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(54) Title: TRANSGENIC ANIMALS AND USES THEREOF

(57) Abstract: In general, the invention features genetically modified non-human mammals (e.g., bovines and other ungulates), and method of making these mammals. In particular, the invention features transgenic ungulates having reduced levels of endogenous IgM heavy chain and/or prion protein.

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TRANSGENIC ANIMALS AND USES THEREOF

Background of the Invention

Gene targeting by homologous recombination is a powerful means to specifically modify a gene of interest. The availability of embryonic stem (ES) cells has been instrumental in the study of gene function in mice. In non-murine mammalian species, the lack of ES cells has been circumvented by gene targeting in primary somatic cells, followed by nuclear transfer. In spite of advances in molecular biology techniques, gene targeting in primary cells remains a challenge given the low frequency of homologous recombination (McCreath et al. (2000) Nature 405:1066-1069), the short lifespan of primary cells, and limitations in methods allowing the selection of properly targeted cells. Currently, primary cell gene targeting and production of offspring has mainly been performed with transcriptionally active genes, which are associated with a higher frequency of homologous recombination relative to silent genes (Denning et al. (2001) Nat. Biotechnol. 19:559-562). Furthermore, the selection of correctly targeted cells may be accomplished by having the targeted gene promoter drive the expression of a selection marker, a process which is not applicable to silent genes (Denning et al., *supra*; Lai et al. (2002) Science 295:1089-1092; Yifan et al. (2002) Nat. Biotechnol. 20:251-255; Thomson, A.J., et al., (2003) Reprod. Suppl. 61:495-508).

To fully evaluate the consequences of a genetic modification, both alleles of a gene need to be disrupted. In mice, this is generally accomplished by back crossing from a hemizygous transgenic founder animal to produce a homozygous targeted inbred line. Breeding to homozygosity is extremely time consuming in the mouse and represents an even more severe impediment in species that have a

long generation interval and that are negatively impacted by the consequences of inbreeding. In the pig, two innovative approaches have been used to circumvent this limitation and produce homozygous β(1,3)-galactocyltransferase knockout animals. Hemizygous targeted primary cells were selected *in vitro* for the lack of the enzymatic activity resulting either from a spontaneous point mutation in the second allele of the gene (Denning et al., (2003) Reproduction 126:1-11) or for mitotic recombinants (Piedrahita (2000) Theriogenology 53: 105-16) and cloned offspring were made from the homozygous knockout cell lines. Unfortunately, these approaches are not generally useful for silent genes nor widely applicable for active genes. Thus, improved methods to study gene function in non-human mammals are desirable.

Antibody production in genetically modified animals

In 1890, Shibasaburo Kitazato and Emil Behring reported an experiment with extraordinary results; particularly, they demonstrated that immunity can be transferred from one animal to another by taking serum from an immune animal and injecting it into a non-immune one. This landmark experiment laid the foundation for the introduction of passive immunization into clinical practice. Today, the preparation and use of human immunoglobulin for passive immunization is standard medical practice. In the United States alone, there is a $1.4B per annum market for human immunoglobulin, and each year more than 16 metric tons of human antibody is used for intravenous antibody therapy. Comparable levels of consumption exist in the economies of most highly industrialized countries, and the demand can be expected to grow rapidly in developing countries. Currently, human antibody for passive immunization is obtained from the pooled serum of human donors. This means that there is an inherent limitation in the amount of human antibody available for therapeutic and prophylactic usage. Already, the demand exceeds the supply and severe shortfalls

in availability have been routine.

In an effort to overcome some of the problems associated with the inadequate supply of human immunoglobulin, various technologies have been developed. For example, the production of human immunoglobulin by recombinant methods in tissue culture is routine. Particularly, the recombinant expression of human immunoglobulin in CHO expression systems is well known, and is currently utilized for the production of several human immunoglobulins and chimeric antibodies now in therapeutic use.

Mice retaining an unrearranged human immunoglobulin gene have also been developed for the production of human antibodies (e.g., monoclonal antibodies) (see, for example, PCT Publication Nos. WO98/24893; WO96/33735; WO 97/13852; WO98/24884; WO97/07671; and U.S. Patent Nos. 5,877,397; 5,874,299; 5,814,318; 5,789,650; 5,770,429; 5,661,016; 5,633,425; 5,625,126; 5,569,825; and 5,545,806).

PCT Publication No. WO00/10383 describes modifying a human chromosome fragment and transferring the fragment into certain cells via microcell fusion. U.S. Patent Nos. 5,849,992 and 5,827,690 describe the production of monoclonal antibodies in the milk of transgenic animals including mice, sheep, pigs, cows, and goats wherein the transgenic animals expressed human immunoglobulin genes under the control of promoters that provide for the expression of the antibodies in mammary epithelial cells. Essentially, this results in the expression of the antibodies in the milk of such animals, for example a cow. U.S. Patent Publication Nos. 2003-0037347-0 describe the expression of xenogenous human immunoglobulins in cloned, transgenic ungulates.

Notwithstanding the foregoing, further improved methods for producing ungulates that are amenable to being used as hosts for xenogenous antibody production are of great value to this industry.

Production of prion protein-deficient bovines

The cellular prion protein PrP^C is a ubiquitously expressed, plasma membrane, glycosylphosphatidylinositol-anchored glycoprotein. This protein plays a crucial role in the pathogenesis of transmittable spongiform encephalopathies such as BSE, in cattle, and Creutzfeldt-Jakob disease (CJD), in humans. Its disease-associated, protease-resistant isoform, PrP^{Sc}, has the ability to convert normal PrP^C to PrP^{Sc} and is considered essential for the pathogenesis and transmittance of spongiform encephalopathies. Although the accumulation of PrP^{Sc} in neurons is associated with fatal neurodegeneration, the normal physiological function of the protein remains unclear. Mice with disruptions restricted to the coding region of the PrP gene have been generated and show only minor phenotypic deficits. Importantly, they are resistant to infection by PrP^{Sc}, suggesting that PrP^C is necessary for pathogenesis of prion diseases (Prusiner et al. (1993) Proc. Natl. Acad. Sci. USA 90:10608-10612; Weissmann et al. (1994) Ann. NY Acad. Sci. 724:235-240).

BSE was first recognized in 1986 in the United Kingdom and now has been spread to many countries throughout the world. BSE can be transmitted from cattle to humans by direct consumption of contaminated beef products, resulting in a variant form of CJD (vCJD). Currently, there is no cure for the fatal disease. To reduce risk of exposure to the disease-causing PrP^{Sc} protein, expensive testing programs have been implemented and efforts to remove bovine components from a wide variety of products have been initiated in many countries. Cattle with homozygous null mutation in the PrP gene alleles could be used to alleviate concerns about BSE-contaminated bovine products. Furthermore, these animals could be useful as a model, in addition to the mouse, for investigating the involvement of PrP in BSE.

Summary of the Invention

In general, the invention features genetically modified non-human mammals (e.g., bovines and other ungulates), and methods of making these mammals. In particular, the invention features transgenic ungulates having reduced levels of endogenous IgM heavy chain and/or prion protein.

Transgenic ungulates having reduced IgM heavy chain protein

We discovered that bovines have two IgM heavy chain-encoding genes, each of which is expressed and undergoes VDJ rearrangement. Additionally, we have used homologous recombination to disrupt each of these genes in bovine fibroblasts, which can then be used in cloning methods to make transgenic bovines in which functional IgM is reduced, substantially eliminated, or eliminated.

Although only one IgM-encoding gene has been identified in other ungulates, it is likely, in view of our discovery, that additional such genes are present.

The transgenic ungulates of the invention are useful, for example, for the production of xenogeneic immunoglobulins and xenogeneic hematopoietic stem cells, and for the maintenance of xenogeneic tissues and organs *in vivo*.

Accordingly, the invention features a transgenic ungulate that is producing less than 10% of endogenous IgM heavy chain, relative to a control ungulate, and whose genome comprises a mutation of a gene encoding IgM heavy chain (e.g., bovine IgμU or bovine IgμAY). Desirably, the ungulate is producing less than 5%, 2%, or even 1%, relative to a matching control ungulate. Most desirably, the ungulate is producing no IgM heavy chain or IgM at levels below detection by western blot.

In one embodiment, the ungulate is a transgenic ungulate ungulate whose genome comprises a mutation in each of two genes encoding IgM heavy chain. Desirably, at least one mutation is a homozygous mutation, and more desirably

both mutations are homozygous mutations. The mutation may be an insertion, deletion, or substitution, but most desirably is achieved by insertion of an exogenous nucleic acid, e.g., by homologous recombination. While the ungulate may still make some functional IgM heavy chain, it is most desirable that all functional IgM heavy chain is lost as a result of the mutations.

The invention is exemplified in a bovine, but is equally applicable to other ungulates (e.g., ovines, porcines, and caprines). In the case of bovines, the two IgM-encoding genes are IgμU and IgμAY.

The ungulate may be an adult ungulate, a fetal ungulate, or an ungulate embryo.

The invention also features (i) a transgenic ungulate somatic cell whose genome comprises a mutation of two genes encoding IgM heavy chain, as described above, and (ii) a transgenic bovine somatic cell whose genome comprises a hemizygous or homozygous mutation of IgμAY. Suitable cells include fibroblasts, epithelial cells, endothelial cells, neural cells, keratinocytes, hematopoietic cells, melanocytes, chondrocytes, lymphocytes (B-cells and T-cells), macrophages, monocytes, mononuclear cells, cardiac muscle cells, other muscle cells, and epidermal cells.

