 1998,
and a continuation-in-part of application No. 08/812,851,
filed on Mar. 6, 1997, and a continuation-in-part of appli-
cation No. 08/812,031, filed on Mar. 6, 1997.
(60) Provisional application No. 60/073,019, filed on Jan. 29,
1998.**(51) Int. Cl.⁷ C12P 21/00; A01K 67/027;**
C12N 15/00**(52) U.S. Cl. 800/7; 800/15; 800/16;**
800/17; 800/24**(58) Field of Search 800/24, 8, 14,**
800/15, 16, 17, 18, 4; 435/69.1**(56) References Cited****U.S. PATENT DOCUMENTS**4,227,381 A 10/1980 Sullivan et al. 62/223
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Primary Examiner—Deborah Crouch**(74) Attorney, Agent, or Firm—Foley & Lardner****(57)****ABSTRACT**The present invention relates to cloning technologies. The
invention relates in part to immortalized and totipotent cells
useful for cloning animals, the embryos produced from these
cells using nuclear transfer techniques, animals that arise
from these cells and embryos, and materials, methods, and
processes for establishing such cells, embryos, and animals.**16 Claims, 4 Drawing Sheets**

METHOD OF PRODUCING A POLYPEPTIDE IN AN UNGULATE

This application is a continuation of U.S. application Ser. No. 09/239,922, Jan. 28, 1999 U.S. Pat. No. 6,011,197 which claims benefit of provisional application 60/073,019, filed January 29, 1998, entitled "Cloning of Biological Organisms from Immortalized Totipotent Cells" (pending); and a CIP of U.S. application Ser. No. 08/812,851, filed Mar. 6, 1997, entitled "Method of Cloning Animals" (pending); and a CIP of application Serial No. PCT/US 98/04345, filed Mar. 5, 1998, entitled "Method of Cloning Animals" (pending); and a CIP of U.S. application Ser. No. 08/812,031, filed Mar. 6, 1997, entitled "Method of Cloning Bovines" (pending), each of which is hereby incorporated by reference in its entirety including any drawings, and from each of which priority is claimed.

FIELD OF THE INVENTION

The invention relates to the cloning of animals.

BACKGROUND OF THE INVENTION

The following discussion of the background of the invention is merely provided to aid the reader in understanding the invention and is not admitted to describe or constitute prior art to the present invention.

Researchers have been developing methods for cloning mammalian animals over the past two decades. These reported methods typically include the steps of (1) isolating a cell, most often an embryonic cell; (2) inserting the cell or nucleus isolated from the cell into an enucleated oocyte (e.g., the oocyte's nucleus was previously extracted), and (3) allowing the embryo to mature in vivo.

The first successful nuclear transfer experiment using mammalian cells was reported in 1983, where the pronuclei isolated from a murine (mouse) zygote were inserted into an enucleated oocyte and resulted in like offspring(s). McGrath & Solter, 1983, *Science* 220:1300-1302. Subsequently, others described the production of chimeric murine embryos (e.g., embryos that contain a subset of cells having significantly different nuclear DNA from other cells in the embryo) using murine primordial germ cells (PGC). These cells are and can give rise to pluripotent cells (e.g., cells that can differentiate into other types of cells but do not differentiate into a grown animal). Matsui et al., 1992, *Cell* 70:841-847 and Resnick et al., 1992, *Nature* 359:550; Kato et al., 1994, *Journal of Reproduction and Fertility Abstract Series*, Society For the Study of Fertility, Annual Conference, Southampton, 13:38.

Some publications related to murine pluripotent cells stress the importance of steel factor for converting precursor cells into pluripotent cells. U.S. Pat. Nos. 5,453,357 and 5,670,372, entitled "Pluripotent Embryonic Stem Cells and Methods of Making Same," issued to Hogan. These same publications indicate that murine pluripotent cells exhibit strong, uniform alkaline phosphatase staining.

Although murine animals were never clearly cloned from nuclear transfer techniques using embryonic cells, some progress was reported in the field of cloning ovine (sheep) animals. One of the first successful nuclear transfer experiments utilizing ovine embryonic cells as nuclear donors was reported in 1986. Willadsen, 1986, *Nature* 320:63-65. A decade later, others reported that additional lambs were cloned from ovine embryonic cells. Campbell et al., 1996, *Nature* 380:64-66 and PCT Publication WO 95/20042. Recently, another lamb was reported to be cloned from ovine

somatic mammary tissue. Wilmut et al., 1997, *Nature* 385:810-813. Some methods for cloning ovine animals focused upon utilizing serum deprived somatic ovine cells and cells isolated from ovine embryonic discs as nuclear donors. PCT Publications WO 96/07732 and WO 97/07669. Other methods for cloning ovine animals involved manipulating the activation state of an in vivo matured oocyte after nuclear transfer. PCT Publication WO 97/07668.

While few lambs were produced, publications that disclose cloned lambs report a cloning efficiency that is, at best, approximately 0.4%. Cloning efficiency, as calculated for the previous estimate, is a ratio equal to the number of cloned lambs divided by the number of nuclear transfers used to produce that number of cloned lambs.

Despite the slower progress endemic to the field of cloning bovine animals, a bovine animal was cloned using embryonic cells derived from 2-64 cell embryos. This bovine animal was cloned by utilizing the nuclear transfer techniques set forth in U.S. Pat. Nos. 4,994,384 and 5,057,420. Others reported that cloned bovine embryos were formed by nuclear transfer techniques utilizing the inner cell mass cells of a blastocyst stage embryo. Sims & First, 1993, *Theriogenology* 39:313 and Keefer et al., 1994, *Mol. Reprod. Dev.* 38:264-268. In addition, another publication reported that cloned bovine embryos were prepared by nuclear transfer techniques that utilized PGCs isolated from fetal tissue. Delhaise et al., 1995, *Reprod. Fert. Develop.* 7:1217-1219; Lavoie 1994, *J. Reprod. Dev.* 37:413-424; and PCT application WO 95/10599 entitled "Embryonic Stem Cell-Like Cells." However, the reports demonstrated that cloned PGC-derived bovine embryos never clearly developed past the first trimester during gestation. Similarly, embryonic stem cell (e.g., cell line derived from embryos which are undifferentiated, pluripotent, and can establish a permanent cell line which exhibits a stable karyotype), ESC, derived bovine embryos never developed past fifty-five days, presumably due to incomplete placental development. Stice et al., 1996, *Biol. Reprod.* 54: 100-110.

Despite the progress of cloning ovine and bovine animals, there remains a great need in the art for methods and materials that increase cloning efficiency. In addition there remains a great need in the art to expand the variety of cells that can be utilized as nuclear donors, especially expanding nuclear donors to non-embryonic cells. Furthermore, there remains a long felt need in the art for karyotypically stable permanent cell lines that can be used for genome manipulation and production of transgenic cloned animals.

SUMMARY

The present invention relates to cloning technologies. The invention relates in part to immortalized, totipotent cells useful for cloning animals, the embryos produced from these cells using nuclear transfer techniques, animals that arise from these cells and embryos, and the methods and processes for creating such cells, embryos, and animals.

The present invention provides multiple advantages over the tools and methods currently utilized in the field of mammalian cloning. Such features and advantages include:

(1) Production of cloned animals from virtually any type of cell. The invention provides materials and methods for reprogramming non-totipotent cells into totipotent cells. These non-totipotent cells may be of non-embryonic origin. This feature of the invention allows for the ability to assess the phenotype of an existing animal and then readily establish a permanent cell line for cloning that animal.

(2) Creation of permanent cell lines from virtually any type of cell. Permanent cell lines provide a nearly unlimited

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cloned embryo is subject to manipulation; (3) the manipulation comprises the step of disaggregating at least one individual cell from a cloned embryo; (4) the manipulation comprises the step of utilizing the individual cell as a nuclear donor in a nuclear transfer procedure; (5) the individual cell is disaggregated from the inner cell mass of a blastocyst stage embryo; (6) the individual cell is disaggregated from a pre-blastocyst stage embryo; (7) the manipulation comprises the process of re-cloning; (8) the re-cloning process comprises the steps of: (a) separating the embryo into one or more individual cells, and (b) performing at least one subsequent nuclear transfer between (i) an individual cell of (a), and (ii) an oocyte; (9) the oocyte utilized for the subsequent nuclear transfer is an aged oocyte; (10) the individual cell is placed in the perivitelline space of the enucleated oocyte for the subsequent nuclear transfer; (11) the subsequent nuclear transfer comprises at least one of the steps of translocation, injection, fusion, and activation of the individual cell and/or the enucleated oocyte; (12) one or more cells of the cloned mammalian embryo arising from the subsequent nuclear transfer comprises modified nuclear DNA; and (13) the cloned mammalian embryo arising from the subsequent nuclear transfer may be subject to a subsequent manipulation, where the subsequent manipulation is any of the manipulation steps defined previously herein in relation to immortalized cells and/or cloned embryos.

The term "individual cells" as used herein refers to cells that have been isolated from a cloned mammalian embryo of the invention. An individual single cell can be isolated from the rest of the embryonic mass by techniques well known to those skilled in the art. See, U.S. Pat. Nos. 4,994,384 and 5,957,420, previously incorporated herein by reference in their entireties.

The term "subsequent nuclear transfer" as described herein is also referred to as a "re-cloning" step. Preferably, a re-cloning step can be utilized to enhance nuclear reprogramming during nuclear transfer, such that the product of nuclear transfer is a live born animal. The re-cloning step is distinct, since previous efforts towards re-cloning have been directed to multiplying embryo number and not for enhancement of nuclear reprogramming. The number of subsequent nuclear transfer steps is discussed in greater detail hereafter.

Any of the preferred embodiments related to the translocation, injection, fusion, and activation steps described previously herein relate to the subsequent nuclear transfer step.

The term "inner cell mass" as used herein refers to the cells that gives rise to the embryo proper. The cells that line the outside of a blastocyst are referred to as the trophoblast of the embryo. The methods for isolating inner cell mass cells from an embryo are well known to a person of ordinary skill in the art. See, Sims and First, 1993, *Theriogenology* 39:313; and Keefer et al., 1994, *Mol. Reprod. Dev.* 38:264-268, hereby incorporated by reference herein in their entireties, including all figures, tables, and drawings. The term "pre-blastocyst" is well known in the art and is referred to previously.

The term "aged oocyte" as used herein refers to an oocyte that has been matured in vitro or ovulated in vivo for more than 28 hours since the onset of maturation or ovulation. An aged oocyte can be identified by its characteristically homogenous ooplasm. This appearance is to be contrasted with the pycnotic appearance of young oocytes as described previously herein. The age of the oocyte can be defined by the time that has elapsed between the time that the oocyte is placed in a suitable maturation medium and the time that the

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oocyte is activated. The age of the oocyte can dramatically enhance the efficiency of nuclear transfer.

The term "ovulated in vivo" as used herein refers to an oocyte that is isolated from an animal a certain number of hours after the animal exhibits characteristics that it is in estrus. The characteristics of an animal in estrus are well known to a person of ordinary skill in the art, as described in references disclosed herein.

In another aspect the invention relates to a method for preparing a cloned mammalian embryo. The method comprises the step of a nuclear transfer between: (a) a totipotent mammalian cell, where the cell is immortalized; and (b) an oocyte, where the oocyte is at a stage allowing formation of the embryo. In preferred embodiments, any of the embodiments of the invention concerning cloned mammalian embryos apply to methods for preparing cloned mammalian embryos.

Cloned Fetuses of the Invention

In another aspect, the invention features cloned mammalian fetuses arising from totipotent embryos of the invention. Preferably, the mammalian fetuses are ungulate fetuses, and more preferably, the ungulate fetuses are bovine fetuses. A fetus may be isolated from the uterus of a pregnant female animal.

In preferred embodiments, (1) one or more cells of the fetuses harbor modified nuclear DNA (defined previously herein); and (2) the fetuses may be subject to any of the manipulations defined herein. For example, one manipulation may comprise the steps of isolating a fetus from the uterus of a pregnant female animal, isolating a cell from the fetus (e.g., a primordial germ cell), and utilizing the isolated cell as a nuclear donor for nuclear transfer.

Other aspects of the invention feature (1) a cloned mammalian fetus prepared by a process comprising the steps of (a) preparation of a cloned mammalian embryo defined previously, and (b) manipulation of the cloned mammalian embryo such that it develops into a fetus; (2) a method for preparing a cloned mammalian fetus comprising the steps of (a) preparation of a cloned mammalian embryo defined previously, and (b) manipulation of the cloned mammalian embryo such that it develops into a fetus; (3) a method of using a cloned fetus of the invention comprising the step of isolating at least one cell type from a fetus (e.g., for creating a feeder cell layer); and (4) a method of using a cloned fetus of the invention comprising the step of separating at least one part of a fetus into individual cells (e.g., for establishing a feeder cell layer).

Cloned Animals of the Invention

In another aspect the invention features a cloned mammalian animal arising from a cloned embryo of the invention. The embryo is totipotent and can arise from any of the processes or methods described previously herein.

In preferred embodiments, the cloned mammalian animal (1) is preferably a cloned ungulate animal and more preferably a cloned bovine animal; and (2) is equal in age or older than an animal selected from the group consisting of pre- and post-pubertal animals.

In yet another aspect the invention relates to a cloned mammalian animal, where the animal is one member of a plurality of animals, and where the plurality of animals have a substantially similar nuclear DNA sequence. The term "substantially similar" in relation to nuclear DNA sequences is defined previously herein.