The invention further features methods for making xenogenous antibodies. One such method includes the steps of: (a) providing a transgenic ungulate of the invention, the ungulate having engrafted xenogenous hematopoietic stem cells; and (b) recovering xenogenous antibodies from this ungulate (e.g., from the serum or milk).

Another method for producing xenogenous antibodies includes the steps of: (a) providing a transgenic ungulate of the invention, the ungulate having a nucleic acid encoding all or part of a xenogenous immunoglobulin gene that undergoes rearrangement and expresses a xenogenous immunoglobulin; (b) administering one or more antigens of interest to the ungulate; and (c) recovering xenogenous

antibodies from the ungulate.

The invention also features a method of expanding xenogenous hematopoietic stem cells by (a) providing a transgenic ungulate of the invention and having engrafted xenogenous hematopoietic stem cells; and (b) allowing the xenogenous hematopoietic stem cells to expand in said transgenic ungulate. If desired, the expanded hematopoietic stem cells can be collected from the transgenic ungulate.

The invention also features a method for maintaining a desired tissue or organ *in vivo* by: (a) providing a transgenic ungulate of the invention; (b) engrafting desired allogeneic or xenogeneic tissue or organ (e.g., skin, heart, lung, pancreatic, liver or kidney tissue) into the ungulate; and (c) maintaining the tissue or organ in the animal.

In any of the foregoing aspects of the invention, a transgenic ungulate may optionally have one or more nucleic acids encoding all or part of a xenogenous immunoglobulin gene that undergoes rearrangement and expresses one or more xenogenous immunoglobulins. In a preferred embodiment, the nucleic acid encoding all or part of a xenogenous gene is substantially human. Preferably, the nucleic acid encodes a xenogenous antibody, such as a human antibody or a polyclonal antibody. In various embodiments, the immunoglobulin chain or antibody is expressed in serum and/or milk. In other embodiments, the nucleic acid is contained within a chromosome fragment, such as a ΔHAC(FERM BP-7582, the International Patent Organism Depository, National Institute of Advanced Industrial Science and Technology, Tsukuba Central 6, 1-1, Higashi 1-Chome Tsukuba-shi, Ibaraki-ken, 305-8566, Japan) or a ΔΔHAC (FERM BP-7581). In yet other embodiments, the nucleic acid is maintained in an ungulate cell independently from the host chromosome.

In still other embodiments of any of the aspects of the invention, the ungulate has a mutation of one or both alleles of an endogenous alpha-(1,3)-

galactosyltransferase gene, PrP gene, and/or J chain gene. In other preferred embodiments, the ungulate has a nucleic acid encoding an exogenous J chain, such as a human J chain. Preferably, the mutation reduces or eliminates the expression of the endogenous alpha-(1,3)-galactosyltransferase enzyme, galactosyl(α1,3)galactose epitope, prion protein, and/or J chain. In still other preferred embodiments, the ungulate contains a xenogenous J chain nucleic acid, such as a human J chain nucleic acid. Preferably, the ungulate produces human IgA or IgM molecules containing human J chain. In various embodiments of the invention, the nucleic acid used to mutate an endogenous ungulate nucleic acid (e.g., a knockout cassette which includes a promoter operably linked to a nucleic acid encoding a selectable marker and operably linked to a nucleic acid having substantial sequence identity to the gene to be mutated) is not contained in a viral vector, such as an adenoviral vector or an adeno-associated viral vector. For example, the nucleic acid may be contained in a plasmid or artificial chromosome that is inserted into an ungulate cell, using a standard method such as transfection or lipofection that does not involve viral infection of the cell. In yet another embodiment, the nucleic acid used to mutate an endogenous ungulate nucleic acid (*e.g.*, a knockout cassette which includes a promoter operably linked to a nucleic acid encoding a selectable marker and operably linked to a nucleic acid having substantial sequence identity to the gene to be mutated) is contained in a viral vector, such as an adenoviral vector or an adeno-associated viral vector. According to this embodiment, a virus containing the viral vector is used to infect an ungulate cell, resulting in the insertion of a portion or the entire viral vector into the ungulate cell.

Preferably, the ungulate is a bovine, ovine, porcine, or caprine. Preferably, the transgenic ungulate expresses an immunoglobulin chain or antibody from another genus, such as an antibody from any other mammal. Particularly preferred ungulates are sheep, big-horn sheep, goats, buffalos, antelopes, oxen, horses,

donkeys, mule, deer, elk, caribou, water buffalo, camels, llama, alpaca, pigs, and elephants.

The invention also features a method of producing a transgenic ungulate (for example, a transgenic bovine) that rearranges and expresses a xenogenous (e.g., human) immunoglobulin gene locus. This may be accomplished, for example, by stably introducing a human chromosome fragment containing immunoglobulin genes into the ungulate, in order to produce a transgenic ungulate having B-cells that produce xenogenous immunoglobulins, in addition to or in lieu of endogenous immunoglobulins. This may also be accomplished by integrating a nucleic acid encoding a xenogenous immunoglobulin chain or xenogenous antibody into a chromosome of an ungulate. The transgenic ungulate has a mutation in at least two genes encoding IgM, such that the expression of endogenous IgM has been reduced or eliminated. In one embodiment, the invention features a method of producing a transgenic ungulate (for example, a transgenic bovine) in which at least two mu constant regions have been disrupted, and an artificial chromosome containing a gene locus encoding another species' immunoglobulin, preferably human, has been stably incorporated.

The invention also features a method of producing an ungulate somatic or embryonic stem (ES) cell, preferably a fibroblast or B-cell, wherein one or both alleles of at least two the endogenous IgM heavy chain genes have been disrupted, for example, by homologous recombination.

The invention also features a method of inserting, into an ungulate in which at least two the endogenous IgM heavy chain genes have been disrupted, a nucleic acid (for example, an artificial chromosome) that contains genes sufficient for the functional expression of non-ungulate immunoglobulins or their heavy or light chains. Preferably, these immunoglobulins are human immunoglobulins produced by introduction of nucleic acid encoding these immunoglobulins or immunoglobulin chains into an ungulate somatic cell, preferably a fibroblast, and

producing cloned ungulates in which the nucleic acid is transmitted into the germ line.

The invention also features a method for introducing an artificial chromosome, preferably a human artificial chromosome (HAC), that contains genes that provide for immunoglobulin expression into the aforementioned homozygous knockout cells and generate ungulates that express non-ungulate immunoglobulins, preferably human immunoglobulins, in response to immunization and which undergo affinity maturation.

The invention also features methods for producing hybridomas and monoclonal antibodies using B-cells derived from the above-described transgenic ungulates (for example, transgenic bovines).

The invention also features methods for producing ungulate antiserum or milk that includes polyclonal human immunoglobulin by providing a transgenic ungulate described above that is producing polyclonal human immunoglobulins, and collecting ungulate antiserum or milk from the ungulate. Such human immunoglobulin, preferably human IgG, may be used as intravenenous immunoglobulin (IVIG) for the treatment or prevention of disease in humans. The polyclonal human immunoglobulins are preferably reactive against an antigen of interest.

Transgenic ungulates having reduced prion protein

The present invention also features a bovine or bovine fetus that includes a non-naturally occurring mutation in one or both alleles of an endogenous prion nucleic acid. The mutation reduces, substantially eliminates, or eliminates the expression of functional prion protein, and may be hemizygous or homozygous. In addition, the bovine or bovine fetus may carry a further mutation that reduces the expression of an endogenous antibody. This mutation, for example, may reduce or substantially eliminate the expression of functional IgM heavy chain.

Preferably, the bovine fetus is at least 30 days post-fertilization but may be any age up to birth. Preferred bovines include newborns and calves that are at least 1 week of age, and, more preferably, at least 2 months of age.

In a related aspect, the invention further features a product produced from a bovine or bovine fetus of the invention and a method of producing a product that substantially lacks prion protein, the method involving manufacturing the product in, or obtaining the product from, a bovine or bovine fetus of the invention. Exemplary products include milk, gelatin, collagen, and serum obtained from the bovine or bovine fetus, as well as a recombinant protein produced in the bovine or bovine fetus.

Methods for producing non-human mammals and cells containing multiallelic or multigenic mutations

The invention provides a method of producing a cell having two genetic modifications (e.g., mutations or insertions of transgenes or artificial chromosomes). The method includes the steps of: (a) providing a non-human mammalian somatic cell having a first genetic modification; (b) inserting the cell or a progeny thereof, a chromatin mass from said cell or progeny thereof, or a nucleus from the cell or progeny thereof into a nucleated or enucleated oocyte; (c) transferring the oocyte obtained from step (b) or an embryo formed from the oocyte into a recipient; (d) isolating a cell from the embryo, or from a fetus or juvenile produced therefrom, wherein the cell contains the first genetic modification; and (e) introducing a second genetic modification into the genome of the cell of step (d) or a progeny thereof, thereby producing a cell having two genetic modifications.

The present invention thus provides methods for producing non-human mammals and cells containing multiallelic or multigenic mutations. Examples of such mammals are ungulates (e.g., bovines, ovines, porcines, caprines, equines, or

buffaloes), rabbits, mice, rats, or primates (e.g., monkeys, baboons, or gorillas). Using the rejuvenation techniques provided herein, the lifespan of cells is increased, thereby allowing the genetic manipulation of cells on a long-term basis.

In one aspect, the present invention provides for producing a bovine cell containing multiallelic or multigenic mutations, involving the steps of: (a) providing a somatic ungulate cell having a mutation in a first allele of an endogenous gene; (b) inserting the cell or a progeny thereof, a chromatin mass from the cell or a progeny thereof, or a nucleus from the cell or a progeny thereof into a nucleated or enucleated oocyte; (c) transferring the oocyte obtained from step (b) or an embryo formed from this oocyte into the oviduct or uterus of a bovine; (d) allowing the transferred oocyte or the embryo of step (c) to develop into a fetus; (e) isolating a fetal cell from the bovine at between 25 and 90 days of gestation; and (f) introducing a mutation into the second allele of the first endogenous gene or into an allele of a different, second endogenous gene, thereby producing a bovine cell containing multiallelic or multigenic mutations. If desired, the method of the invention further involves, beginning with the cell obtained in step (f) or a progeny thereof, repeating the above method one or more times, thereby introducing mutations in additional alleles or genes.

The invention further provides a method for producing a non-human mammal (e.g., an ungulate) containing two genetic modifications, involving the steps of: (a) providing a non-human mammalian cell containing multiallelic or multigenic mutations (e.g., any one of the cells obtained above); (b) inserting the cell or a progeny thereof, a chromatin mass from the cell or a progeny thereof, or a nucleus from the cell or a progeny thereof into a nucleated or enucleated oocyte; (c) transferring the oocyte obtained from step (b) or an embryo formed from this oocyte into a non-human mammal; (d) allowing the transferred oocyte or the embryo of step (c) to develop into a non-human mammal, thereby producing a non-human mammal containing two genetic modifications. If desired, prior to

step (b), the cell provided in step (a) may be permeabilized under conditions that allow chromatin condensation.

Exemplary non-human mammals of the invention are ungulates (e.g., bovines, ovines, porcines, caprines, equines, or buffalos), primates (e.g., monkeys, baboons, or gorillas), rabbits, mice, and rats. Desirably, a bovine (e.g., *Bos taurus* or *Bos indicus*) containing two genetic odifications is produced using the cloning methods described herein.

In all foregoing aspects of the invention, any somatic cell may be used (e.g., a cell from an embryo, fetus, calf, or adult). Exemplary cells include fibroblasts, epithelial cells, neural cells, epidermal cells, keratinocytes, hematopoietic cells, melanocytes, chondrocytes, B-lymphocytes, T-lymphocytes, erythrocytes, macrophages, monocytes, placental cells, and muscle cells. If the non-human mammal is a bovine, desirably, somatic cells are isolated from a fetus at between 25 to 90 days of gestation, between 35 to 60 days of gestation, between 35 and 50 days, between 35 and 45 days, between 38 and 43 days, and most preferably at about 40 days of gestation. Optionally, somatic cells may be permeabilized as described herein prior to the their insertion into oocytes.

One type of genetic modification is a mutation. Mutations may be introduced into transcriptionally active genes or transcriptionally silent genes. Exemplary endogenous genes in which mutations may be introduced are antibody-encoding genes (e.g., J chain or Igμ genes such as IgμU or IgμAY), genes encoding α-(1, 3)-galactosyltransferase, or prion genes.

Typically, a mutation is introduced by the insertion of a polynucleotide (e.g., positive selection marker such as an antibiotic resistance gene) into an endogenous gene. Optionally, a mutation that is introduced in an endogenous gene reduces the expression of this gene. Alternatively, a mutation may also include the insertion of xenogenous nucleic acid molecule encoding for a polypeptide, such as an antibody. If desired, the polynucleotide may also contain

recombinase sites flanking the positive selection marker, such as loxP sites, so that the positive selection marker may be removed by a recombinase (e.g., Cre recombinase).

Another type of genetic modification is a transgene that has been inserted into the cell's genome without introducing a mutation. One desirable transgene is an artificial chromosome such as a human artificial chromosome. The use of artificial chromosomes for producing xenogenous antibody (e.g., human antibody) is described herein.

As used herein, by "allele" is meant one member of a DNA pair that occupies a specific position on a specific chromosome.

By "artificial chromosome" is meant a mammalian chromosome or fragment thereof which has an artificial modification such as the addition of a selectable marker, the addition of a cloning site, the deletion of one or more nucleotides, the substitution of one or more nucleotides, and the like. By "human artificial chromosome (HAC)" is meant an artificial chromosome generated from one or more human chromosome(s). An artificial chromosome can be maintained in the host cell independently from the endogenous chromosomes of the host cell. In this case, the HAC can stably replicate and segregate along side endogenous chromosomes. Alternatively, it may be translocated to, or inserted into, an endogenous chromosome of the host cell. Two or more artificial chromosomes can be introduced to the host cell simultaneously or sequentially. For example, artificial chromosomes derived from human chromosome #14 (comprising the Ig heavy chain gene), human chromosome #2 (comprising the Ig kappa chain gene), and human chromosome #22 (comprising the Ig lambda chain gene) can be introduced. Alternatively, an artificial chromosome(s) comprising both a xenogenous Ig heavy chain gene and Ig light chain gene, such as ΔHAC, ΔΔHAC, or κHAC may be introduced. Preferably, the heavy chain loci and the light chain

loci are on different chromosome arms (i.e., on different side of the centromere). In still other preferred embodiments, the total size of the HAC is less than or equal to approximately 12, 10, 9, 8, or 7 megabases.

By a "bovine fetus" is meant a bovine in utero that is at least 30 days post-fertilization.

By "cells derived from an embryo" is meant cells that result from the cell division of cells in the embryo.

By "chimeric embryo" is meant an embryo formed from cells from two or more embryos. The resulting fetus or offspring can have cells that are derived from only one of the initial embryos or cells derived from more than one of the initial embryos. If desired, the percentage of cells from each embryo that are incorporated into the placental tissue and into the fetal tissue can be determined using standard FISH analysis or analysis of a membrane dye added to one embryo.

By "chimeric ungulate" is meant an ungulate formed from cells from two or more embryos. The ungulate can have cells that are derived from only one of the initial embryos or cells derived from more than one of the initial embryos. If desired, the percentage of cells from each embryo that are incorporated into the placental tissue and into the fetal tissue can be determined using standard FISH analysis or analysis of a membrane dye added to one embryo.

By "chromatin mass" is meant more than one chromosome not enclosed by a membrane. Preferably, the chromatin mass contains all of the chromosomes of a cell. An artificially induced chromatin mass containing condensed chromosomes may be formed by exposure of a nucleus to a mitotic reprogramming media (e.g., a mitotic extract) as described herein. Alternatively, an artificially induced chromatin mass containing decondensed or partially condensed chromosomes may be generated by exposure of a nucleus to one of the following, as described herein: a mitotic extract containing an anti-NuMA antibody, a detergent and/or salt solution, or a protein kinase solution. A chromatin mass may contain discrete

chromosomes that are not physically touching each other or may contain two or more chromosomes that are in physical contact.

If desired, the level of chromosome condensation may be determined using standard methods by measuring the intensity of staining with the DNA stain, DAPI. As chromosomes condense, this staining intensity increases. Thus, the staining intensity of the chromosomes may be compared to the staining intensity for decondensed chromosomes in interphase (designated 0% condensed) and maximally condensed chromosomes in mitosis (designated 100% condensed). Based on this comparison, the percent of maximal condensation may be determined. Preferred condensed chromatin masses are at least 20, 30, 40, 50, 60, 70, 80, 90, or 100% condensed. Preferred decondensed or partially condensed chromatin masses are less than 10% condensed.

By "days of gestation" is meant the days from the time that the oocyte or embryo is transferred into a uterus.

By "donor cell" is meant a cell from which a nucleus or chromatin mass is derived, or a permeabilized cell.

By "embryo" or "embryonic" is meant a developing cell mass that has not implanted into the uterine membrane of a maternal host. Hence, the term "embryo" may refer to a fertilized oocyte; an oocyte containing a donor chromatin mass, nucleus, or reprogrammed cell; a pre-blastocyst stage developing cell mass; or any other developing cell mass that is at a stage of development prior to implantation into the uterine membrane of a maternal host and prior to formation of a genital ridge. An embryo may represent multiple stages of cell development. For example, a one cell embryo can be referred to as a zygote; a solid spherical mass of cells resulting from a cleaved embryo can be referred to as a morula, and an embryo having a blastocoel can be referred to as a blastocyst. An "embryonic cell" is a cell isolated from or contained in an embryo.

By "embryo cloning" is meant the process in which an embryo is produced from a cell or cellular materials from another animal. Embryo cloning may be performed, for example, by inserting or fusing a donor cell, nucleus, or chromatin mass with an oocyte. The resulting oocyte or the embryo formed from this oocyte is then transferred into the uterus of an animal, thereby producing a cloned animal.

By "enrichment or depletion of a factor" is meant the addition or removal of a naturally-occurring or recombinant factor by at least 20, 40, 60, 80, or 100% of the amount of the factor originally present in a reprogramming media (e.g., a cell extract). Alternatively, a naturally-occurring or recombinant factor that is not naturally present in the reprogramming media may be added. Preferred factors include proteins such as DNA methyltransferases, histone deacetylases, histones, protamines, nuclear lamins, transcription factors, activators, and repressors; membrane vesicles, and organelles. In one preferred embodiment, the factor is purified prior to being added to the reprogramming media, as described below. Alternatively, one of the purification methods described below may be used to remove an undesired factor from the reprogramming media.

By "fetus" is meant a developing cell mass that has implanted into the uterine membrane of a maternal host. A "fetal cell" is any cell isolated from or contained in a fetus at any stage of gestation including birth.