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In preferred embodiments, (1) the plurality consists of five or more animals, ten or more animals, one-hundred or more animals, and one-thousand or more animals; and (2) the plurality of animals can have an identical nuclear DNA sequence. The term "identical" in reference to nuclear DNA sequences is described previously herein.

In another aspect, the invention relates to a cloned mammalian animal having one or more cells that comprise modified nuclear DNA. All of the preferred embodiments relating to modified nuclear DNA described previously apply to cloned bovine animals of the invention.

In yet another aspect, the invention features a method of using a cloned mammalian animal, comprising the step of isolating at least one component from the mammalian animal.

The term "component" as used herein refers to any portion of an animal. A component can be selected from the group consisting of fluid, biological fluid, cell, tissue, organ, gamete, embryo, and fetus. Precursor cells may arise from fluids, biological fluids, cells, tissues, organs, gametes, embryos, and fetuses isolated from cloned organisms of the invention.

The term "gamete" as used herein refers to any cell participating, directly or indirectly, to the reproductive system of an animal. Examples of gametes are spermatocytes, spermatogonia, oocytes, and oögonia. Gametes can be present in fluids, tissues, and organs collected from animals (e.g., sperm is present in semen). For example, methods of collecting semen for the purposes of artificial insemination are well known to a person of ordinary skill in the art. See, e.g., *Physiology of Reproduction and Artificial Insemination of Cattle* (2nd edition), Salisbury et al., copyright 1961, 1978, WH Freeman & Co., San Francisco. However, the invention relates to the collection of any type of gamete from an animal.

The term "tissue" is defined previously. The term "organ" refers to any organ isolated from an animal or any portion of an organ. Examples of organs and tissues are neuronal tissue, brain tissue, spleen, heart, lung, gallbladder, pancreas, testis, ovary and kidney. These examples are not limiting and the invention relates to any organ and any tissue isolated from a cloned animal of the invention.

In a preferred embodiment, the invention relates to (1) fluids, biological fluids, cells, tissues, organs, gametes, embryos, and fetuses can be subject to manipulation; (2) the manipulation can comprise the step of cryopreserving the gametes, embryos, and/or fetal tissues; (3) the manipulation can comprise the step of thawing the cryopreserved items; (4) the manipulation can comprise the step of separating the semen into X-chromosome bearing semen and Y-chromosome bearing semen; (5) the manipulation comprises methods of preparing the semen for artificial insemination; (6) the manipulation comprises the step of purification of a desired polypeptide(s) from the biological fluid or tissue; (7) the manipulation comprises concentration of the biological fluids or tissues; and (8) the manipulation can comprise the step of transferring one or more cloned cells, cloned tissues, cloned organs, and/or portions of cloned organs to a recipient organism (e.g., the recipient organism may be of a different specie than the donor source).

The term "separating" as used herein in reference to separating semen refers to methods well known to a person skilled in the art for fractionating a semen sample into sex-specific fractions. This type of separation can be accomplished by using flow cytometers that are commercially available. Methods of utilizing flow cytometers from sepa-

rating sperm by genetic content are well known in the art. In addition, semen can be separated by its sex-associated characteristics by other methods well known to a person of ordinary skill in the art. See, U.S. Pat. Nos. 5,439,362, 5,346,990, and 5,021,244, entitled "Sex-Associated Membrane Proteins and Methods for Increasing the Probability that Offspring Will Be of a Desired Sex," Spaulding, issued on Aug. 8, 1995, Sep. 13, 1994, and Jun. 4, 1991 respectively, all of which are incorporated herein by reference in their entireties including all figures, tables, and drawings.

Semen-preparation methods are well known to someone of ordinary skill in the art. Examples of these preparative steps are described in *Physiology of Reproduction and Artificial Insemination of Cattle* (2nd. edition), Salisbury et al., copyright 1961, 1978, W. H. Freeman & Co., San Francisco.

The term "purification" as used herein refers to increasing the specific activity of a particular polypeptide or polypeptides in a sample. In preferred embodiments, specific activity is expressed as the ratio between the activity of the target polypeptide and the concentration of total polypeptide in the sample. Activity can be catalytic activity and/or binding activity, for example. In other preferred embodiments, specific activity is expressed as the ratio between the concentration of the target polypeptide and the concentration of total polypeptide. Purification methods include dialysis, centrifugation, and column chromatography techniques, which are well-known procedures to a person of ordinary skill in the art. See, e.g., Young et al., 1997, "Production of biopharmaceutical proteins in the milk of transgenic dairy animals," *BioPharm* 10(6): 34-38.

The term "transferring" as used herein refers to shifting a group of cells, tissues, organs, and/or portions of organs to an animal. The cells, tissues, organs, and/or portions of organs can be, for example, (a) developed in vitro and then transferred to an animal, (b) removed from an animal and transferred to another animal of a different specie, (c) removed from an animal and transferred to another animal of the same specie, (d) removed from one portion of an animal (e.g., the leg of an animal) and then transferred to another portion of the same animal (e.g., the brain of the animal), and/or (e) any combination of the foregoing. The term "transferring" refers to adding cells, tissues, and/or organs to an animal and can also relate to removing cells, tissues, and/or organs from an animal and replacing them with cells, tissues, and/or organs from another source.

The term "transferring" as used herein also refers to implanting one or more cells, tissues, organs, and/or portions of organs from the cloned mammalian animal into another organism. For example, neuronal tissue from a cloned mammalian organism can be grafted into an appropriate area in the human nervous system to treat neurological diseases such as Alzheimer's disease. Alternatively, cloned cells, tissues, and/or organs originating from a porcine organism may be transferred to a human recipient. Surgical methods for accomplishing this preferred aspect of the invention are well known to a person of ordinary skill in the art. Transferring procedures may include the step of removing cells, tissues, or organs from a recipient organism before a transfer step.

In other aspects the invention features (1) a cloned mammalian animal prepared by a process comprising the steps of: (a) preparation of a cloned mammalian embryo by any one of the methods described herein for producing such a cloned mammalian embryo, and (b) manipulation of the

cloned mammalian embryo such that it develops into a live born animal; (2) a process comprising the steps of: (a) preparation of a cloned mammalian embryo by any one of the methods described herein for preparing such a cloned mammalian embryo, and (b) manipulation of the cloned mammalian embryo such that it develops into a live born animal; and (3) a cloned mammalian animal, comprising the steps of: (a) preparation of a cloned mammalian embryo by any one of the methods for producing such an embryo described herein, and (b) manipulation of the cloned mammalian embryo such that it develops into a live born animal.

In preferred embodiments, (1) the live born animal is preferably an ungulate animal and more preferably a bovine animal; (2) the manipulation can comprise the step of implanting the embryo into a uterus of an animal; (3) the estrus cycle of the animal can be synchronized to the developmental stage of the embryo; and (4) the manipulation can comprise the step of implanting the embryo into an artificial environment.

The summary of the invention described above is not limiting and other features and advantages of the invention will be apparent from the following detailed description of the preferred embodiments, as well as from the claims.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 illustrates multiple embodiments of the invention relating to the generation of immortalized, totipotent cells from precursor cells. The figure indicates that immortalized, totipotent cells can arise from embryonic stem cells, primordial germ cells, and cells isolated from an animal. The precursor cell sources illustrated by FIG. 1 are not limiting and other precursor cell sources are described herein.

FIGS. 2(A-D) illustrates an embodiment of the invention related to cloning. The figure illustrates a cloning procedure in which (a) a precursor cell is reprogrammed into an immortalized, totipotent cell; (b) the immortalized, totipotent cell is utilized as a donor for a first nuclear transfer, which utilizes a young oocyte; (c) the embryo arising from the first nuclear transfer is cultured; (d) a cell isolated from the embryo arising from the first nuclear transfer is utilized as a nuclear donor for a second nuclear transfer, which utilizes an aged oocyte; and (e) the embryo resulting from the second nuclear transfer may be cultured and then allowed to develop into a live born animal. The embryos resulting from the nuclear transfers may be cultured and/or cryopreserved and thawed.

FIG. 3 illustrates multiple embodiments of the invention related to pathways for establishing totipotent cell lines and cloned animals. Fibroblast cells can be isolated from any source described herein. This figure is described in further detail in the Examples section.

FIG. 4 illustrates multiple embodiments of the invention for creating cloned transgenic cell lines and cloned transgenic animals. This figure is described in further detail in the Examples section.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention relates to cloning technologies. The present invention provides multiple advantages over the tools and methods currently utilized in the field of cloning technology. For example, the invention relates in part to immortalized, totipotent cells useful for cloning animals. These immortalized, totipotent cells can give rise to methods of producing cloned animals by utilizing virtually any type

of cell. For example, cells isolated from a live born animal can be reprogrammed into immortalized, totipotent cells. This feature of the invention provides the ability to assess the phenotype of an existing animal and then readily establish a permanent cell line for cloning that animal. As described previously herein, no methods in the art have allowed for such advantages.

In addition, the immortalized, totipotent cells of the invention allow for creating permanent cell lines from virtually any type of cell. This reprogramming method is described previously herein. These permanent cell lines offer a nearly unlimited source of donor cells for nuclear transfer cloning techniques. Moreover, this feature provides the advantage of enhancing cloning efficiency due to the lower differentiation rates of these cell lines than existing cell lines used for cloning. For example, embryonic stem cell lines can harbor multiple colonies of cells that are not totipotent. The totipotent, immortalized cells of the invention harbor a higher percentage of totipotent cells than cell lines previously reported.

Moreover, the methods and processes for creating the immortalized, totipotent cells, totipotent cloned embryos, and cloned animals of the invention demonstrate the enhanced cloning efficiency over cloning tools and techniques previously reported. In particular, the totipotent, immortalized cell lines and the refined nuclear transfer techniques of the invention provide for this enhanced cloning efficiency. This enhanced efficiency satisfies a long felt need in the art.

I. Immortalized and Totipotent Cells

A. Generation of Immortalized and Totipotent Cells

Immortalized, totipotent cells of the invention can be produced from virtually any type of precursor cell. Preferred embodiments of the invention relate to the following types of precursor cells: (1) embryos arising from the union of two gametes in vitro or in vivo; (2) embryonic stem cells (ESCs) arising from embryos (e.g., pre-blastocyst cells and inner cell mass cells); (3) cultured and non-cultured cells derived from the inner cell mass of embryos; (4) cultured and non-cultured cells arising from a fetus; (5) primordial germ cells arising from a developing cell mass (e.g., genital ridge cells); (6) immortalized cultured cells arising from primordial germ cells, where the immortalized cells are referred to as embryonic germ cells (EGCs) in the art; (7) cultured and non-cultured cells obtained from amniotic fluid; (8) cultured and non-cultured cells arising from an ovarian follicle (e.g., cumulus cells); (9) cultured and non-cultured cells arising from a liver (e.g., hepatocytes); and (10) cultured and non-cultured cells isolated from an animal.

ESCs and EGCs can be readily generated from methods known in the art. See, e.g., Stice et al., 1996, *Biology of Reproduction* 54: 100-110, hereby incorporated by reference herein in its entirety including all figures, tables, and drawings. See also, Strelchenko, 1996, *Theriogenology* 45: 130-141. ESCs have been demonstrated to give rise to fetuses, from which primordial germ cells and EGCs can be derived. Therefore, ESCs are a nearly unlimited source for primordial germ cells and EGCs.

Cells derived from an animal can be isolated from nearly any type of tissue. For example, an ear-punch can be taken from an animal, the cells from the sample can be separated, and the separated cells can be subsequently cultured in vitro by using cell culture techniques well known to a person of ordinary skill in the art. Preferably, cells of the invention are extracted from bovine animals. Examples of materials and methods for reprogramming primary culture cells into

immortalized, totipotent cells are described in exemplary embodiments hereafter.

Although exemplary embodiments of the invention are directed to bovine animals, materials and methods of the invention can be applied to the generation of immortalized, totipotent cells using precursor cells isolated from any mammal. Preferably immortalized, totipotent cells are extracted from ungulates. Examples of preferred ungulates envisioned for the invention are described previously.

Immortalized, totipotent cells of the invention are preferably generated from the examples of cells indicated in the preceding paragraph after treatment with a receptor ligand cocktail. Examples of receptor ligands are well known to a person of ordinary skill in the art. Cytokines and/or growth factors are preferred receptor ligands of the invention. See, e.g., R&D Systems Catalog, 614 McKinley Place N.E., Minneapolis, Minn. 55413. In exemplary embodiments, varying amounts of human recombinant leukemia inhibitory factor (hrLIF) and basic bovine fibroblast growth factor (bFGF) can be added to the culture medium to reprogram the precursor cells into immortalized, totipotent cells. Varying concentrations of these two cytokines can be added to the culture medium, preferably in concentrations of 1–1000 ng/mL, more preferably in concentrations between 10–500 ng/mL, and most preferably about 100 ng/mL. Exogenous soluble and membrane-associated forms of steel factor are not required for converting precursor cells into totipotent, immortalized cells.

These examples are not meant to be limiting and any cytokine or combination of cytokines can be added or deleted from those described in exemplary embodiments described hereafter. Preferred cytokines for generating immortalized, totipotent cells can be selected from the group consisting of fibroblast growth factor (FGF), leukemia inhibitor factor (LIF), cardiotrophin 1 (CT-1), ciliary neurotrophic factor (CNTF), stem cell factor (SCF), oncostatin M (OSM), and any member of the interleukin (IL) family, including IL-6, IL-11, and IL-12.