By "fragment" is meant a polypeptide having a region of consecutive amino acids that is identical to the corresponding region of an antibody of the invention but is less than the full-length sequence. The fragment has the ability to bind the same antigen as the corresponding antibody based on standard assays, such as those described herein. Preferably, the binding of the fragment to the antigen is at least 20, 40, 60, 80, or 90% of that of the corresponding antibody.

By "gene" is meant a hereditary unit consisting of a sequence of DNA that occupies a specific location on a chromosome and determines a particular characteristic in an organism. A gene typically has two alleles.

By "hemizygous mutation" is meant that one allele of an endogenous gene has been mutated and the other allele has not been mutated.

By "homozygous mutation" is meant that two alleles of an endogenous gene have been mutated. According to this invention, the mutation-introducing event at both alleles may or may not be the same. Accordingly, two alleles of an endogenous gene genetically targeted by two different targeting vectors would be considered a homozygous mutation.

By "homozygous knock-out non-human mammal" is meant a mammal other than a human in which the two alleles of an endogenous gene have been genetically targeted, resulting in the marked reduction or elimination of expression of a functional gene product. According to this invention, the genetic targeting event at both alleles may or may not be the same. Accordingly, a non-human mammal, in which the two alleles of an endogenous gene have been genetically targeted by two different targeting vectors resulting in the null expression of the endogenous gene, would be considered as being a homozygous knock-out non-human mammal.

By "immortalized" is meant capable of undergoing at least 25, 50, 75, 90, or 95% more cell divisions than a naturally-occurring control cell of the same cell type, genus, and species as the immortalized cell or than the donor cell from which the immortalized cell was derived. Preferably, an immortalized cell is capable of undergoing at least 2-, 5-, 10-, or 20-fold more cell divisions than the control cell. More preferably, the immortalized cell is capable of undergoing an unlimited number of cell divisions. Immortalized cells include cells that naturally acquire a mutation *in vivo* or *in vitro* that alters their normal growth-regulating process. Still other preferred immortalized cells include cells that have been genetically modified to express an oncogene, such as ras, myc, abl, bcl2, or neu, or that have been infected with a transforming DNA or RNA virus, such as Epstein Barr virus or SV40 virus (Kumar et al. (1999) Immunol. Lett. 65:153-159; Knight et al.

(1988) Proc. Natl. Acad. Sci. USA 85:3130-3134; Shammah et al. (1993) J. Immunol. Methods 160:19-25; Gustafsson and Hinkula (1994) Hum. Antibodies Hybridomas 5:98-104; Kataoka et al. (1997) Differentiation 62:201-211; Chatelut et al. (1998) Scand. J. Immunol. 48:659-666). Cells can also be genetically modified to express the telomerase gene (Roques et al. (2001) Cancer Res. 61:8405-8507).

By "knock-in mutation" is meant the insertion of an exogenous nucleic acid, optionally, encoding a polypeptide, into the chromosome of a cell.

By "mutation" is meant an alteration in a naturally-occurring or reference nucleic acid sequence, such as an insertion, deletion, frameshift mutation, silent mutation, nonsense mutation, or missense mutation. Preferably, the amino acid sequence encoded by the nucleic acid sequence has at least one amino acid alteration from a naturally-occurring sequence. Examples of recombinant DNA techniques for altering the genomic sequence of a cell, embryo, fetus, or mammal include inserting a DNA sequence from another organism (e.g., a human) into the genome, deleting one or more DNA sequences, and introducing one or more base mutations (e.g., site-directed or random mutations) into a target DNA sequence. Examples of methods for producing these modifications include retroviral insertion, artificial chromosome techniques, gene insertion, random insertion with tissue specific promoters, homologous recombination, gene targeting, transposable elements, and any other method for introducing foreign DNA. All of these techniques are well known to those skilled in the art of molecular biology. A "non-naturally occurring mutation" is one that is introduced artificially, for example, by recombinant means.

By "non-human mammal comprising multiallellic mutations" or "multiallelic non-human mammal" is meant a mammal other than a human in which two alleles of an endogenous gene have been mutated. The mutations in the two alleles may or may not be in the same location, and may or may not be due to

the same type of alteration. For example, the alleles of the gene in a multiallelic non-human mammal may be mutated by the insertion of two different polynucleotide sequences.

By "non-human mammal comprising multigenic mutations" or "multigenic non-human mammal" is meant a mammal other than a human in which two or more different genes have been mutated. The mutations in the two genes may or may not be in the same location and may or may not be due to the same type of alteration. For example, two genes in a multigenic non-human mammal may be mutated by the insertion of two different polynucleotide sequences.

By "nucleus" is meant a membrane-bounded organelle containing most or all of the DNA of a cell. The DNA is packaged into chromosomes in a decondensed form. Preferably, the membrane encapsulating the DNA includes one or two lipid bilayers or has nucleoporins.

By "permeabilization" is meant the formation of pores in the plasma membrane or the partial or complete removal of the plasma membrane.

By "placenta" is meant the membranous vascular organ that develops in female mammals during pregnancy, lining the uterine wall and partially enveloping the fetus, to which it is attached by the umbilical cord.

By "purified" is meant separated from other components that naturally accompany it. Typically, a factor is substantially pure when it is at least 50%, by weight, free from proteins, antibodies, and naturally-occurring organic molecules with which it is naturally associated. Preferably, the factor is at least 75%, more preferably, at least 90%, and most preferably, at least 99%, by weight, pure. A substantially pure factor may be obtained by chemical synthesis, separation of the factor from natural sources, or production of the factor in a recombinant host cell that does not naturally produce the factor. Proteins, vesicles, and organelles may be purified by one skilled in the art using standard techniques such as those described by Ausubel et al. Current Protocols in Molecular Biology, John Wiley &

Sons, 1995). The factor is preferably at least 2, 5, or 10 times as pure as the starting material, as measured using polyacrylamide gel electrophoresis, column chromatography, optical density, HPLC analysis, or western blot analysis (Ausubel et al., *supra*). Preferred methods of purification include immunoprecipitation, column chromatography such as immunoaffinity chromatography, magnetic bead immunoaffinity purification, and panning with a plate-bound antibody.

By "recloned" is meant used in a subsequent (e.g., second) round of cloning. In particular, a cell from an embryo, fetus, or adult generated from the methods of the invention may be incubated in a mitotic reprogramming media (e.g., a mitotic cell extract) to form a chromatin mass for insertion into an enucleated oocyte, as described above. Alternatively, the cell may be permeabilized, incubated in a reprogramming media, and inserted into an enucleated oocyte, as described above. Performing two or more rounds of cloning may result in additional reprogramming of the donor chromatin mass or donor cell, thereby increasing the chance of generating a viable offspring after the last round of cloning.

By "reducing the expression of an endogenous antibody" is meant reducing the amount of endogenous, functional antibodies produced by a B-cell or a population of B-cells. This reduction in the amount of endogenous antibodies may be due to a decrease in the amount of endogenous antibodies produced per B-cell, a decrease in the number of functional endogenous B-cells, or a combination thereof. Preferably, the amount of an endogenous antibody secreted by a B-cell or expressed on the surface of a B-cell expressing or secreting endogenous antibody is reduced by at least 25, 50, 75, 90, or 95%. In another preferred embodiment, the number of endogenous B-cells in a sample from the recipient mammal, such as a blood sample, is reduced by at least 25, 50, 75, 90, or 95%.

By "reprogramming media" is meant a solution that allows the removal of a factor from a cell, nucleus, chromatin mass, or chromosome or the addition of a factor from the solution to the cell, nucleus, chromatin mass, or chromosome. Preferably, the addition or removal of a factor increases or decreases the level of expression of an mRNA or protein in the donor cell, chromatin mass, or nucleus or in a cell containing the reprogrammed chromatin mass or nucleus. In another embodiment, incubating a permeabilized cell, chromatin mass, or nucleus in the reprogramming media alters a phenotype of the permeabilized cell or a cell containing the reprogrammed chromatin mass or nucleus relative to the phenotype of the donor cell. In yet another embodiment, incubating a permeabilized cell, chromatin mass, or nucleus in the reprogramming media causes the permeabilized cell or a cell containing the reprogrammed chromatin mass or nucleus to gain or lose an activity relative to the donor cell.