Other cytokines and other molecules besides cytokines can be added or deleted from the receptor ligand cocktail described in the exemplary embodiments described hereafter to create immortalized, totipotent cells from any of the cells described in the previous paragraph. Any of the conditions for generating immortalized, totipotent cells can be modified from those described herein. The ability of these modified conditions to generate immortalized, totipotent cells can be monitored by methods defined in the section "Identification of Immortalized and Totipotent Cells" described hereafter.

B. Culturing Immortalized and Totipotent Cells

A variety of methods for culturing cells exist in the art. See, e.g., *Culture of animal cells: a manual of basic technique* (2nd. edition), Freshney, copyright 1987, Alan R. Liss, Inc., New York. Particularly the cells that are precursor cells for immortalized, totipotent cells, as well as the immortalized, totipotent cells themselves, can be grown on feeder layers. Examples of feeder layers are well known to a person of ordinary skill in the art, and can arise from a number of different cells that are cultured *in vitro*. See, e.g., Strelchenko, 1996, *Theriogenology* 45: 130–141, as well as exemplary embodiments described hereafter. However, precursor cells for immortalized, totipotent cells as well as the immortalized, totipotent cells themselves need not be grown on feeder layers.

C. Identification of Immortalized and Totipotent Cells

Identification of Immortalized Cells

Immortalized cells can be identified as those that are not confined to the Hayflick limit. The Hayflick limit is defined

by cells that divide for more than 60 cell divisions. Hence, cells that have divided for more than 60 cell divisions are immortalized cells. In addition, immortalized cells typically can be passaged at lower cell densities than non-immortalized cells.

The materials and methods described above (e.g. culturing the cells with cytokines) can convert non-immortalized cells into immortalized cells. Other methods exist in the art for generating immortalized cell lines from primary cells. For example, manipulating the activity of telomerase within the cells can immortalize cells. See, e.g., U.S. Pat. No. 5,645,986, entitled "Therapy and Diagnosis of Conditions Related to Telomere Length and/or Telomerase Activity," West et al., issued Jul. 8, 1997, and hereby incorporated by reference herein in its entirety including all figures, drawings, and tables.

Moreover, cellular immortality can be determined by identifying both low molecular weight and macromolecular markers that are specific for immortalized cells. The existence or lack of existence of a marker can be a determination of cell immortalization. In addition, a phenomenon associated with a marker can be an indication of immortality. For example, if the marker is an enzyme, an indication of the presence of the enzyme and/or a certain level of catalytic activity of that enzyme may be a suitable indication that a certain cell type is immortalized.

Low molecular weight markers include specific nucleosides, lipid associated sialic acids, polyamines, and pseudouridine. These examples are not limiting and the invention relates to any other low molecular weight markers known in the art.

Macromolecular markers can be separated into several classes including nucleic acid polymers, peptides, polypeptides, proteins, enzymes, growth factors, growth factor receptors, hormones, hormone receptors, oncogenes, oncogene products, and specific glycoproteins. Macromolecular markers can be selected from the group consisting of extracellular proteins, membrane associated proteins, and/or intracellular proteins, which may be membrane associated or soluble. One such marker for immortalized cells is telomerase or its associated activity, for example. See, U.S. Pat. No. 5,645,986, *supra*. Other examples of markers specific for immortalized cells can be selected from the following list:

- 1) Epidermal growth factor (EGF) and its receptor (EGF-R)
- 2) Transforming growth factor-alpha (TGF-alpha) and its receptor
- 3) c-erbB2 receptor tyrosine kinase (HER2 product)
- 4) Hyaluronan receptor (probably CD44, an integral membrane glycoprotein)
- 5) Carcinoembryonic antigen (CEA) family of tumor markers (for example T1, a glycosylated protein)
- 6) Telomerase, a ribonucleoprotein which maintains telomere length in immortalized cells
- 7) Phosphatases: placental alkaline phosphatase (PLAP), germ cell alkaline phosphatase, prostate acid phosphatase (PAS)
- 8) Cathepsin D (catalyzes degradation of laminin).
- 9) Ornithine decarboxylase (ODC) (catalyzes the rate-limiting step in polyamine synthesis)
- 10) Beta-glucuronidase
- 11) Alpha-6 integrin
- 12) Keratin K8
- 13) Oncogene products: ras oncogenes (k-ras, Ha-ras, p21), v-src, c-myc
- 14) Cyclin D1, cyclin A, and Retinoblastoma Gene Protein (Rb)

- 15) Changes in p53 expression or p53 mutations
- 16) Heterogeneous ribonucleoprotein-A2 (hnRNP-A2) overexpression
- 17) L-plastin
- 18) Ganglioside fucosyl-GM1
- 19) Mob-1 expression (mob-1) (homology to proinflammatory cytokines) These examples are not limiting and the invention relates to any markers specific for immortalized cells that are known in the art.

In addition to markers for immortalization known in the art, markers for immortalization can be identified using methods well known in the art. For example, immortalization markers can be identified by analyzing particular molecules (e.g., nucleic acid molecules and polypeptide molecules) that are unique to specific cell types.

In examples pertaining to nucleic acid immortalization markers, immortalized and non-immortalized cells may be subjected to analysis for nucleic acid sequence content (e.g., hybridization techniques with nucleic acid probes). Nucleic acid samples from particular immortalized cells and nucleic acid samples from particular non-immortalized cells can be screened for particular nucleic acid sequences. If samples from non-immortalized cells lack a nucleic acid sequence present in immortalized cells, then this nucleic acid sequence could be a marker for distinguishing immortalized cells from non-immortalized cells. Similarly, if samples from non-immortalized cells harbor a nucleic acid sequence that immortalized cells lack, this nucleic acid sequence could be a marker for distinguishing immortalized cells from non-immortalized cells. Similar methods can elucidate polypeptide markers by utilizing polypeptide analytical techniques (e.g., PAGE, SDS-PAGE, procedures comprising antibodies, and HPLC techniques known in the art).

Identification of Totipotent Cells

Totipotent cells can be identified by a number of tests. Examples of these tests include:

- (1) identifying a marker specific for totipotent cells;
- (2) performing one or more nuclear transfer cycles with a cell (as described hereafter) and developing the resulting embryo into an animal.

Markers can be utilized to distinguish totipotent cells from non-totipotent cells. Markers can be selected from the group of low molecular weight markers, macromolecular markers, cell surface markers, and intracellular markers. Examples of markers that may be suitable for identifying totipotent cells can be selected from the group consisting of alkaline phosphatase, cytokeratin, vimentin, laminin, and c-kit. These markers are well known to a person of ordinary skill in the art and these examples are not meant to be limiting.

Some of these markers have been tested for cultured bovine cells being identified for totipotency. As noted previously, totipotent, immortalized bovine cells of the invention generally do not appreciably stain for alkaline phosphatase. Therefore the cells of the invention are to be contrasted with pluripotent cells discussed in previously referenced publications. It should be noted that some of the exemplary markers listed previously may not be specific for totipotent cells as some of these markers may exist in pluripotent cells as well as in totipotent cells. For example, although immortalized, totipotent bovine cells do not appreciably stain for alkaline phosphatase, immortalized, totipotent porcine cells may appreciably stain for alkaline phosphatase. The invention relates to any markers specific for totipotent cells that are known to a person of ordinary skill in the art.

Markers for totipotency that are not clearly defined in the art can be elucidated by processes defined in the previous section, which illustrates methods for elucidating immortalization cell markers.

- 5 A preferred test for determining totipotency of cells is determining whether cells give rise to totipotent embryos and eventually cloned animals. This test represents a definitive test for cellular totipotency. An example of such a test includes the following steps: (1) utilizing a potentially totipotent cell for nuclear transfer with an enucleated oocyte; (2) allowing the resulting cybrid to develop; (3) separating an embryo that developed from the cybrid into individual cells and subjecting one or more of the individual cells to a second round of nuclear transfer; (4) allowing a resulting cybrid from step (3) to develop into an embryo; (5) implanting the embryo from step (2) or (4) into a uterine environment; and (6) allowing the embryo to develop. If the ensuing fetus develops past the first trimester of pregnancy then the cells initially used for nuclear transfer are most likely totipotent cells. If the cells utilized for nuclear transfer develop into a live born cloned animal then the cells are definitively totipotent. Examples of the techniques utilized for this exemplary test (e.g., enucleation of oocytes and nuclear transfer) are described completely in the art and in exemplary embodiments defined hereafter.

Hence, the materials and methods provided herein are the first to feature immortalized, totipotent cells for cloning a bovine animal. As described above these materials and methods can be applied to other ungulates due to the high degree of nuclear DNA sequence homology among ungulates. Using the tests for identifying immortalized, totipotent cells, the methods and materials described herein can be modified by a person of ordinary skill in the art to produce immortalized, totipotent cells from any type of precursor cell. Hence, the invention covers any of the materials and methods described herein as well as modifications to these methods for generating immortalized, totipotent cells, since a person of ordinary skill in the art can readily produce immortalized, totipotent cells by utilizing the materials and methods described herein in conjunction with methods for identifying immortalized, totipotent cells.

II. Transgenic Immortalized and Totipotent Cells

Materials and methods readily available to a person of ordinary skill in the art can be utilized to convert immortalized, totipotent cells of the invention into transgenic immortalized, totipotent cells. Once the nuclear DNA is modified in the immortalized, totipotent cells of the invention, embryos and animals arising from these cells can also comprise the modified nuclear DNA. Hence, materials and methods readily available to a person of ordinary skill in the art can be applied to the immortalized, totipotent cells of the invention to produce transgenic animals and chimeric animals. See, e.g., EPO 264 166, entitled "Transgenic Animals Secreting Desired Proteins Into Milk"; WO 94/19935, entitled "Isolation of Components of Interest From Milk"; WO 93/22432, entitled "Method for Identifying Transgenic Pre-implantation Embryos"; and WO 95/17085, entitled "Transgenic Production of Antibodies in Milk," all of which are incorporated by reference herein in their entirety including all figures, drawings and tables.

Methods for generating transgenic cells typically include the steps of (1) assembling a suitable DNA construct useful for inserting a specific DNA sequence into the nuclear genome of a cell; (2) transfecting the DNA construct into the cells; (3) allowing random insertion and/or homologous recombination to occur. The modification resulting from this

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process may be the insertion of a suitable DNA construct(s) into the target genome; deletion of DNA from the target genome; and/or mutation of the target genome.

DNA constructs can comprise a gene of interest as well as a variety of elements including regulatory promoters, insulators, enhancers, and repressors as well as elements for ribosomal binding to the RNA transcribed from the DNA construct. DNA constructs can also encode ribozymes and anti-sense DNA and/or RNA, identified previously herein. These examples are well known to a person of ordinary skill in the art and are not meant to be limiting.

Due to the effective recombinant DNA techniques available in conjunction with DNA sequences for regulatory elements and genes readily available in data bases and the commercial sector, a person of ordinary skill in the art can readily generate a DNA construct appropriate for establishing transgenic cells using the materials and methods described herein.

Transfection techniques are well known to a person of ordinary skill in the art and materials and methods for carrying out transfection of DNA constructs into cells are commercially available. Materials typically used to transfect cells with DNA constructs are lipophilic compounds, such as Lipofectin™ for example. Particular lipophilic compounds can be induced to form liposomes for mediating transfection of the DNA construct into the cells.

Target sequences from the DNA construct can be inserted into specific regions of the nuclear genome by rational design of the DNA construct. These design techniques and methods are well known to a person of ordinary skill in the art. See, U.S. Pat. No. 5,633,067, "Method of Producing a Transgenic Bovine or Transgenic Bovine Embryo," DeBoer et al., issued May 27, 1997; U.S. Pat. No. 5,612,205, "Homologous Recombination in Mammalian Cells," Kay et al., issued Mar. 18, 1997; and PCT publication WO 93/22432, "Method for Identifying Transgenic Pre-Implantation Embryos," both of which are incorporated by reference herein in their entirety, including all figures, drawings, and tables. Once the desired DNA sequence is inserted into the nuclear genome, the location of the insertion region as well as the frequency with which the desired DNA sequence has inserted into the nuclear genome can be identified by methods well known to those skilled in the art.

Once the transgene is inserted into the nuclear genome of the immortalized, totipotent cell, that cell can be used as a nuclear donor for cloning a transgenic animal. A description of the embodiments related to transgenic animals are described in more detail hereafter.

A. Diseases and Parasites

Desired DNA sequences can be inserted into the (nuclear cellular) genome to enhance the resistance of a cloned transgenic animal to particular parasites and diseases. Examples of parasites include worms, flies, ticks, and fleas. Examples of infectious agents include bacteria, fungi, and viruses. Examples of diseases include Johnes's, BVD, tuberculosis, foot and mouth, BLV, BSE and brucellosis. These examples are not limiting and the invention relates to any disease or parasite or infectious agent known in the art. See, e.g., *Hagan & Bruners Infectious Diseases of Domestic Animals* (7th edition), Gillespie & Timoney, copyright 1981, Cornell University Press, Ithaca N.Y.

A transgene can confer resistance to a particular parasite or disease by completely abrogating or partially alleviating the symptoms of the disease or parasitic condition, or by producing a protein which controls the parasite or disease.