Exemplary reprogramming media include solutions, such as buffers, that do not contain biological molecules such as proteins or nucleic acids. Such solutions are useful for the removal of one or more factors from a nucleus, chromatin mass, or chromosome. Other preferred reprogramming medias are extracts, such as cellular extracts from cell nuclei, cell cytoplasm, or a combination thereof. Exemplary cell extracts include extracts from oocytes (e.g., mammalian, vertebrate, or invertebrate oocytes), male germ cells (mammalian, vertebrate, or invertebrate germ cells such as spermatogonia, spermatocyte, spermatid, or sperm), and stem cells (e.g., adult or embryonic stem cells). Yet other reprogramming media are solutions or extracts to which one or more naturally-occurring or recombinant factors (e.g., nucleic acids or proteins such as DNA methyltransferases, histone deacetylases, histones, protamines, nuclear lamins, transcription factors, activators, repressors, chromatin remodeling proteins, growth factors, interleukins, cytokines, or other hormones) have been added, or extracts from which one or more factors have been removed. Still other reprogramming

media include solutions of detergent (e.g., 0.01% to 0.1%, 0.1% to 0.5%, or 0.5% to 2% ionic or non-ionic detergent such as one or more of the following detergents: SDS, Triton X-100, Triton X-114, CHAPS, Na-deoxycholate, n-octyl glucoside, Nonidet P40, IGEPAL, Tween 20, Tween 40, or Tween 80), salt (e.g., ~0.1, 0.15, 0.25, 0.5, 0.75, 1, 1.5, or 2 M NaCl or KCl), polyamine (e.g., ~1 μM, 10 μM, 100 μM, 1 mM or 10 mM spermine, spermidine, protamine, or poly-L-lysine), a protein kinase (e.g., cyclin-dependent kinase 1, protein kinase C, protein kinase A, MAP kinase, calcium/calmodulin-dependent kinase, CK1 casein kinase, or CK2 casein kinase), and/or a phosphatase inhibitor (e.g., ~10 μM, 100 μM, 1 mM, 10 mM, 50 mM, 100 mM of one or more of the following inhibitors: Na-orthovanadate, Na-pyrophosphate, Na-fluoride, NIPP1, inhibitor 2, PNUTS, SDS22, AKAP149, or ocadaic acid) or nuclioplasmin. In some embodiments, the reprogramming medium contains an anti-NuMA antibody. If desired, multiple reprogramming media may be used simultaneously or sequentially to reprogram a donor cell, nucleus, or chromatin mass.

By "reprogrammed cell" is meant a cell that has been exposed to a reprogramming media. Preferably, at least 1, 5, 10, 15, 20, 25, 50, 75, 100, 150, 200, 300, or more mRNA or protein molecules are expressed in the reprogrammed cell that are not expressed in the donor or permeabilized cell. In another preferred embodiment, the number of mRNA or protein molecules that are expressed in the reprogrammed cell, but not expressed in the donor or permeabilized cell, is between 1 and 5, 5 and 10, 10 and 25, 25 and 50, 50 and 75, 75 and 100, 100 and 150, 150 and 200, or 200 and 300, inclusive. Preferably, at least 1, 5, 10, 15, 20, 25, 50, 75, 100, 150, 200, 300, or more mRNA or protein molecules are expressed in the donor or permeabilized cell that are not expressed in the reprogrammed cell. In yet another preferred embodiment, the number of mRNA or protein molecules that are expressed in the donor or permeabilized cell, but not expressed in the reprogrammed cell, is between 1 and 5, 5 and 10, 10 and 25, 25 and 50, 50 and 75,

75 and 100, 100 and 150, 150 and 200, or 200 and 300, inclusive. In still another preferred embodiment, these mRNA or protein molecules are expressed in both the donor cell (i.e., the donor or permeabilized starting cell) and the reprogrammed cell, but the expression levels in these cells differ by at least 2, 5, 10, or 20-fold, as measured using standard assays (see, for example, Ausubel et al., *supra*).

By "substantially identical" is meant having a sequence that is at least 80, 90, 95, 98, or 100% identical to that of another sequence. Sequence identity is typically measured using BLAST® (Basic Local Alignment Search Tool) or BLAST® 2with the default parameters specified therein (see, Altschul et al. (1990) J. Mol. Biol. 215:403-410; and Tatiana et al. (1999) FEMS Microbiol. Lett. 174:247-250). These software programs match similar sequences by assigning degrees of homology to various substitutions, deletions, and other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine, valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

By "viable offspring" is meant an animal that survives *ex utero*. Preferably, the mammal is alive for at least one second, one minute, one hour, one day, one week, one month, six months, or one year from the time it exits the maternal host. The animal does not require the circulatory system of an *in utero* environment for survival.

Other features and advantages of the invention will be apparent from the following detailed description and from the claims.

Brief Description of the Drawings

FIGURE 1 is a schematic diagram depicting sequential gene targeting in bovine primary fibroblasts. Holstein fetal fibroblasts (#6939) were targeted, after which wells containing targeted cells were selected and cloned, using a chromatin

transfer system to generate IgμU$^{-/+}$ fetuses. The IgμU$^{-/+}$ cell line (#3287) was then used for the production of calves and for targeting the second allele of IgμU. Once again, cells were selected and regenerated by production of fetuses. Fetuses were harvested for the production of IgμU-/- cell lines, gene expression analysis, and production of calves. An IgμU$^{-/-}$ cell line (#4658) was transfected with a Cre-recombinase expression plasmid to remove both the neo and puro genes simultaneously, followed by a third round of chromatin transfer to generate cloned fetuses and cell lines, in which both neo and puro selection marker genes were excised. One Cre-excised IgμU$^{-/-}$ fibroblast cell line (#1404) was used for a third round of gene targeting to produce triple targeted, Cre/IgμU$^{-/-}$/PrP$^{-/+}$ fetuses and cell lines. One cell line (#8334) was subjected to the fourth round of gene targeting to produce double homozygous KO (cre/IgμU$^{-/-}$/PrP$^{-/-}$) fetuses and cell lines and for the evaluation of PrP gene expression.

FIGURE 2A is a schematic diagram representing the structure of IgμU constant region locus in #6939, the puro vector used for the first round of targeting, and the genomic PCR assay used for the targeting event. The targeting vector was composed of a 5' homologous arm (7.2 kb), a 3' homologous arm (2.0 kb), STOP cassette containing transcriptional and translational stop sequences, DT-A (diphtheria toxin A gene), and a floxed puro gene. The vector was designed to insert the knockout cassette into exon 2 of the IgμU constant region locus. In #6939 fibroblasts, polymorphic sequences were found to distinguish allele A and allele B, as indicated. Primer pairs, puroF2 x puroR2 were used to identify the first targeting event. PCR and sequencing product showed that the vector was integrated into allele B in cell line #3287 and in allele A in #2184-1 and 2 cell lines, based on the polymorphic sequences presented in the PCR product.

FIGURE 2B is a photograph representing the identification of IgμU$^{-/+}$ fetuses by genomic PCR with puroF2 x puroR2 primers. N is a negative control (mixture of the 1st KO vector and #6939 genomic DNA) and P is a positive

control (mixture of about 10^4 copies/μl of plasmid DNA covering puroF2-puroR2 region and #6939 genomic DNA). Cell lines #2184-1, #2184-2 and #3287 were $Ig\mu U^{-/+}$.

FIGURE 2C is a photograph representing the genotyping of $Ig\mu U^{-/+}$ calves by genomic PCR with puroF2 x puroR2 primers. N is a negative control (mixture of the 1st KO vector and #6939 genomic DNA) and P is a positive control (mixture of about 10^4 copies/μl of plasmid DNA covering puroF2-puroR2 region and #6939 genomic DNA). Out of 13 $Ig\mu U^{-/+}$ calves born (also shown in FIGURE 2C), five were genotyped and found to be positive to the first targeting event.

FIGURE 3A is a schematic diagram depicting the structure of $Ig\mu U^{-/+}$ #3287 alleles, the *neo* vector used for targeting the second allele, and the genomic PCR assay for the targeting events. Primer pairs, neoF3 x neoR3 were used to identify the *neo* targeting event at allele A. BCμf x BCμr is a primer pair used to confirm the absence of wild-type alleles. The primers would not amplify sequence from the targeted alleles because of the presence of STOP cassettes.

FIGURE 3B is a series of photographs representing the identification of $Ig\mu U^{-/-}$ fetuses and fibroblasts by genomic PCR with puroF2 x puroR2, neoF3 x neoR3, and BCμf x BCμr primers. "P" is a positive control (mixture of about 10^4 copies/μl of plasmid DNA covering either puroF2-puroR2 or neoF3-neoR3 region and #6939 genomic DNA). "N" is a negative control (mixture of either the 1st KO or 2nd KO vector and #6939 genomic DNA), and #6939 is the original fibroblast cell line. Cell lines #4658, #3655, #5109, #5139, and #4554 were positive for the targeting events both at allele A (neo-targeting) and B (puro-targeting), but negative for wild-type alleles.

FIGURE 3C is a photograph showing RT-PCR analysis of IgμU expression in mRNA extracted from spleen in day 90 fetuses. Clear expression from a positive control "P" (commercially available polyA+ bovine spleen RNA) and the wild-type (#6939) fetuses (#1, #2), but not from $Ig\mu U^{-/-}$ fetuses, was

detected.

FIGURE 3D is a series of photographs showing the genotyping of Igμ$U^{-/-}$ calves by genomic PCR with puroF2 x puroR2, neoF3 x neoR3 and BCμf x BCμr primers. N is a negative control (mixture of either the 1st KO or 2nd KO vector and #6939 genomic DNA) and P is a positive control (mixture of about 10^4 copies/μl of plasmid DNA covering either puroF2-puroR2 or neoF3-neoR3 region and #6939 genomic DNA). The two Igμ$U^{-/-}$ calves born (one of which is shown in FIGURE 3D) were genotyped and were positive for targeting events at both allele B and A of Igμ U gene but were negative for the wild-type allele.

FIGURE 4A is a schematic diagram representing the structure of Igμ$U^{-/-}$ #4658 alleles and the genomic PCR assay for Cre-loxP mediated removal of selection marker genes. Amplification from primer pairs, CreExF x CreExR, results in a 2.5 kb fragment from the puro targeted allele, a 4.3 kb from the neo targeted allele, or a short 0.4 kb fragment when both selection marker genes are excised.

FIGURE 4B is a photograph showing the identification of Cre/Igμ$U^{-/-}$ fetuses and fibroblasts by genomic PCR with CreExF x CreExR primers. In #4658 cell line, prior to introduction of Cre, 2.5 kb (puro) and 4.3 kb (neo) PCR products are detected. In the five Cre/Igμ$U^{-/-}$ fetuses and cell lines, these bands completely disappear and, instead, a 0.4 kb (without puro and neo) band is detected.