B. Elements of DNA Constructs and Production of DNA Constructs

A wide variety of transcriptional and translational regulatory sequences may be employed, depending upon the nature of the host. The transcriptional and translational regulatory signals may be derived from viral sources, such as adenovirus, bovine papilloma virus, cytomegalovirus, simian virus, or the like, whereas the regulatory signals are associated with a particular gene sequence possessing potential for high levels of expression. Alternatively, promoters from mammalian expression products, such as actin, casein, alpha-lactalbumin, uroplakin, collagen, myosin, and the like, may be employed. Transcriptional regulatory signals may be selected which allow for repression or activation, so that expression of the gene product can be modulated. Of interest are regulatory signals which can be repressed or initiated by external factors such as chemicals or drugs. Other examples of regulatory elements are described herein.

C. Examples of Preferred Recombinant Products

A variety of proteins and polypeptides can be encoded by a gene harbored within a DNA construct suitable for creating transgenic cells. Those proteins or polypeptides include hormones, growth factors, enzymes, clotting factors, apolipoproteins, receptors, drugs, pharmaceuticals, bioceuticals, nutraceuticals, oncogenes, tumor antigens, tumor suppressors, cytokines, viral antigens, parasitic antigens, bacterial antigens and chemically synthesized polymers and polymers biosynthesized and/or modified by chemical, cellular and/or enzymatic processes. Specific examples of these compounds include proinsulin, insulin, growth hormone, androgen receptors, insulin-like growth factor I, insulin-like growth factor II, insulin growth factor binding proteins, epidermal growth factor, TGF- α , TGF- β , dermal growth factor (PDGF), angiogenesis factors (acidic fibroblast growth factor, basic fibroblast growth factor and angiogenin), matrix proteins (Type IV collagen, Type VII collagen, laminin), oncogenes (ras, fos, myc, erb, src, sis, jun), E6 or E7 transforming sequence, p53 protein, cytokine receptor, IL-1, IL-6, IL-8, IL-2, α , β , or γ IFN, GM-CSF, G-CSF, viral capsid protein, and proteins from viral, bacterial and parasitic organisms. Other specific proteins or polypeptides which can be expressed include: phenylalanine hydroxylase, α -1-antitrypsin, cholesterol-7 α -hydroxylase, truncated apolipoprotein B, lipoprotein lipase, apolipoprotein E, apolipoprotein A1, LDL receptor, scavenger receptor for oxidized lipoproteins, molecular variants of each, VEGF, and combinations thereof. Other examples are clotting factors, apolipoproteins, drugs, tumor antigens, viral antigens, parasitic antigens, monoclonal antibodies, and bacterial antigens. One skilled in the art readily appreciates that these proteins belong to a wide variety of classes of proteins, and that other proteins within these classes can also be used. These are only examples and are not meant to be limiting in any way.

It should also be noted that the genetic material which is incorporated into the cells from DNA constructs includes (1) nucleic acid sequences not normally found in the cells; (2) nucleic acid molecules which are normally found in the cells but not expressed at physiological significant levels; (3) nucleic acid sequences normally found in the cells and normally expressed at physiological desired levels; (4) other nucleic acid sequences which can be modified for expression in cells; and (5) any combination of the above.

In addition, DNA constructs may become incorporated into the nuclear DNA of cells, where the incorporated DNA can be transcribed into ribonucleic acid molecules that can cleave other RNA molecules at specific regions. Ribonucleic

acid molecules which can cleave RNA molecules are referred to in the art as ribozymes, which are RNA molecules themselves. Ribozymes can bind to discrete regions on a RNA molecule, and then specifically cleave a region within that binding region or adjacent to the binding region. Ribozyme techniques can thereby decrease the amount of polypeptide translated from formerly intact message RNA molecules.

Furthermore, DNA constructs can be incorporated into the nuclear complement of cells and when transcribed produce RNA that can bind to both specific RNA or DNA sequences. The nucleic acid sequences are utilized in anti-sense techniques, which bind to the message (mRNA) and block the translation of these messages. Anti-sense techniques can thereby block or partially block the synthesis of particular polypeptides in cells.

III. Nuclear Transfer

Nuclear transfer (NT) techniques using non-immortalized and non-totipotent cells are well known to a person of ordinary skill in the art. See, U.S. Pat. No. 4,994,384 (Prather et al.) and U.S. Pat. No. 5,057,420 (M4assey et al.). All of the advantages inherent to using the immortalized, totipotent cells as described above are also advantages for NT techniques, specifically the fact that the immortalized, totipotent cells are a nearly unlimited source of nuclear donors and that these cells increase the efficiency of NT. Exemplary embodiments define a two-cycle NT technique that provides for efficient production of totipotent bovine embryos. This technique can be applied to any mammal, preferably ungulates.

A. Nuclear Donors

Immortalized, totipotent cells of the invention can be used as nuclear donors in a NT process for generating a cloned embryo. As described above, the immortalized, totipotent cells can be generated from nearly any type of cell. For NT techniques, a donor cell may be separated from a growing cell mass or isolated from a cell line. The entire cell may be placed in the perivitelline space of a recipient oocyte or may be directly injected into the recipient oocyte by aspirating the nuclear donor into a needle, placing the needle into the recipient oocyte, releasing the nuclear donor and removing the needle without significantly disrupting the plasma membrane of the oocyte. Alternatively, a nucleus (karyoplast) may be isolated from a nuclear donor and placed into the perivitelline space of or injected directly into the recipient oocyte, for example.

B. Recipient Oocytes

A recipient oocyte is typically an oocyte with a portion of its ooplasm removed, where the removed ooplasm comprises the oocyte nucleus. Enucleation techniques are well known to a person of ordinary skill in the art. See e.g., U.S. Pat. Nos. 4,994,384 and 5,057,420.

Oocytes can be isolated from either oviducts and/or ovaries of live animals by oviductal recovery procedures or transvaginal oocyte recovery procedures well known in the art and described herein. Furthermore, oocytes can be isolated from deceased animals. For example, ovaries can be obtained from abattoirs and the oocytes aspirated from these ovaries. The oocytes can also be isolated from the ovaries of a recently sacrificed animal or when the ovary has been frozen and/or thawed.

Oocytes can be matured in a variety of media well known to a person of ordinary skill in the art. One example of such a medium suitable for maturing oocytes is depicted in an exemplary embodiment described hereafter. Oocytes can be successfully matured in this type of medium within an

environment comprising 5% CO₂ at 39° C. Oocytes may be cryopreserved and then thawed before placing the oocytes in maturation medium. Cryopreservation procedures for cells and embryos are well known in the art as discussed herein.

The nuclear donor may be incorporated into either a young or an aged oocyte. The age of the oocyte can be determined by the time that has elapsed since the oocyte was placed in maturation medium and the time it was activated. A young oocyte can be defined as an oocyte that is cultured in vitro less than 28 hours before activation. An aged oocyte is defined as an oocyte that is cultured in vitro for more than 28 hours before activation.

The age of the oocytes can be functionally identified by the appearance of their ooplasm. For example, because certain cellular materials have not yet dispersed within the ooplasm of a young oocyte, young oocytes have a pycnotic appearance. Aged oocytes, in comparison, are characterized by a more homogeneous cytoplasm. A publication discussing the use of aged oocytes for NT is WO 97/07662, entitled "Inactivated Oocytes as Cytoplast Recipients for Nuclear Transfer."

The nuclear donor cell and the recipient oocyte can arise from the same specie or different species. For example, a bovine immortalized, totipotent cell can be inserted into a bovine enucleated oocyte. Alternatively, an immortalized, totipotent cell derived from a bison can be inserted into a bovine enucleated oocyte. Any nuclear donor/recipient oocyte combinations are envisioned by the invention. Preferably the nuclear donor and recipient oocyte arise from one specie or different species of ungulates. Cross-species NT techniques can be utilized to produce cloned animals that are endangered.

The oocytes can be activated by electrical and/or non-electrical means before, during, and/or after fusion of the nuclear donor and recipient oocyte. For example, the oocyte can be placed in a medium containing one or more components suitable for non-electrical activation prior to fusion. Alternatively, a fused cybrid can be placed in a medium containing one or more components suitable for non-electrical activation. The activation process is discussed in greater detail hereafter.

C. Injection/Fusion

A nuclear donor can be translocated into an oocyte using a variety of materials and methods that are well known to a person of ordinary skill in the art. In one example, a nuclear donor may be directly injected into a recipient oocyte. This direct injection can be accomplished by gently pulling a nuclear donor into a needle, piercing a recipient oocyte with that needle, releasing the nuclear donor into the oocyte, and removing the needle from the oocyte without significantly disrupting its membrane. Appropriate needles can be fashioned from glass capillary tubes, as defined in the art and specifically by publications incorporated herein by reference.

In another example, at least a portion of plasma membrane from a nuclear donor and recipient oocyte can be fused together using techniques well known to a person of ordinary skill in the art. See, Willadsen, 1986, *Nature* 320:63-65, hereby incorporated herein by reference in its entirety including all figures, tables, and drawings. Typically, lipid membranes can be fused together by electrical and chemical means, as defined previously and in other references incorporated by reference herein.

Other examples of non-electrical means of cell fusion involve incubating cybrids in solutions comprising polyethylene glycol (PEG), and/or Sendai virus. Various molecular weights of PEG can be utilized for cell fusion.

Although the efficiency of NT as a process is sensitive to minor modifications, other variables for fusion can be determined without undue experimentation. For example, modifications to cell fusion techniques can be monitored for their efficiency by viewing the degree of cell fusion under a microscope. The resulting embryo can then be cloned and identified as a totipotent embryo by the same methods as those previously described herein for identifying immortalized, totipotent cells, which can include tests for selectable markers and/or tests for developing an animal.

D. Activation

Methods of activating oocytes and cybrids are known to those of ordinary skill in the art. See, U.S. Pat. No. 5,496,720, "Parthenogenic Oocyte Activation," Susko-Parrish et al., issued on March 5, 1996, hereby incorporated by reference herein in its entirety including all figures, tables, and drawings.

Both electrical and non-electrical means can be used for activating the cybrids. Although use of a non-electrical means for activation is not always necessary, non-electrical activation can enhance the developmental potential of cybrids, particularly when young oocytes are utilized as recipients.

Examples of electrical techniques for activating cells are well known in the art. See, U.S. Pat. Nos. 4,994,384 and 5,057,420. Non-electrical means for activating cells can include any method known in the art that increases the probability of cell division. Examples of non-electrical means for activating a nuclear donor and/or recipient can be accomplished by introducing cells to ethanol; inositol triphosphate (IP_3); Ca^{++} ionophore and protein kinase inhibitors such as 6-dimethylaminopurine; temperature change; protein synthesis inhibitors (e.g., cyclohexamide); phorbol esters such as phorbol 12-myristate 13-acetate (PMA); mechanical techniques, thapsigargin, and sperm factors. Sperm factors can include any component of a sperm. Other non-electrical methods for activation include subjecting the cell or cells to cold shock and/or mechanical stress.

Examples of preferred protein kinase inhibitors are protein kinase A, G, and C inhibitors such as 6-dimethylaminopurine (DMAP), staurosporin, 2-aminopurine, sphingosine. Potentially, tyrosine kinase inhibitors may also be utilized to activate cells.

Although the NT process is sensitive to minor modifications, other variables for activation can be determined without undue experimentation. Other activation materials and methods can be identified by modifying the specified conditions defined in the exemplary protocols described hereafter and in U.S. Pat. No. 5,496,720.

The result of any modifications upon efficiency and totipotency of the activated embryo can be identified by the methods described previously in the section entitled "Identification of Immortalized and Totipotent Cells." Methods for identifying totipotent embryos can include one or more tests, such as (a) identifying specific markers for totipotent cells in embryos, and (b) by determining whether the embryos are totipotent by allowing them to develop into an animal. Therefore, the invention relates to any modifications to the activation procedures described herein even though these modifications may not be explicitly stated herein.

F. Manipulation of Embryos Resulting from Nuclear Transfer

An embryo resulting from a NT can be manipulated in a variety of manners. The invention relates to cloned embryos that arise from at least one NT.

Exemplary embodiments of the invention demonstrate that two or more NT procedures may enhance the efficiency

for the production of totipotent embryos. The exemplary embodiments indicate that incorporating two or more NT procedures into methods for producing cloned totipotent embryos may enhance placental development. In addition, increasing the number of NT cycles involved in a process for producing totipotent embryos may represent a necessary factor for converting non-totipotent cells into totipotent cells. The effect of incorporating two or more NT cycles on the totipotency of resulting embryos is a surprising result, which was not previously identified or explored in the art.

Incorporating two or more NT cycles into methods for cloned totipotent embryos can provide another advantage. Incorporation of multiple NT procedures into methods for creating cloned totipotent embryos provides a method for multiplying the number of cloned totipotent embryos.

When multiple NT procedures are utilized for the formation of a cloned totipotent embryo, young or aged oocytes can be utilized as recipients in the first, second or subsequent NT procedures. For example, if a first NT and then a second NT are performed, the first NT can utilize a young enucleated oocyte as a recipient and the second NT may utilize an aged enucleated oocyte as a recipient. Alternatively, the first NT may utilize an aged enucleated oocyte as a recipient and the second NT may utilize a young enucleated oocyte as a recipient for the same two-cycle model for NT. In addition, both NT cycles may utilize young enucleated oocytes as recipients or both NT cycles may utilize aged enucleated oocytes as recipients in the two-cycle NT example.

For NT techniques that incorporate two or more NT cycles, one or more of the NT cycles may be preceded, followed, and/or carried out simultaneously with an activation step. As defined previously herein, an activation step may be accomplished by electrical and/or non-electrical means as defined herein. Exemplified embodiments described hereafter describe NT techniques that incorporate an activation step after one of the NT cycles. However, activation steps may also be carried out in conjunction with NT cycles (e.g., simultaneously with the NT cycle) and/or activation steps may be carried out prior to a NT cycle.

A preferred embodiment of the invention, for example, relates to a first NT utilizing a young enucleated oocyte as a recipient followed by activation. This, in turn, is followed by a second NT utilizing an aged enucleated oocyte as a recipient. This second NT procedure is not followed by activation. This example is not meant to be limiting and the invention relates to any number of NT cycles that are optionally preceded by, followed by, simultaneously carried out with an activation procedure.

NT techniques may utilize virtually any cell as a nuclear donor. For example, in a preferred embodiment, a first NT may utilize an immortalized, totipotent cell of the invention as a nuclear donor and a second NT may utilize an embryonic cell as a nuclear donor. The second NT cycle in this example may utilize a blastomere (a cell isolated from an embryo), a cell isolated from a fetus (e.g. a primordial germ cell) as a nuclear donor, a cell isolated from a cell line, or a synchronized cell (described herein). The invention pertains in part to utilizing nearly any type of cell as a nuclear donor in any NT. The effect of using different nuclear donors on the overall efficiency for producing cloned totipotent embryos can be tested by practicing the tests for totipotency described in the preceding section entitled "Identification of Immortalized and Totipotent Cells."

The cloned totipotent embryos resulting from NTs can be (1) disaggregated or (2) allowed to develop further.

If the embryos are disaggregated, these embryonic derived cells can be utilized to establish cultured cells. Any

type of embryonic cell can be utilized to produce cultured cells. These cultured cells are sometimes referred to as embryonic stem cells or embryonic stem-like cells in the scientific literature. The embryonic stem cells can be derived from early embryos, morulae, and blastocyst stage embryos. Multiple methods are known to a person of ordinary skill in the art for producing cultured embryonic cells. These methods are enumerated in specific references previously incorporated by reference herein.

If the embryos are allowed to develop in utero, cells isolated from the developing fetus can be utilized to produce cultured cells. In preferred embodiments, primordial germ cells are isolated from the genital ridge of 28 to 75 day old developing cell masses for the establishment of cell lines. These cultured cells are sometimes referred to as embryonic germ cells (EG). These cultured cells can be generated using methods well known to a person of ordinary skill in the art. The methods are enumerated in references previously incorporated by reference herein.

The cloned totipotent embryos resulting from NT can also be manipulated by cryopreserving and/or thawing the embryos. See, U.S. Pat. No. 5,160,312, entitled "Cryopreservation Process for Direct Transfer of Embryos," Voelkel, and issued on Nov. 3, 1992; and U.S. Pat. No. 4,227,381, entitled "Wind Tunnel Freezer," Sullivan et al., issued on Oct. 14, 1980, all of which are hereby incorporated by reference herein in their entireties including all tables, figures, and drawings. Other embryo manipulation methods include culturing, performing embryo transfer, dissociating for NT, dissociating for establishing cell lines for use in NT, splitting, aggregating, sexing, and biopsying the embryos resulting from NT, which are described hereafter. The exemplary manipulation procedures are not meant to be limiting and the invention relates to any embryo manipulation procedure known to a person of ordinary skill in the art.

IV. Development of Cloned Embryos

A. Totipotency

Totipotent embryos can be identified by the methods described in the section "Identification of Immortalized and Totipotent Cells." Individual cells can be isolated and subjected to these similar tests. The tests relate to similar markers for identifying totipotent cells, as well as a test for determining totipotency by allowing an embryo to develop until it passes the second trimester of gestation, or preferably, gives rise to a live born animal. Methods for identifying other markers for totipotency are also described in that section.

B. Culture of Embryos in vitro

Methods for culturing embryos in vitro are well known to those skilled in the art. See, U.S. Pat. No. 5,213,979, entitled "In vitro Culture of Bovine Embryos," First et al., issued on May 25, 1993, and U.S. Pat. No. 5,096,822, entitled "Bovine Embryo Medium," Rosenkrans, Jr. et al., issued on Mar. 17, 1992, both of which are incorporated by reference herein in its entirety, including all figures, tables, and drawings. In addition, exemplary embodiments for media suitable for culturing cloned embryos in vitro are described hereafter. Feeder cell layers may or may not be utilized for culturing cloned embryos in vitro. Feeder cells are described previously and in exemplary embodiments hereafter.

The present invention is superior to existing materials and methods for cloning organisms, because embodiments of the invention allow for culturing all cells and embryos in vitro prior to implantation. For example, cloning methods described for cloning ovine organisms require an in vivo development step in the oviducts of an ovine host animal before the embryos are implanted in a suitable host. Because

embodiments of the present invention do not require in vivo development steps prior to implantation into the uterus, the materials and methods of the present invention represent an inventive step over cloning methods previously described by others.

C. Development of Embryos in utero

Cloned embryos can be cultured in an artificial or natural uterine environment after NT procedures. Examples of artificial development environments are being developed and some are known to those skilled in the art. Components of the artificial environment can be modified with little experimentation, for example, by modifying one component and monitoring the growth rate of the embryo.

Methods for implanting embryos into the uterus of an animal are also well known in the art. Preferably, the developmental stage of the embryo(s) is correlated with the estrus cycle of the animal.

Embryos from one specie can be placed into the uterine environment of an animal from another specie. For example it has been shown in the art that bovine embryos can develop in the oviducts of sheep. Stice & Keefer, 1993, "Multiple generational bovine embryo cloning," *Biology of Reproduction* 48: 715-719. The invention relates to any combination of ungulate embryo in any other ungulate uterine environment. The cross-species relationship between embryo and uterus can allow for efficient production of cloned animals of an endangered species. For example, a bison embryo can develop in the uterus of a domestic bovine. In another example, a big-horn sheep embryo can develop in the uterus of a large domesticated sheep.

Once the embryo is placed in the uterus of an animal, the embryo can develop to term. Alternatively, the embryo can be allowed to develop in the uterus and then can be removed at a chosen time. Surgical methods are well known in the art for removing fetuses from uteri before they are born.

V. Cloned Animals

A. Bovine Cloned Animals

As described previously herein, the invention provides the advantages of being able to assess the phenotype of an animal before cloning. This is an advantage of the invention since previous reports have only allowed the cloning of bovine animals from blastomeres, a method that does not allow for phenotype assessment.

Multiple products can be isolated from a cloned animal. For example, semen can be collected from an animal, such as a bovine bull. Semen can be cryopreserved as well as separated sperm into sex-specific fractions. See, U.S. Pat. Nos. 5,439,362, 5,346,990, and 5,021,244, entitled "Sex-associated Membrane Proteins and Methods for Increasing the Probability that Offspring Will be of a Desired Sex," Spaulding, and issued on Aug. 8, 1995, Sep. 13, 1994, and Jun. 4, 1991, respectively, all of which are hereby incorporated by reference herein in their entireties including all figures, drawings, and tables. Methods of collecting semen are well known to a person of ordinary skill in the art. *Physiology of Reproduction and Artificial Insemination of Cattle* (2nd. edition), Salisbury et al., copyright 1961, 1978, W. H. Freeman & Co., San Francisco.

The invention relates in part to any products collected from a cloned animal, preferably a cloned bovine animal. The products can be any body fluids or organs isolated from the animal, or any products isolated from the fluids or organs. In preferred embodiments, products such as milk and meat may be collected from cloned animals, preferably cloned bovine animals. In another embodiment, the invention relates to determining the phenotype of a bovine steer, which is a neutered animal, and then cloning this animal.

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such that the cloned animals are reproductively functional and can be used to produce ~~semen~~. Other preferred embodiments of the invention relate to such products as xenograft materials, sperm, embryos, oocytes, any type of cells, and offspring harvested from cloned animals of the invention, preferably cloned bovine animals.

Xenograft materials, which are described previously herein, can relate to any cellular material extracted from one organism and placed into another organism. Medical procedures for extracting the cellular material from one organism and grafting it into another organism are well known to a person of ordinary skill in the art. Examples of preferable xenograft cellular materials can be selected from the group consisting of liver, lung, heart, nerve, gallbladder, and pancreas cellular material.

B. Non-Bovine Cloned Animals

Due to the high DNA sequence homology between bovine animals and other ungulates, the materials and methods of the invention can be utilized to clone other ungulates. The materials and methods of the invention are the most efficient means for cloning a mammal as known in the state of the art.

In preferred embodiments the materials and methods of the invention can be utilized to clone endangered species, such as bison. In addition, the materials and methods of the invention can be utilized to clone commercially relevant ungulates, such as pigs. Due to the methods for reprogramming primary cells isolated from an animal into immortalized, totipotent cells, the more closely related the animal species is to cattle, the higher probability that the cloning methods of the invention will have greater success. Exemplary embodiments are described hereafter for cloning non-bovine animals.

C. Cloned Animals with Modified Nuclear DNA

As discussed in a previous section, transgenic animals can be generated from the methods of the invention by using transgenic techniques well known to those of ordinary skill in the art. Preferably, cloned transgenic bovine animals are produced from these methods. These cloned transgenic animals can be engineered such that they are resistant or partially resistant to diseases and parasites endemic to such animals. Examples of these diseases and parasites are outlined in a preceding section.

Moreover, the cloned transgenic animals can be engineered such that they produce a recombinant product. Examples of recombinant products are outlined in a preceding section. The expression of these products can be directed to particular cells or regions within the cloned transgenic animals by selectively engineering a suitable promoter element and other regulatory elements to achieve this end.

For example, human recombinant products can be expressed in the urine of cattle by operably linking a uroplakin promoter to the DNA sequence encoding a recombinant product. Alternatively, examples are well known to a person of ordinary skill in the art for selectively expressing human recombinant products in the milk of a bovine animal.

Once the recombinant product or products have been expressed in a particular tissue or fluid of the cloned transgenic animal, the suitable tissue or fluid can be collected using methods well known in the art. Recombinant products can be purified from that fluid or tissue by using standard purification techniques well known to a person of ordinary skill in the art.

EXAMPLES

The examples below are non-limiting and are merely representative of various aspects and features of the present invention.

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EXAMPLE 1

Feeder Layer Preparation

A feeder cell layer was prepared from mouse fetuses that were from 10 to 20 days gestation. The head, liver, heart and alimentary tract were removed and the remaining tissue washed and incubated at 37° C. in 0.025% trypsin-0.02% EDTA (Difco, Cat # 0153-61-1). Loose cells were cultured in tissue culture dishes containing MEM-alpha supplemented with penicillin, streptomycin, 10% fetal calf serum and 0.1 mM 2-mercaptoethanol. The feeder cell cultures were established over a two to three week period at 37.4° C., 3.5% CO₂ and humidified air. Before being used as feeder cells, mouse fibroblasts were pre-treated with mitomycin C (Calbiochem, Cat # 47589) at a final concentration of 10 µg/ml for 3 hours and washed 5 times with PBS before pre-equilibrated growth media was added.

Feeder cells can be established from bovine fetuses from 30 to 70 days using the same procedure. Bovine fetal cells may be optionally treated with mitomycin C.

EXAMPLE 2

Establishing Cultured Cells From Non-Embryonic Tissue

One advantage provided by the materials and methods defined herein is the ability to create an immortalized and totipotent cell from virtually any type of precursor cell. These precursor cells can be embryonic cells, cultured embryonic cells, primordial germ cells, fetal cells, and cells isolated from the tissues of adult animals, for example. Cells isolated from the kidney and ear of an adult grown bovine have been utilized as precursor cells for the generation of immortalized, totipotent cells.

After cells are isolated from their respective tissues, the cells can be subjected to the materials and methods defined in Example 3.

A first step towards generating immortalized, totipotent cells from tissues of grown animals includes a primary culture of isolated cells. A protocol for culturing cells isolated from the tissues of grown animals is provided hereafter. Although the illustrative protocol relates to ear punch samples, this protocol can apply to cells isolated from any type of tissue.

The following steps are preferably performed utilizing sterile procedures:

- 1) Wash each ear sample twice with 2 mL of trypsin/EDTA solution in two separate 35 mm Petri dishes. Process each ear sample separately. Mince the ear sample with sterile scissors and scalpel in a 35 mm Petri dish containing 2 mL of trypsin/EDTA solution. The minced pieces are preferably less than 1 mm in diameter.
- 2) Incubate minced ear pieces in the trypsin/EDTA solution for 40-50 min. in a 37° C. incubator with occasional swirling. The trypsin/EDTA solution is described in more detail hereafter. The dish may be wrapped with a stretchable material, such as Parafilm®, to reduce CO₂ accumulation.
- 3) Transfer digested ear pieces to a 15 mL sterile tube. Wash the dish from which the digested ear pieces were recovered with 2 mL of the trypsin/EDTA solution and transfer this wash solution to the sterile tube.
- 4) Vortex the tube at high speed for 2 min.
- 5) Add 5 mL of media (defined below) to inactivate the trypsin.
- 6) Centrifuge the 15 mL tube at 280xg for 10 minutes.