FIGURE 5A is a schematic diagram illustrating the structure of PrP locus in Cre/Igμ$U^{-/-}$ #1404 cell line, the targeting vector for PrP gene, and the genomic PCR assay for the targeting event. The vector was composed of a 5' homologous arm (1.2 kb), a 3' homologous arm (8.3 kb), STOP cassette, DT-A gene and floxed *neo* gene. The vector was designed to insert the knockout cassette just behind its initial ATG codon located in exon 3 of the PrP locus. A single base pair polymorphism was found between allele C and allele D as indicated. Primer pair,

neoF7 x neoR7, was designed to show the neo targeting event and include the polymorphic base. PCR and sequencing products showed that the vector was integrated into allele C.

FIGURE 5B is a series of photographs showing the identification of the triple targeted fetuses and fibroblast cell lines ($Ig\mu U^{-/-}/PrP^{-/+}$) by genomic PCR with positive PrP primer pair, neoF7 x neoR7, and negative IgμU primer pair, BCμf x BCμr. P is a positive control (mixture of about 10^4 copies/μl of plasmid DNA covering neoF7-neoR7 region and #6939 genomic DNA) and #1404 is a negative control. Cell lines derived from fetus #8103, 1661, 8375, 8112 and 8443 were positive for the PrP targeting and negative for wild-type IgμU alleles, demonstrating that they were triple targeted cell lines ($Ig\mu U^{-/-}/PrP^{-/+}$).

FIGURE 6A is a schematic diagram showing the structure of $Ig\mu U^{-/-}/PrP^{-/+}$ #8443 alleles, the puro vector used for targeting the second allele and the genomic PCR assay for the targeting events. Primer pairs, puroF14 x puroR14 were used to identify the puro targeting event at allele D. BPrPex3F x BPrPex3R is a primer pair used to confirm the absence of wild-type alleles. The primers would not amplify sequence from the targeted alleles because of the disruption of annealing sites of BPrPex3F primer on the both alleles of PrP gene caused by the homozygous insertion.

FIGURE 6B is a series of photographs showing the identification of double homozygous KO ($Ig\mu U^{-/-}/PrP^{-/-}$) fetuses and fibroblasts by genomic PCR with puroF14 x puroR14, neoF7 x neoR7 and BPrPex3F x BPrPex3R primers. "P" is a positive control (mixture of about 10^4 copies/μl of plasmid DNA covering either puroF14-puroR14 or neoF7-neoR7 region and #6939 genomic DNA). "N" is a negative control (mixture of either the 3rd KO or 4th KO vector and #6939 genomic DNA) and the $Ig\mu U^{-/-}$ fetal cell line (#4658) and the $Ig\mu U^{-/-}/PrP^{-/+}$ cell line (#8443) are indicated. Cell line #8454, 8400 and 6397 are three of the double homozygous KO ($Ig\mu U^{-/-}/PrP^{-/-}$) fetuses. They were positive to the 3rd and 4th

targeting event at allele C (neo-targeting) and D (puro-targeting) of the PrP gene but negative to wild-type alleles.

FIGURE 6C is a photograph showing RT-PCR analysis on the double homozygous KO ($Ig\mu U^{-/-}/PrP^{-/-}$) fetuses. To detect PrP mRNA, PrPmF3 x PrPmR3 primers were used. Clear expression from the #4658 ($Ig\mu U^{-/-}$) and the #8443 ($Ig\mu U^{-/-}/PrP^{-/+}$) fetuses, but no expression from the double homozygous KO ($Ig\mu U^{-/-}/Pr\mathit{P}^{-/-}$) fetuses was observed.

FIGURE 7 shows the polynucleotide sequence of GenBank accession no. U63637.

FIGURE 8 shows the polynucleotide sequence of *Bos taurus* IgμU (GenBank accession no. U636372.2).

FIGURE 9 shows a partial polynucleotide sequence of *Bos taurus* IgμAY (GenBank accession no. AY221099).

FIGURE 10 is a schematic illustration showing that IgμAY could be detected by PCR in $Ig\mu U^{-/-}$ cells.

FIGURE 11 is an illustration of a sequence trace showing that both IgμAY and IgμU undergo VDJ rearrangement and are expressed.

FIGURE 12 is a schematic illustration showing the genomic organization of IgμAY.

FIGURE 13 shows the sequence of the *AY* and *ay* alleles of IgμAY.

FIGURE 14 is a schematic illustration showing the AY KO vector and the ay KO vector.

FIGURE 15 illustrates $PrP^{+/+}$, $PrP^{-/+}$ (341), $PrP^{-/-}$ (342) calves born simultaneously.

FIGURES 16A-16D illustrate genotyping of the $PrP^{-/-}$ $Ig\mu U^{-/-}$ calf 342. Figure 16A shows a fibroblast cell line established from an ear biopsy of $PrP^{-/-}$ $Ig\mu U^{-/-}$ calf 342. Figure 16B shows verification of the $PrP^{-/-}$ $Ig\mu U^{-/-}$ genotype in

the ear biopsy fibroblasts by genomic PCR. P, positive control; N, negative control. As indicated, using puroF14 x puroR14 and neoF7 x neoR7 primers, calf 342 was shown to be PCR-positive for targeting markers at both alleles of the PrP gene. Figure 16C shows the absence of PrP wild type-derived alleles in the $PrP^{-/-}$ $Ig\mu U^{-/-}$ calf. Calf 342 was PCR-negative for wild-type alleles of the PrP gene using BPrPex3F x BPrPex3R primers. Figure 16D shows the additional absence of IgμU wild type-derived alleles in the $PrP^{-/-}$ $Ig\mu U^{-/-}$ calf using BCμf x BCμr primers.

FIGURES 17A-17B illustrate functional inactivation of the PrP gene in $PrP^{-/-}$ $Ig\mu U^{-/-}$ calf 342. Figure 17A shows disruption of mRNA expression in fibroblasts from $PrP^{-/-}$ $Ig\mu U^{-/-}$ calf 342. RT-PCR analysis on $PrP^{-/-}$ $Ig\mu U^{-/-}$ calf 342 was carried out. To detect PrP mRNA, PrPmF3 x PrPmR3 primers were used. Clear expression was detected in the $PrP^{+/+}$ $Ig\mu U^{-/-}$ and $PrP^{-/+}$ $Ig\mu U^{-/-}$ (calf 341) calves, but no expression was detected in $PrP^{-/-}$ $Ig\mu U^{-/-}$ calf 342. As an internal positive control, bovine β-actin mRNA expression was also evaluated using primer pair bBAF x bBAR. Figure 17B shows the absence of PrP protein in fibroblasts of $PrP^{-/-}$ $Ig\mu U^{-/-}$ calf 342. Western blot analysis on $PrP^{-/-}$ $Ig\mu U^{-/-}$ calf 342 was performed. As a positive control, a $PrP^{+/+}$ $Ig\mu U^{-/-}$ calf was analyzed. As a negative control, protein extracts from mouse fibroblasts were used as the monoclonal antibody utilized for this analysis was stated to be specific for bovine PrP protein. The presence of a 33-35 kDa protein band (the size of bovine PrP) was detected in protein extracts from the $PrP^{+/+}$ $Ig\mu U^{-/-}$ and $PrP^{-/+}$ $Ig\mu U^{-/-}$ calves, but no positive band was detected in protein extracts from $PrP^{-/-}$ $Ig\mu U^{-/-}$ calf 342. The same blot was stained with anti-CDC2 monoclonal antibody as an internal positive control.

FIGURE 18 illustrates the absence of PrP protein in the brain stems of $PrP^{-/-}$ calves. Western blot analysis on the brain stems of $PrP^{-/-}$ calves 354 and

282 was performed as described herein. No band for PrP protein was detected in the $PrP^{-/-}$ calves.

FIGURE 19 illustrates the absence of PrP protein in peripheral blood lymphocytes (PBLs) of exemplary $PrP^{-/-}$ calf 352 cloned from a different $PrP^{-/-}$ fibroblast cell line. Western blot analysis of PBLs from $PrP^{-/-}$ calf 352 was performed. No positive band for the 33-35 kDa bovine PrP protein was detected.

Detailed Description

In general, the invention features transgenic non-human mammals (e.g., bovines and other ungulates), and methods of making these mammals. In particular, the invention features transgenic ungulates having reduced levels of endogenous IgM heavy chain and/or prion protein. The invention is described in more detail below.

Methods for producing non-human mammals and cells containing multiallelic or multigenic mutations

The present invention provides rapid methods to sequentially target transcriptionally silent and transcriptionally active genes in non-human mammals, such as cattle (e.g., bovine). More specifically, we have discovered a sequential gene targeting system that we first used to knock out both alleles of a silent gene, the bovine immunoglobulin μU (IgμU) gene, producing heterozygous and homozygous knockout calves. IgμU targeting was followed by sequential knockout targeting of both alleles of a transcriptionally active gene in fibroblasts, the bovine prion protein (PrP) gene, in the $Ig\mu U^{-/-}$ cell line to produce double homozygous knockout fetuses. Using the methods of the invention, gene targeting can be performed sequentially in somatic cells, resulting in the production of multiallelic or multigenic non-human mammals. The present invention further employs cloning methods to rejuvenate cell lines following each round of

targeting, as described in U.S. Patent Application Publication No. 2003-0046722, hereby incorporated by reference.