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- 7) Decant the supernatant and re-suspend the cell pellet in residual solution by gently taping the side of the tube.
 - 8) Add 2 mL of media to the tube and then centrifuge as described in step (6).
 - 9) Decant the supernatant, re-suspend the pellet as described in step (7), then add 2 mL of media.
 - 10) Keep 2-3 pieces of the ear for DNA analysis and store at -20° C. in a 15 mL tube.
 - 11) Transfer resuspended cells into a 35 mm Nunc culture dish and incubate at 37° C. in a humidified 5% CO₂/95% air atmosphere.
 - 12) Change media every 2 days.
- Trypsin/EDTA Solution:
 0.025% trypsin (w/v) (Bacto trypsin, Difco #cat 0153-61-1)
 0.02% EDTA (Sigma) (w/v)
 Add the trypsin and EDTA to Ca²⁺-free and Mg²⁺-free Dulbecco's phosphate-buffered saline (PBS) (Gibco cat# 450-1600EA) and sterilize by filtration through a 0.2 µm filter.

Media:

Combine Alpha minimum essential medium (MEM) (Biowhittaker) with 10% fetal bovine serum (Hyclone), 4 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin B (Fungizone).

This protocol has been also successfully utilized to establish cultures of kidney and liver cells isolated from grown bovine animals. As discussed above, the protocol can be utilized to create cell cultures from any type of cell isolated from a grown animal, for any species or family of animals.

EXAMPLE 3**Reprogramming and Establishment of Immortalized and Totipotent Cells from Precursor Cells**

The reprogramming procedures described hereafter can utilize any cell type of cells as precursor cells for the generation of immortalized, totipotent cells. As an example, the cell cultures described previously can be utilized as precursor cells for the reprogramming procedures described below. As another example, the following procedure describes one embodiment of the invention, where primordial germ cells were utilized as precursor cells for the generation of immortalized, totipotent cells. An embodiment of the reprogramming process is illustrated in FIG. 2.

A bovine fetus approximately 40 days old was obtained from a pregnant animal. The genital ridges were located at the caudo-ventral part of the abdominal cavity. Genital ridges were removed aseptically and washed in phosphate buffered saline (PBS) (Gibco, Cat # 14287-015) with 500 U/mL penicillin/500 µg/ml streptomycin. The tissue was sliced into 1-1.5 mm pieces and placed into a solution containing pronase E (3 mg/ml; Sigma Cat # P6911) in Tyrodes Lactate (TL) HEPES (Biowhittaker, Cat # 04-616F) for 30-45 minutes at 35-37° C. The proteolytic action of pronase E disaggregated the slices of genital ridges to a cell suspension. Pronase E was removed by dilution and centrifugation in TL HEPES solution. After this step, the cell suspension was frozen and stored at -196° C.

A thawed cell suspension (final concentration 100,000 cells/ml) was placed into a 35 mm Petri dish containing a murine primary embryonic fibroblast feeder layer. The culture media used was MEM alpha (Biowhittaker, Cat # 12-169F) supplemented with 0.1 mM 2-mercaptoethanol (Gibco, Cat # 21985-023), 4 mM glutamine, 100 ng/ml human recombinant leukemia inhibitory factor (hrLIF; R&D System, Cat # 250-L), 100 ng/ml bovine basic fibroblast growth factor (bFGF; R&D System, Cat # 133-FB) and 10%

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fetal calf serum (FCS, HyClone, Cat # A1111D) at 37.5° C. and 3.5% CO₂. Exogenous steel factor (e.g., membrane associated steel factor and soluble steel factor) was not added to the culture media. After 24 hours, and again at 48 hour intervals, supplemented culture media was replaced. After an E As initial culture of 6 days, concentrations of hrLIF and bFGF were lowered to 40 ng/ml, respectively. After nine days in culture, hrLIF and bFGF were removed from the medium entirely.

At the beginning of in vitro culture of genital ridge cells, simple embryonic bodies were occasionally observed. These bodies eventually disappeared with subsequent passages. The rate of establishing immortalized, totipotent cell lines from genital ridge cells was 100% and did not appear to be sex dependent. Table 1 contains data from establishment of seven immortalized, totipotent cell lines. Established immortalized, totipotent cell lines were maintained in MEM-alpha supplemented with 10% FCS which was replaced every second or third day. High density population cells were passaged every week at a dilution ratio of 1:4 to 1:8. Cells were passaged by incubating with 0.025% trypsin-0.02% EDTA mixture and preparing new cultures in fresh growth medium. The growth promoting capacity of MEM-alpha media for immortalized, totipotent cells was enhanced by adding insulin-transferrin, sodium selenite supplement, diluted to 1:100 (Sigma Cat # 11884). As a preventive measure against mycoplasma contamination, short term cultivation with tylosine tartrate (Sigma, Cat T3151) was carried out. Before NT, cell lines were tested for presence of mycoplasma by PCR performed with DNA primers specific for mycoplasma sequences (Stratagene, Cat 302007).

TABLE 1

Characterization of Established Bovine Immortalized and Totipotent Cell Lines			
Cell line	Weight of fetus (gm)	Days in culture	Sex of Cell line
EG	14.2	>400	male
EG-1	20.2	>300	male
EG-2	3.9	>30	female
EG-3	4.8	>30	male
EG-4	39.6	>100	female
EG-5	3.9	>250	male
EG-6	8.6	>30	male

EXAMPLE 4**Embryo Construction**

The following embodiment of the invention describes materials and methods utilized to produce totipotent embryos of the invention. Immortalized embryos of the invention can be produced by utilizing immortalized and totipotent cells of the invention as nuclear donors in NT procedures. As described previously, multiple NT procedures can be utilized to create a totipotent embryo. The following two examples describe a multiple NT procedure, which describes the use of two NTs.

Mycoplasma free immortalized, totipotent cells used in the NT procedure, were prepared by cutting out a group of immortalized, totipotent cells from the feeder layer using a glass needle. The isolated immortalized, totipotent cells were then incubated in a TL HEPES solution containing from 1 to 3 mg/ml pronase E at approximately 32° C. for 1 to 4 hours, the amount of time which was needed in this example to disaggregate the cells. Once the cells were in a single cell suspension they were used for NT within a 2-3 hour period.

Oocytes aspirated from ovaries were matured overnight (16 hours) in maturation medium. Medium 199 (Biowhittaker, Cat #12-119F) supplemented with luteinizing hormone 10IU/ml (LH; Sigma, Cat # L9773), 1 mg/ml estradiol (Sigma, Cat # E8875) and 10% FCS or estrus cow serum, was used. Within 16 hours of maturation, the cumulus layer expanded and the first polar bodies were extruded.

In the first NT procedure, young oocytes were stripped of their cumulus cell layers and nuclear material stained with Hoechst 33342 5mg/ml (Sigma, Cat # 2261) in TL HEPES solution supplemented with cytochalasin B (7 μ g/ml, Sigma, Cat # C6762) for 15 min. Oocytes were then enucleated in TL HEPES solution under mineral oil. A single immortalized, totipotent cell of optimal size (12 to 15 μ m) was then inserted from a cell suspension and injected into the perivitelline space of the enucleated oocyte. The immortalized, totipotent cell and oocyte membranes were then induced to fuse by electrofusion in a 500 μ m chamber by application of an electrical pulse of 90V for 15 μ s.

Cybrid activation was induced by a 4 min exposure to 5 μ M calcium ionophore A23187 (Sigma Cat. # C-7522) or ionomycin Ca-salt in HECM (hamster embryo culture medium) containing 1 mg/ml BSA followed by a 1:1000 dilution in HECM containing 30 mg/ml BSA for 5 min. For HECM medium, see, e.g., Seshagiri & Barister, 1989, "Phosphate is required for inhibition of glucose of development of hamster eight-cell embryos in vitro," *Biol. Reprod.* 40: 599-606. This step is followed by incubation in CR2 medium containing 1.9 mM 6-dimethylaminopurine (DMAP; Sigma product, Cat # D2629) for 4 hrs followed by a wash in HECM and then cultured in CR2 media with BSA (3 mg/ml) under humidified air with 5% CO₂ at 39° C. For CR2 medium, see, e.g., Rosenkrans & First, 1994, "Effect of free amino acids and vitamins on cleavage and developmental rate of bovine zygotes in vitro," *J. Anim. Sci.* 72: 434-437. Mitotic divisions of the cybrid formed an embryo. Three days later the embryos were transferred to CR2 media containing 10% FCS for the remainder of their in vitro culture.

Table 2 shows the effect of oocyte age on blastocyst development. The data was obtained utilizing blastomeres from in vitro produced embryos or immortalized, totipotent cells as donor nuclei in the NT procedure. Developmental potential was measured in young versus aged oocytes.

TABLE 2

Effect of Oocyte Timing for Different Cell Sources		
oocyte age (hours)	Immortalized and Totipotent Cells (n = 174)	Blastomeres (n = 192)
16-28 (n = 175)	17.9% blastocyst (n = 140)	no development (n = 35)
28-48 (n = 191)	no development (n = 34)	17.3% blastocyst (n = 157)

The data presented in Table 2 shows that oocytes maintained in culture for 16-28h were more suitable recipients for immortalized, totipotent cells, while aged oocytes maintained in culture for 28-48 h were a more suitable recipient for blastomeres derived from embryos. In addition, activation procedures differed between young and aged oocytes. Young oocytes, when used in the NT procedure, appear to require chemical activation with ionomycin and DMAP from these studies. Aged oocytes, on the other hand, appear to be easily activated by electrofusion according to these studies.

EXAMPLE 5

Second Nuclear Transfer (Recloning)

Cells obtained from fetuses and embryos produced by the NT procedures described herein can be used in a second NT, or recloning, procedure. For example, a fetus can be harvested from a maternal host, the head, viscera, and genital ridge removed, and the remaining fetal cells used to establish a cell line to provide nuclear donor material for a subsequent NT procedure. The following example describes obtaining and using a blastomere from an NT embryo as a nuclear donor in a recloning procedure.

Embryos from the first generation NT at the morula stage were disaggregated either by pronase E (1-3 mg/ml in TL HEPES) or mechanically after treatment with cytochalasin B. Single blastomeres were placed into the perivitelline space of enucleated aged oocytes (28-48 hours of incubation). Aged oocytes were produced by incubating matured "young" oocytes for an additional time in CR2 media with 3 mg/ml BSA in humidified air with 5% CO₂ at 39° C.

A blastomere from an embryo produced from an immortalized, totipotent cell was fused into the enucleated oocyte via electrofusion in a 500 μ m chamber with an electrical pulse of 105V for 15 μ s in an isotonic sorbitol solution (0.25 M) at 30° C. Aged oocytes were simultaneously activated with a fusion pulse, not by chemical activation as with young oocytes.

After blastomere-oocyte fusion, the cybrids from second generation NT were cultured in CR2 media supplemented with BSA (3 mg/ml) under humidified air with 5% CO₂ at 39° C. On the third day of culture, developing embryos were evaluated and cultured further until day seven in CR2 media containing 10% FCS. Morphologically good to fair quality embryos were non-surgically transferred into recipient females. Table 3 shows the increased gestation length achieved by use of recloned (double NT) immortalized, totipotent cells.

TABLE 3

Development of Immortalized and Totipotent Cells Derived Fetuses after Double NT			
Expt #	No. of embryos transferred	No. of recipients transferred into	No. of pregnant recipients after 140 days
Expt #1	15	5	1
Expt #2	18	6	1 (two fetuses)

EXAMPLE 6

Cloning Non-Bovine Ungulates

The specification provides for methods of cloning non-bovine ungulates. Examples of such ungulates can be selected from the group consisting of bovids, ovids, cervids, suids, equids and camelids, such as bison, sheep, big-horn sheep, caribou, antelope, deer, goat, water buffalo, camel, and pig.

Immortalized, totipotent cell lines can be prepared from multiple types of cells isolated from the non-bovine ungulate by using the methods described in previous examples relating to bovine animals, or by using the screening procedures for these methods as described in the specification. Virtually any type of cell isolated from the non-bovine ungulate can

be utilized to establish an immortalized, totipotent cell line. For example, an ear-punch sample taken from a bison can be cultured in vitro using a variety of cell culture media such as MEM-alpha medium.

Bison-derived primary cells can then be converted or reprogrammed into immortalized, totipotent bison cells by supplementing the cell culture medium with hrLIF and bFGF as described in previous examples and in the specification. Alternatively, the bison-derived primary cells can be converted into immortalized, totipotent cells by supplementing the growth medium with other types of molecules identified by methods for identifying such reprogramming molecules as described in the specification. The reprogrammed bison-derived cells can then be tested for totipotency by analyzing selected markers, such as alkaline phosphatase, lamini, and c-kit. In addition, the bison-derived cells can be considered permanent if the number of cell divisions exceeds the Hayflick limit and/or if the cells can grow to confluency after being replated under conditions where the cells are not in physical contact with one another, for example.

Once totipotent, immortalized cells have been established as nuclear donors, proper enucleated oocytes can be prepared for NT. Oocytes from the same or different specie as the nuclear donor can be used for NT. For example, a bison-derived nuclear donor cell can be fused or directly injected into a bison-derived enucleated oocyte or an enucleated oocyte from another specie, such as a bovine.

As described in the specification, the oocytes can be derived from any ungulate in a variety of ways, such as sacrificing an animal and retrieving oocytes from its oviducts, or spaying the animals by ovarian hysterectomy and isolating the oocytes from the oviducts or ovaries. Oocytes can also be obtained from live animals by utilizing such methods as transvaginal oocyte recovery. The oocytes can then be enucleated by using methods described herein as applied to sheep or cattle. These methods can be easily applied to oocytes derived from other ungulates.

Nuclear transfer techniques can be performed after enucleated oocytes and nuclear donor cells are prepared. Young or aged oocytes can be utilized for the NT procedure, and the number of NTs can vary as described in the specification. In addition the parameters that define the fusion step for a NT may be varied as described herein. An activation step can be applied to one or more of the NT cycles. For example, the NT cycles defined in a previous exemplary embodiment can be applied to the generation of cloned bison. The embryo resulting from the NT can be tested for totipotency by utilizing tests for one or more markers, such as alkaline phosphatase, cytokeratin, vimentin, laminin, and c-kit. In addition, the embryo can be tested for totipotency by implanting it into the uterus of an animal and allowing development to term.

Once a cloned totipotent embryo is produced from the methods described above for a non-bovine ungulate, the embryo can be further manipulated. Such manipulations include cryopreserving, thawing, culturing, disaggregating the embryo into single cells, and implanting the embryo. The embryo may be cultured in an artificial development environment (as described previously) or may be placed in utero of a properly synchronized female animal. An embryo derived from one specie may be placed in a uterus of the same or different specie. For example, a bison-derived embryo can be placed in the uterus of a bovine. The embryo can be allowed to develop until term, or may be retrieved from the uterine environment before birth.

EXAMPLE 7

Multiple Pathways for Cloning Animals

FIG. 3 illustrates multiple embodiments of the invention. Animals can be cloned from cells that are reprogrammed into totipotent and immortalized cells.

Fibroblast cell cultures were prepared as defined above from ear punches extracted from an adult bovine animal. However, the cell cultures could be established from any type of differentiated cell. Individual cells isolated from these cultures were utilized as nuclear donors in a nuclear transfer process, labeled as step 2 in FIG. 3. Although one nuclear transfer cycle was utilized to obtain embryos (labeled as step 3 in FIG. 3), multiple nuclear transfer cycles could be applied to obtain these embryos. Also optional is (1) the addition of a stimulus before or after nuclear transfer, and (2) an activation step before or after nuclear transfer.

The embryo of step 3 in FIG. 3 was implanted into a recipient bovine female as described herein and a fetus (step 7) was isolated from that female. Cells isolated from embryos of step 3 may be utilized to establish embryonic stem cell cultures (step 4). In addition, the embryos of step 3 may be implanted into a female host and allowed to develop into a cloned animal (step 5).

The steps labelled 8, 9, 10, 11, and 12 in FIG. 3 were performed to establish totipotent and immortalized cells. The fetus of step 7 was manipulated in three manners. The manipulation in step 8 involved the isolation of genital ridge cells, specifically primordial germ cells, from the fetus of step 7. In step 9, the primordial germ cells were placed in co-culture with feeder cells. The feeder cells were either established from mouse fibroblast cells or from the rest of the fetus from which the primordial germ cells were extracted. Example 1 defines a method for establishing feeder cells. The head region and body cavity contents were removed from the fetus before the fetus was digested into a consistency suitable for establishing feeder cells. However, the fetus may be digested before the head region and contents of the body cavity are removed. In addition, feeder cells may be established from a fetus other than the fetus from which the primordial germ cells are isolated.

In step 10, a cell culture was established with a digested fetus from which the primordial germ cells, head region, and body cavity contents were removed. Step 11 illustrates that cell cultures may be established utilizing fetuses from which no cell types have been removed.

In step 12, cell cultures were either (1) subjected to a mechanical stimulus, or (2) not subjected to a mechanical stimulus. When applied, the mechanical stimulus was effected by supplementing the culture medium with a receptor ligand cocktail comprising 100 ng/ml human recombinant leukemia inhibitory factor (hrLIF; R&D System, Cat # 250-L) and 100 ng/ml bovine basic fibroblast growth factor (bFGF; R&D System, Cat # 133-FB). After step 12, cells were isolated from the cell cultures and utilized as nuclear donors in nuclear transfer processes, which are defined previously. Although one nuclear transfer cycle was utilized for step 13, more than one nuclear transfer cycle could be utilized.

Embryos developed after the nuclear transfer process of step 13. The embryos of step 14 may be implanted into a bovine recipient female and develop into a cloned bovine animal.

Cells isolated from any of the developing cell masses of steps 1, 3, 4, 5, 7, 8, 9, 10, 11, 12, 13, and 16 in FIG. 3 may

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be transfected with a DNA construct to form transgenic cells suitable for cloning transgenic animals. One embodiment for cloning transgenic animals is defined in the next example.

EXAMPLE 8

Cloning Transgenic Animals

Transgenic cells suitable for creating a cloned transgenic animal can be prepared from cells isolated from an adult animal. FIG. 4 illustrates processes that can be utilized to create such transgenic cells. Although transgenic cells can be created from nearly any cell type by using the teachings of the invention, FIG. 4 illustrates procedures for establishing transgenic embryonic stem cells and transgenic immortalized and totipotent cells.

Fibroblast cell cultures can be established from ear punches extracted from a bovine animal as defined previously. Individual cells can be isolated from this cell culture and utilized as nuclear donors in a nuclear transfer process. A single nuclear transfer cycle or multiple nuclear transfer cycles can be applied. Other optional steps are defined in the previous example.

Pre-blastocyst stage embryos and/or blastocyst stage embryos developed from the nuclear transfer process can be

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Cells can then be transfected with a DNA construct. Cells can be transfected at multiple steps, as indicated in FIG. 4. Materials and methods for preparing transgenic cells are defined in publications referenced previously. ~~Immortalized and totipotent cells of the invention were successfully transfected with a DNA construct comprising (a) a neomycin gene, which encodes a product that renders cells resistant to a compound designated G418; (b) a gene encoding the enzyme α -glucosidase; and (c) a casein promoter element.~~ The transfected cells were selected for transgenic modification by selecting for transgenic cells in cell culture conditions harboring G418. The transgenic cells are then screened for transgenic modification by utilizing one or more screening techniques. Examples of these techniques are: (1) polymerase chain reaction, (2) Southern blotting, and (3) FISH-filter procedures. These techniques are well known to a person of ordinary skill in the art. The latter two techniques are utilized to determine the number of copies of an inserted gene sequence in embryonic stem cell nuclear DNA.

These screening procedures can be applied to transfected cells at any of the steps indicated in FIG. 4. Cloned transgenic animals may be created from transgenic fetuses. Table 4 shows the cloned bovine animals produced by the methods described in Examples 1-8.

TABLE 4

Development of Immortalized and Totipotent Cells Bovine Animals Produced						
Date of Birth	Gender	Breed	Age of Fetus Harvested (days)	Type of Cell Used (1° NT)	Type of Cell Used (2° NT)	Stimulus
2/6/97	M	H	60 (est)	EG	Blastomere	LIF, FGF
7/7/97 (*)	M	H	60 (est)	EG	Blastomere	LIF, FGF
7/7/97 (*)	M	H	60 (est)	EG	Blastomere	LIF, FGF
2/7/98	F	H	55 (est)	EG	Blastomere	LIF, FGF, FSK
2/26/98	F	H	55 (est)	EG	Blastomere	LIF, FGF, FSK
10/26/98	F	H	55 (est)	EG	Blastomere	LIF, FGF, FSK
10/28/98	F	H	58	EG	Blastomere	LIF, FGF, FSK
12/2/98	F	BS	58	EG	n/a	LIF, FGF, FSK
12/6/98	F	H	58	EG	n/a	Culture
12/22/98 (*)	F	H	58	EG	n/a	Culture
12/22/98	F	H	58	EG	n/a	Culture
12/22/98 (*)	F	H	58	EG (t)	n/a	LIF, FGF
12/30/98	F	H	58	EG	n/a	Culture
12/30/98	F	H	58	EG	n/a	Culture
12/31/98	F	H	58	EG	n/a	Culture
1/6/99	F	H	58	EG (t)	n/a	LIF, FGF
1/7/99	F	H	58	EG	n/a	Culture
1/7/99	F	H	58	EG	n/a	Culture
1/15/99	F	H	58	EG	n/a	Culture
1/15/99	F	H	58	EG (t)	n/a	LIF, FGF
1/19/99	F	H	58	EG (t)	n/a	LIF, FGF

(*) - Stillborn

H - Holstein

BS - Brown Swiss

Age of fetus harvested - Age (in days) of fetus used as a source of precursor cells in 1° NT

(t) - transgenic nuclear donor: a nuclear donor cell transfected with a DNA construct having a human α -glucosidase gene

EGF - Epidermal growth factor

LIF - Leukemia inhibitor factor

FSK - Forskolin

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utilized to establish embryonic stem cells. Materials and methods for preparing embryonic stem cells are described by Stice et al., 1996, *Biology of Reproduction* 54: 100-110, hereby incorporated by reference herein in its entirety, including all figures, tables, and drawings. Immortalized and totipotent cells can be established according to the procedures defined in previous examples.

While the invention has been described and exemplified in sufficient detail for those skilled in this art to make and use it, various alternatives, modifications, and improvements should be apparent without departing from the spirit and scope of the invention.

One skilled in the art readily appreciates that the present invention is well adapted to carry out the objects and obtain

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the ends and advantages mentioned, as well as those inherent therein. The cell lines, embryos, animals, and processes and methods for producing them are representative of preferred embodiments are exemplary, and are not intended as limitations on the scope of the invention. Modifications therein and other uses will occur to those skilled in the art. These modifications are encompassed within the spirit of the invention and are defined by the scope of the claims.

It will be readily apparent to a person skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

All patents and publications mentioned in the specification are indicative of the levels of those of ordinary skill in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising", "consisting essentially of" and "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group. For example, if X is described as selected from the group consisting of bromine, chlorine, and iodine, claims for X being bromine and claims for X being bromine and chlorine are fully described.

Other embodiments are set forth within the following claims.

What is claimed is:

1. A method of producing a polypeptide in an ungulate, the method comprising:

(a) forming a nuclear transfer embryo by translocating a cultured ungulate nonembryonic cell, or a nucleus thereof, into an enucleated oocyte obtained from the same species as the ungulate nonembryonic cell, wherein said ungulate nonembryonic cell is, or is obtained by culture of, a cell transfected in vitro with a DNA sequence encoding said polypeptide operably linked to a milk protein promoter, such that said DNA sequence integrates into the genome of said cell;

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(b) before, during, or after said forming step, activating said oocyte, whereby an activated nuclear transfer embryo is provided following said forming and activating steps; and

(c) transferring said activated nuclear transfer embryo, or a recloned embryo thereof, into the uterus of a host ungulate of the same species as the ungulate nonembryonic cell so as to produce a fetus that undergoes full fetal development and parturition to generate said ungulate, wherein said ungulate expresses said DNA sequence and produces said polypeptide in milk, blood, serum, or plasma in detectable quantities.

2. A method according to claim 1, wherein said ungulate is a bovine.

3. A method according to claim 1, wherein said ungulate nonembryonic cell is a fetal cell.

4. A method according to claim 3, wherein said fetal cell is an embryonic germ cell.

5. A method according to claim 1, wherein said activating step comprises increasing intracellular levels of divalent cations and reducing phosphorylation of cellular proteins in said oocyte.

6. A method according to claim 5, wherein said activating step comprises incubating said oocyte in a medium comprising ionomycin.

7. A method according to claim 5, wherein said activating step comprises incubating said oocyte in a medium comprising 6-dimethylaminopurine.

8. A method according to claim 5, wherein said activating step comprises incubating said oocyte in a medium comprising ionomycin and 6-dimethylaminopurine.

9. A method according to claim 1, wherein said milk protein promoter is selected from the group consisting of α -S1-casein promoter, whey acidic protein promoter, β -casein promoter, and α -lactalbumin promoter.

10. A method according to claim 1, wherein said polypeptide is a human polypeptide.

11. A method according to claim 1, wherein said ungulate nonembryonic cell is not serum starved.

12. A method according to claim 1, wherein said activated nuclear transfer embryo is cultured prior to said transferring step (c).

13. A method of obtaining a polypeptide, the method comprising

(a) producing a polypeptide in an ungulate according to the method of claim 1; and

(b) isolating said polypeptide from the milk, blood, serum, or plasma of said ungulate.

14. A method according to claim 13, wherein said ungulate is a bovine.

15. A method of producing a polypeptide in an ungulate, the method comprising: providing a second ungulate by sexual reproduction or clon of a first ungulate, wherein said first ungulate is produced by the method of claim 1, and wherein said second ungulate expresses said DNA sequence and produces said polypeptide in milk, blood, serum, or plasma in detectable quantities.

16. The method of claim 15, wherein said first ungulate and said second ungulate are bovines.

* * * * *



US005741957A

United States Patent [19]

Deboer et al.

[11] Patent Number: **5,741,957**[45] Date of Patent: **Apr. 21, 1998**[54] **TRANSGENIC BOVINE**

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 Netherlands; **Herbert L. Heyneker**,
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 Netherlands

[73] Assignee: **Pharming B.V.**, Leiden, Netherlands[21] Appl. No.: **461,333**[22] Filed: **Jun. 5, 1995****Related U.S. Application Data**

[60] Division of Ser. No. 154,019, Nov. 16, 1993, Pat. No. 5,633,076, which is a continuation-in-part of Ser. No. 77,788, Jun. 15, 1993, abandoned, which is a continuation-in-part of Ser. No. 898,956, Jun. 15, 1992, abandoned, which is a continuation-in-part of Ser. No. 619,131, Nov. 27, 1990, abandoned, which is a continuation-in-part of Ser. No. 444,745, Dec. 1, 1989, abandoned.