The disruption of a gene of interest first involves the production of hemizygous gene knockout cells and the production of a fetus by embryonic cloning. Genetically targeted cells are next harvested from the resulting fetuses at any time during gestation. In a bovine, such cell harvesting desirably occurs at between 25 to 90 days of gestation, at between 35 to 60 days of gestation, at between 35 to 50 days of gestation, preferably at between 35 to 45 days, more preferably at between 38 to 43 days, and most preferably at about 40 days of gestation. Next, the second allele of the same gene locus, or alternatively, an allele of a different endogenous gene is targeted in the harvested cells. These cells are next used to derive fetuses, from which somatic cells such as fibroblasts may further be isolated and used for further rounds of cloning. The above steps may then be repeated until cells containing the desired multiallelic or multigenic mutations are generated. If desired, these cells may be used to produce non-human mammals, such as ungulates.

Using the methods of the present invention, we show that each targeting event required approximately 2.5 months from transfection to establishment of regenerated cell lines, such that a single homozygous genetic modification could be made in calves in 14 months (including five months for targeting two alleles and nine months gestation) and a double homozygous modification could be made in 19 months. In contrast, breeding a heterozygous founder to produce homozygous calves would require approximately five years, making the generation of double homozygous calves from two heterozygous founders impractical. Using the sequential targeting strategy described herein, complex genetic modifications in large animal species are not only feasible but also straightforward and useful for many applications.

Genetically modified large animals are, in many cases, more suitable models for the study of human disease than mice, such as for the study of human cystic fibrosis, for example (Harris (1997) Hum. Mol. Genet. 6:2191-2194). Homozygous knockout targeting may also be useful, for example, for the elimination of bovine antibodies when attempting to produce human polyclonal antibodies in a genetically modified cow (Kuroiwa (2002) Nature Biotechnol. 20:889-894). Furthermore, the inactivation of the IgμU gene, for example, may be useful in investigating bovine immunology, such as the mechanism of B-cell development, which is known to be substantially different from that of humans or mice (Butler (1997) Rev. Sci. Tech. 17:43-70). Pigs carrying multiple KOs, not only for α-1,3-galactosyltransferase gene (Phelps et al. (2003) Science 299:411-414), but also for other genes, such as major histocompatibility complex (MHC) genes, may also be a more suitable source of organs and tissue for xenotransplantion. In agriculture, homozygous knockout targeting could be useful in improving the safety and disease-resistance of animals, such as the inactivation of the bovine prion gene, and elimination of the "mad-cow disease" threat. The methods of the invention are therefore useful for complex genetic modifications in animals for gene-functional analysis, as well as biomedical and agricultural applications, without the necessity of germ line transmission.

Transgenic ungulates having reduced IgM heavy chain protein

We have determined that there are two genes encoding bovine IgM heavy chains. The first published sequence asserted to encode bovine IgM is shown in FIGURE 7. This sequence was first published in 1996 as GenBank accession no. U63637 (gi:1575489) by Hammarstrom and colleagues, and subsequently described by them in Immunology (93: 581-588, 1998). U63637 was replaced on February 27, 2003 with a second sequence (GenBank accession no. U63637.2; gi:2859209; FIGURE 8). Later, Hammarstrom and colleagues submitted a third

sequence, encoding *Bos taurus* heavy chain constant region (GenBank accession no. AY221099; gi:33413901; FIGURE 9). In the paper accompanying this third submission (Zhao et al. (2003) J. Biol. Chem. 278:35024-35032), the authors concluded that the differences between the second and third sequences (the first having been retracted) were due to polymorphisms.

We now demonstrate that bovines contain and express two Igμ genes, which we refer to as IgμU and IgμAY. As both genes are expressed, to produce a bovine having no IgM heavy chain protein requires mutation of both genes.

Although only one IgM-encoding gene has been identified in other ungulates, it is likely, in view of our discovery of two Igμ genes in bovines, that additional such genes are present. These additional genes can be identified using standard techniques, such as those described herein.

To alter immunoglobulin genes of other ungulates, targeting vectors are designed to contain three main regions. The first region is homologous to the locus to be targeted. The second region is a drug selection marker that specifically replaces a portion of the targeted locus. The third region, like the first region, is homologous to the targeted locus but is not contiguous with the first region in the wild type genome. Homologous recombination between the targeting vector and the desired wild type locus results in deletion of locus sequences between the two regions of homology represented in the targeting vector and replacement of that sequence with a drug resistance marker. In preferred embodiments, the total size of the two regions of homology is approximately 6 kilobases, and the size of the second region that replaces a portion of the targeted locus is approximately 2 kilobases. This targeting strategy is broadly useful for a wide range of species from prokaryotic cells to human cells. The uniqueness of each vector used is in the locus chosen for gene targeting procedures and the sequences employed in that strategy. This approach may be used in all ungulates, including, without limitation, goats (*Capra hircus*), sheep (*Ovis aries*), and the pig (*Sus scrofa*), as

well as cattle (*Bos taurus* and *Bos indicus*).

The use of electroporation for targeting specific genes in the cells of ungulates may also be broadly used in ungulates. The general procedure described herein is adaptable to the introduction of targeted mutations into the genomes of other ungulates. Modification of electroporation conditions (voltage and capacitance) may be employed to optimize the number of transfectants obtained from other ungulates.

In addition, the strategy used herein to target the heavy chain locus in cattle (i.e., removal of all coding exons and intervening sequences using a vector containing regions homologous to the regions immediately flanking the removed exons) may also be used equally well in other ungulates. For example, extensive sequence analysis has been performed on one immunoglobulin heavy chain locus of sheep (*Ovis aries*), and the sheep locus is highly similar to the bovine locus in both structure and sequence (GenBank accession nos. Z71572, Z49180 through Z49188, M60441, M60440, AF172659 through AF172703). In addition to the large number of cDNA sequences reported for rearranged *Ovis aries* immunoglobulin chains, genomic sequence information has been reported for the heavy chain locus, including the heavy chain 5' enhancer (GenBank accession no. Z98207), the 3' mu switch region (GenBank accession no. Z98680) and the 5' mu switch region (GenBank accession no. Z98681). The complete mRNA sequence for the sheep secreted form of the heavy chain has been deposited as GenBank accession no. X59994. This deposit contains the entire sequence of four coding exons, which are very similar to the corresponding bovine sequence.

Thus, the present invention relates to the production of a transgenic ungulate, preferably a transgenic cow, in which endogenous IgM expression has been reduced or eliminated by the mutation of two or more Igμ genes. Optionally, a nucleic acid (e.g., an artificial chromosome) has been stably introduced that comprises genes that are sufficient for the production of functional antibodies of

another species, preferably human.

Transgenic ungulates having reduced prion protein

As described herein, Applicants have successfully produced a healthy PrP-deficient calf by means of sequential gene targeting. Such animals provide an improved population for production of BSE-free bovine-derived agricultural and biomedical materials, including milk, gelatin, collagen, and serum, as well as an improved source for human recombinant protein production for human therapy and medical materials for human xenotransplantation. The bovine or bovine fetuses of the invention may be used to produce any product normally derived agriculturally from such animals. They may also be used to produce any recombinant protein, including, without limitation, human antibodies, for example, by any of the methods described in U.S. Patent Application Publication Nos. 2003-0037347, 2004-0068760, and 2003-0056237, or PCT Publication No. WO2004/044156, all hereby incorporated by reference.

Non-human mammalian cells

As discussed herein, the methods of the present invention involve the introduction of mutations into somatic non-human mammalian cells, such as somatic ungulate cells. Suitable somatic cells include cells from embryos, fetuses, calves, or adult animals. Preferred cells for gene targeting include differentiated cells such as fibroblasts, epithelial cells, neural cells, epidermal cells, keratinocytes, hematopoietic cells, melanocytes, chondrocytes, B-lymphocytes, T-lymphocytes, erythrocytes, macrophages, monocytes, placental, and muscle cells. Preferred cells also include those from any organ, such as the bladder, brain, esophagus, fallopian tube, heart, intestines, gallbladder, kidney, liver, lung, ovaries, pancreas, prostate, spinal cord, spleen, stomach, testes, thymus, thyroid, trachea, ureter, urethra, and uterus. Preferably, the donor cell, donor nucleus,

donor chromatin mass, or reconstituted oocyte is not tetraploid.

Cells may be derived from any non-human mammal, including an ungulate, rabbit, mouse, rat, or primate. Ungulates include members of the orders Perissodactyla and Artiodactyla, such as any member of the genus Bos. Other preferred ungulates include sheep, big-horn sheep, goats, buffalos, antelopes, oxen, horses, donkeys, mule, deer, elk, caribou, water buffalo, camels, llama, alpaca, pigs, and elephants. Most preferably, the non-human mammal is a bovine (e.g., *Bos taurus or Bos indicus*).