[51] Int. Cl.⁶ C12N 5/00; C12N 15/00;
 C12P 21/06

[52] U.S. Cl. 800/2; 800/DIG. 1; 435/69.1;
 935/60; 935/63

[58] Field of Search 800/2, DIG. 1;
 536/23.1, 23.4, 24.1; 435/69.1, 325, 320.1;
 935/36

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(List continued on next page.)

Primary Examiner—Deborah Crouch

Attorney, Agent, or Firm—Townsend & Townsend & Crew

[57]

ABSTRACT

A transgenic bovine is disclosed whose somatic and germ cells contain a transgene, wherein the transgene comprising a mammary gland specific promoter, a mammary gland specific enhancer, a DNA sequence encoding a signal sequence functional in bovine mammary gland secretory cells and a DNA sequence encoding a heterologous polypeptide of interest wherein the transgenic bovine expresses the transgene such that the polypeptide of interest is detectable in milk produced by the transgenic bovine.

36 Claims, 41 Drawing Sheets

PubMedU.S. National Library of Medicine
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Display Settings: Abstract

Performing your original search, *Accurate spatial and temporal transgene expression cerdan*, in PubMed will retrieve **2 records**.

Mol Reprod Dev. 1998 Mar;49(3):236-45.

Accurate spatial and temporal transgene expression driven by a 3.8-kilobase promoter of the bovine beta-casein gene in the lactating mouse mammary gland.

Cerdán MG, Young JJ, Zino E, Falzone TL, Otero V, Torres HN, Rubinstein M.

Instituto de Investigaciones en Ingeniería Genética y Biología Molecular (CONICET-UBA), Buenos Aires, Argentina.

The spatial, temporal, and hormonal pattern of expression of the beta-casein gene is highly regulated and confined to the epithelial cells of the lactating mammary gland. Previous studies have shown that 1.7 kb of the bovine beta-casein promoter were able to drive cell-specific and hormone-dependent expression to a mouse mammary cell line but failed to induce accurate expression to the mammary gland of transgenic mice. We investigated here the ability of 3.8 kb of the bovine beta-casein gene promoter to drive the expression of the human growth hormone (hGH) gene in transgenic mice. A Northern blot analysis using total RNA obtained from different tissues of lactating and nonlactating females revealed the presence of hGH mRNA only in the mammary gland of lactating females. hGH mRNA was not detectable in the mammary gland of virgin females or males. A developmental analysis showed that hGH mRNA only peaked on parturition, resembling more closely the bovine beta-casein temporal expression pattern rather than the murine. In situ hybridization studies performed on mammary gland sections showed that the cellular pattern of hGH expression was homogeneous in all lobules from heterozygous and homozygous transgenic mice. Silver grain counts on the tissue sections highly correlated with the hGH contents in the milk determined by radioimmunoassay ($r = 0.996$). Thus 3.8 kb of the bovine beta-casein promoter direct a high-level expression of a reporter gene to the lactating mammary gland of transgenic mice in a tissue-specific and developmentally regulated manner.

PMID: 9491375 [PubMed - indexed for MEDLINE]

Publication Types, MeSH Terms, Substances

LinkOut - more resources

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado
EMILIO FERRERO
(Apoderado)

ASUNTO

Radicación 06-39628
Trámite 002
Evento 001
Actuación 519
Oficio 3573
Folios 06

Patente de Invención ☒ Patente de Modelo de Utilidad ☐
Vía Nacional ☐
Vía PCT (fase nacional) ☒ Cap. I ☐ Cap. II ☒
No. Sol. Internacional PCT/US2004/031819
Fecha de Presentación Internal. 29 de septiembre de 2004

Como resultado del examen de fondo, realizado con fundamento en el artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le comunica:

1. Documentos estudiados

<u>Descripción:</u>	Folios: 38 a 96	como se radicó inicialmente
<u>Reivindicaciones:</u>	Folios: 97 a 107	como se radicó inicialmente
<u>Figuras:</u>	Folios: 108 a 113	como se radicó inicialmente
<u>Prioridad:</u>	Folio: 1 Fecha: 30/09/2003 País: US Número: 60/506,735	

2. Objeto de la invención

- Título de la solicitud (folio 1): "Un proceso para producir proteína exógena en la leche de mamíferos transgénicos y un proceso para purificar proteínas de ésta manera".
- La presente solicitud se refiere a la producción de proteínas y compuestos a partir de animales transgénicos para su consumo en la dieta de humanos. También, se refiere al proceso de purificación de dichos compuestos.
- El problema planteado en esta solicitud es la necesidad de proporcionar un proceso para la producción de sustancias biológicas tales como polipéptidos eucarióticos a partir de organismos modificados, como animales transgénicos.
- Para solucionar este problema técnico la solicitud en estudio proporciona un método para producir una proteína que comprende a) elaborar un mamífero transgénico no humano que produce una proteína de interés en su leche, b) obtener dicha leche a partir de dicho mamífero transgénico no humano, y c) purificar dicha proteína de interés de dicha leche.
- La Clasificación Internacional de Patentes (IPC) es: C12 N 015/00, C12 P 21/00, A61K 67/027, A61P 5/06.

3. De la solicitud de Patente

Reivindicaciones (artículo 30 D 486)

El capítulo reivindicatorio de la solicitud en estudio presenta los siguientes defectos:

- La reivindicación 1 menciona un método para producir una proteína a partir de un mamífero transgénico pero no especifica que la proteína purificada sea la procedente a partir de la modificación genética realizada en el mamífero. Existe entonces una objeción por que no se entiende si la procedencia de la proteína esta dada a partir del constructo o si se trata de cualquier proteína producida por el mamífero, para lo cual no tendría existiría además sustento en la descripción.
- Las reivindicaciones 22 - 29 y 41 - 46 se encuentran definidas incorrectamente mediante resultados a alcanzar, lo cual no permite precisar las características técnicas del método reivindicado.

Por lo anterior, el capítulo reivindicatorio NO cumple con el requisito de definición, claridad, concisión o sustento por la descripción de acuerdo al art. 30 de la Decisión 486 y/o art. 22 Tratado PCT.

4. Unidad de invención (artículo 25 D 486)

La presente solicitud no tiene unidad de invención, dado que contempla diferentes conceptos inventivos relacionados así:

- Un método para producir una proteína que comprende elaborar un mamífero transgénico no humano que produce una proteína de interés en su leche, obtener dicha leche a partir de dicho mamífero transgénico no humano, y purificar dicha proteína de interés de dicha leche (reivindicaciones 1 – 29).
- Un método para purificar una hormona de crecimiento recombinante de leche de un mamífero transgénico no humano que produce una hormona de crecimiento recombinante que comprende: clarificar la leche y someter dicha leche a cromatografía. Así mismo, las reivindicaciones independientes 36 y 37 obedecen a este mismo concepto inventivo (reivindicaciones 30 a 48).

Entre ellos no existe una relación dado que el método para la producción de la proteína en un animal transgénico es independiente de la purificación de dicha proteína. Los dos procesos pueden ser llevados a cabo por técnicas diferentes, y los resultados de un proceso no involucran las características del otro.

Por lo anterior, se puede concluir que la presente solicitud no cumple con el requisito de unidad de invención.

5. No es una invención (artículo 15 D 486 literal a)-f))

El artículo 15 en su literal b) señala que:

No se consideran invenciones el todo o parte de los seres vivos tal como se encuentran en la naturaleza, los procesos biológicos naturales, el material biológico existente en la naturaleza o aquel que pueda ser aislado inclusive genoma o germoplasma de cualquier ser vivo natural.

- La reivindicación 1 y dependientes enseñan un método para producir una proteína a partir de un mamífero transgénico, que se obtiene a partir de su leche, purificando dicha proteína.

Este despacho considera que dichas reivindicaciones no son una invención debido a que obedecen a un material biológico existente en la naturaleza que puede ser aislado al no especificar que la proteína es codificada por el constructo genético.

Por tal razón las reivindicaciones 1 y dependientes no pueden ser consideradas invenciones.

6. Determinación del Estado de la Técnica

La fecha que se ha tenido en cuenta para determinar el Estado de la Técnica es:

Fecha de presentación de la solicitud prioritaria: 30/09/2003.

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Teniendo en cuenta la fecha indicada se realizó la búsqueda en el estado de la técnica de documentos relacionados con la solicitud encontrándose los siguientes documentos más relevantes:

Nº	Documento	Título	Fecha de publicación*
D1	US 6.395.958	Method of producing a polypeptide in an ungulate	28/05/2002
D2	US 5.741.957	Transgenic bovine	21/04/1998
D3	Cerdán <i>et al.</i> ,	Accurate spatial and temporal transgenes expresión driven by a 3.8 kilobase promoter of the bovine beta-casein gene in the lactating Mouse mammary gland	04/1998

7. Análisis de patentabilidad (artículo 14 D486)

7.1. Novedad (artículo 16 D486)

- La solicitud en estudio divulga, en resumen, un método para producir una proteína que comprende elaborar un mamífero transgénico, obtener la proteína a partir de la leche y purificarla. Las reivindicaciones posteriores divulgan obtener el mamífero transgénico mediante la inserción de un plásmido que posee un gen con un promotor en células somáticas y fusionar dichas células con un oocito eunucleado que resulta en un embrión, transferirlo al útero del mamífero receptivo y monitorear su gestación. El promotor es un promotor de beta caseína y la proteína de interés es la hormona de crecimiento humano (Reivindicaciones 1-3, 7, 11 – 13, 17 - 19).

D1 enseña (ver folios:173 - 181) la obtención de animales clonados (ungulados en general y particularmente bovinos) para la obtención de proteínas como hormonas de crecimiento (col 36, línea 23 y 32) a partir de la manipulación genética a partir de constructos de ADN (col 35, líneas 4 – 8) y de transferencia a oocitos eunucleados con la misma metodología de la solicitud (col 37 – 39) para transferirlo al útero de un mamífero receptivo (col 50, línea 23). El elemento promotor es de caseína y el constructo está unido a un gen de resistencia a neomicina (col 52, línea 6 - 9). Igualmente el compuesto de interés se obtiene a partir de la leche de mamífero transgénico (reivindicación 13).

Los elementos característicos de las composiciones reivindicadas, ya eran conocidos como se evidencia en D1, la forma de realizar el mamífero transgénico y los productos obtenibles a partir de la leche, así como su purificación (col 28, líneas 18 – 32). Por tal razón, los métodos reivindicados no pueden ser considerados novedosos.

- Además de lo mencionado anteriormente, la solicitud en estudio divulga la obtención de un mamífero transgénico a partir de un bovino superovulado con semen de un macho transgénico, para la obtención de la proteína de interés en su leche (reivindicaciones 15-20).

El documento del estado de la técnica D2 (ver folios: 182) enseña la obtención de proteínas como la hormona de crecimiento a partir de bovinos transgénicos en la leche. Este documento menciona además la obtención del mamífero mediante un proceso que comprende superocular el mamífero hembra para su inseminación artificial con semen de un bovino macho transgénico (col 51, ejemplo 25).

De esta forma se establece que las reivindicaciones 1-3, 7, 11 – 13, 17 – 20 no cumplen con el requisito de Novedad según lo divulgado por D1 y las reivindicaciones 15 – 20 según lo divulgado por D2.

7.2. Nivel inventivo (artículo 18 D486)

La única diferencia de la solicitud, es la unión del promotor específico de beta caseína para la obtención del constructo de ADN para la obtención del mamífero transgénico.

El documento del estado de la técnica D3 (ver folios: 183) señala la producción de la hormona de crecimiento humana mediante la construcción de un plásmido con el promotor de beta caseína bovina, obteniendo resultados favorables en la producción de esta hormona en leche de mamífero.

El problema técnico planteado en esta solicitud que era de proporcionar un proceso para la producción de sustancias biológicas tales como polipéptidos eucarióticos a partir de organismos modificados, como animales transgénicos, se había resuelto previamente mediante el mismo tipo de compuestos ya conocidos.

Se considera que la producción de sustancias biológicas como la hormona de crecimiento desarrollado con un constructo de ADN con un promotor de beta caseína como el desarrollado en D3, a partir de un mamífero transgénico como se enseña en D1 o en D2, es evidente para una persona medianamente versada en la materia.

De esta forma se establece que las reivindicaciones 1 a 29 no cumplen con el requisito de Nivel Inventivo.

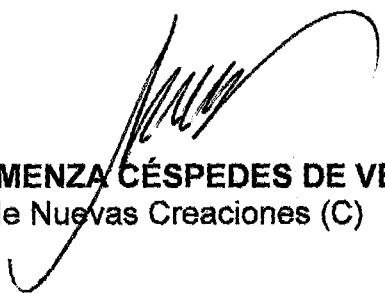
8. Conclusión:

Los requisitos de patentabilidad de la presente solicitud se encuentran afectados así:

Nº	Documento Nº	Reivindicaciones afectadas	Requisito que afecta
D1	US 6.395.958	1-3, 7, 11 – 13, 17 – 20 1 – 29	Novedad Nivel Inventivo
D2	US 5.741.957	15 – 20 1 – 29	Novedad Nivel Inventivo
D3	Cerdán <i>et al.</i> ,	1 – 29	Nivel Inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de 60 días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única N° 10 de 2001.


ALIX CARMENZA CÉSPEDES DE VERGEL
Directora de Nuevas Creaciones (C)

El anterior requerimiento fue notificado por fijación en lista, de fecha

12 MAR 2010

CONCEPTO TÉCNICO No. 818	EXAMINADOR TÉCNICO Código No. 202089
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