If a cell to be genetically targeted is derived from an embryo or a fetus, the cell may be isolated at any time during the gestation period until the birth of the genetically altered non-human mammal. As discussed above, bovine cells are desirably isolated at between 25 to 90 days of gestation, between 35 to 60 days of gestation, between 35 to 50 days, preferably between 35 to 45 days, more preferably between 38 to 43 days, and most preferably at about 40 days of gestation. Ovine cells are desirably isolated at between 25 to 150 days of gestation, between 30 to 100 days, preferably between 35 to 80 days, more preferably between 35 to 60 days, and most preferably at about 40 days of gestation. Equine cells are desirably isolated at between 25 to 300 days of gestation, between 30 to 100 days, preferably between 35 to 80 days, more preferably between 35 to 60 days, and most preferably at about 40 days of gestation. Porcine cells are desirably isolated at between 25 to 110 days of gestation, between 30 to 90 days, preferably between 30 to 70 days, more preferably between 30 to 50 days, and most preferably at about 35 days of gestation. Caprine cells are desirably isolated at between 25 to 150 days of gestation, between 30 to 100 days, preferably between 35 to 80 days, more preferably between 35 to 60 days, and most preferably at about 40 days of gestation. Primate cells are desirably isolated at between 25 to 150 days of estation, between 30 to 100 days, preferably between 35 to 80 days, more

preferably between 35 to 60 days, and most preferably at about 40 days of gestation. Rodent cells are desirably isolated at between 6 to 18 days of gestation, between 8 to 16 days, preferably between 10 to 16 days, more preferably between 12 to 16 days, and most preferably at about 14 days of gestation.

The recipient cell is preferably an oocyte, a fertilized zygote, or a two-cell embryo, all of which may or may not have been enucleated. Typically, the donor and the recipient cell are derived from the same species. However, there has been success reported in achieving development from embryos reconstructed using donor and recipient cells from different species.

Gene targeting

In general, the genetic targeting events of the invention may include inactivation, removal, or modification of a gene; upregulation of a gene; gene replacement; or transgene replacement at a predetermined locus. Examples of genes that may be targeted resulting in their inactivation, removal, or modification are genes encoding antigens which are xenoreactive to humans (e.g., α-1,3 galactosyltransferase); antibody-encoding genes; genes in the PrP locus responsible for the production of the prion protein and its normal counterpart in non-human animals; genes which in humans are responsible for genetic disease and which in modified, inactivated, or deleted form could provide a model of that disease in animals (e.g., the cystic fibrosis transmembrane conductance regulator gene); genes responsible for substances which provoke food intolerance or allergy; genes responsible for the presence of particular carbohydrate residues on glycoproteins (e.g., the cytidine monophospho-N-acetyl neuraminic acid hydroxylase gene in non-human animals); and genes responsible for the somatic rearrangement of immunoglobulin genes, such as RAG1 and RAG2.

Among genes that can be targeted resulting in their upregulation are genes responsible for suppression of complement-mediated lysis (e.g., porcine CD59,

DAF, and MCP). Furthermore and as described further below, replacement of genes may also be performed. Genes that may be replaced include genes responsible for the production of blood constituents (e.g., serum albumin), genes responsible for substances that provoke food intolerance or allergy, immunoglobulin genes, and genes responsible for surface antigens.

Using the methods of the present invention, the time necessary for the identification, isolation, analysis, and expansion of primary cell clones carrying targeted events is significantly minimized. This is an important aspect of the invention because reduction of the time in culture increases the likelihood that cells used as nuclear donors are viable, normal, and euploid.

Targeting constructs

Targeted gene mutation requires generating a nucleic acid construct having regions of homology to the targeted allele in the gene of interest such that integration of the construct into the genomic allele disrupts its expression. Thus, to alter a gene, a targeting vector is designed to contain three main regions. The first region is homologous to the locus to be targeted. The second region is a polynucleotide sequence (e.g., encoding a selection marker such as an antibiotic resistance protein) that specifically replaces a portion of the targeted locus. The third region, like the first region, is homologous to the targeted locus but is not contiguous with the first region in the wild type genome. Homologous recombination between the targeting vector and the desired wild-type locus results in deletion of locus sequences between the two regions of homology represented in the targeting vector and replacement of that sequence, for example, with a drug resistance marker. The uniqueness of each vector used is in the locus chosen for gene targeting procedures and the sequences employed in that strategy. This approach may be used in all mammals, including ungulates such as, goats (*Capra hircus*), sheep (*Ovis aries*), pigs (*Sus scrofa*), and cattle (*Bos taurus or Bos*

indicus). Exemplary vectors for carrying out such targeted mutation are described herein. Methods for constructing vectors that provide for homologous recombination at other targeted sites are well known to those skilled in the art. Moreover, the construction of a suitable vector is within the level of skill in the art.

In order to facilitate homologous recombination, the vectors used to effect homologous recombination and inactivation of a gene of interest, respectively, contain portions of DNA that exhibit substantial sequence identity to the genes to be targeted. Preferably, these sequences have at least 98% sequence identity, more preferably, at least 99% sequence identity, and even 100% sequence identity with the targeted gene loci to facilitate homologous recombination. In preferred embodiments, the total size of the two regions of homology is approximately 9 - 9.5 kilobases and the size of the second region that replaces a portion of the targeted locus is approximately 2 kilobases.

Typically, the construct includes a marker gene that allows for the selection of desired homologous recombinants, for example, cells in which the gene of interest has been disrupted by homologous recombination. Marker genes include antibiotic resistance markers, drug resistance markers, and green fluorescent protein, among others. One neomycin resistance construct was assembled as follows. A construct designated "pSTneoB" (Katoh et al. (1987) Cell Struct. Funct. 12:575; Japanese Collection of Research Biologicals (JCRB) deposit number: VE039) was designed to contain a neomycin resistance gene under the control of an SV40 promoter and TK enhancer upstream of the coding region. Downstream of the coding region is an SV40 terminator sequence. The *neo* cassette was excised from "pSTneoB" as an *Xho*I fragment. After the ends of the fragment were converted to blunt ends using standard molecular biology techniques, the blunt ended fragment was cloned into the *EcoR*V site in the vector, pBS246 (Gibco/Life Technologies). This site is flanked by loxP sites. The new

construct, designated "pLoxP-STNeoR", was used to generate the mu knockout DNA construct. The desired fragment of this construct is flanked by loxP sites and *Not*I sites, which were originally present in the pBS246 cloning vector. The desired *Not*I fragment, which contains loxP-neo-loxP, was used for replacement of the immunoglobulin mu constant region exons. The SV40 promoter operably linked to the neomycin resistance gene activates the transcription of the neomycin resistance gene, allowing cells in which the desired *Not*I fragment has replaced the mu constant region exons to be selected based on their resulting antibiotic resistance.

The strategy used herein to target genes in cattle (i.e., removal of a portion of the coding region and intervening sequences using a vector containing regions homologous to the regions immediately flanking the removed exons) may also be used in other mammals. For example, extensive sequence analysis has been performed on one immunoglobulin heavy chain locus of sheep (*Ovis aries*), and the sheep locus is highly similar to the bovine locus in both structure and sequence (GenBank accession nos. Z71572, Z49180 through Z49188, M60441, M60440, AF172659 through AF172703). In addition to the large number of cDNA sequences reported for rearranged *Ovis aries* immunoglobulin chains, genomic sequence information has been reported for the heavy chain locus, including the heavy chain 5' enhancer (GenBank accession no. Z98207), the 3' mu switch region (GenBank accession no. Z98680) and the 5' mu switch region (GenBank accession no. Z98681). The complete mRNA sequence for the sheep secreted form of the heavy chain has been deposited as GenBank accession no. X59994. This deposit contains the entire sequence of four coding exons, which are very similar to the corresponding bovine sequence. Accordingly, the GenBank sequence may be used to determine areas of high homology with the bovine IgμU sequence for the design of PCR primers. Because non-isogenic DNA was used to target bovine cells, finding areas of high homology with sheep sequence was used

as an indicator that similar conservation of sequences between breeds of cow was likely. Given the similarity between the sequences and structures of the bovine and ovine immunoglobulin loci, the targeting strategies used herein to remove bovine immunoglobulin loci may be applied to the ovine system. In addition, existing information on pigs (*Sus scrofa*; GenBank accession no. S42881) and goats (*Capra hircus*; GenBank accession no. AF140603) indicates that the immunoglobulin loci of both of these species are also sufficiently similar to the bovine loci to utilize the present targeting strategies.

In one method of targeting cells, the targeting construct includes regulatory expression for driving expression of the marker gene, as well as a polyadenylation signal sequence. Such a construct allows for detection of the inserted sequence independent of the expression of the mutagenized gene and thus permits the identification of recombinants in silent genes (i.e., genes that are not expressed in fibroblasts). In order to determine whether the marker gene integrated into the genome by means of homologous recombination rather than through random insertion, one may use standard molecular biology techniques such as Southern blotting, PCR, or DNA sequencing.

Selection of targeted cells

Genetically targeted cells are typically identified using a selectable marker. If a cell already contains a selectable marker however, a new targeting construct containing a different selectable marker may be required. Alternatively, if the same selectable marker is employed, cells may be selected in the second targeting round by raising the drug concentration (for example, by doubling the drug concentration).

Targeting constructs may also contain selectable markers flanked by loxP sites to facilitate the efficient deletion of the marker using the Cre/lox system. Thus, at some point after the gene targeting event and preferably before any

embryo cloning, such excision may be performed to remove portions of genetic material from the cell. This material may be a selectable marker or an introduced genetic transcription activator. This removal may be carried out by procedures described hereinafter, or by other procedures well known in the art.

In one example, fetal fibroblasts carrying the targeting vector are transfected via electroporation with a Cre containing plasmid (e.g., a Cre plasmid that contains a GFPCre fusion gene as described by Gagneten et al. (1997) Nucleic Acids Res. 25:3326-3331). This allows for the rapid selection of all clones that contain a Cre protein. In this regard, cells are selected either by FACS sorting or by manual harvesting of green fluorescing cells via micromanipulation. Cells that are green are expected to carry actively transcribed Cre recombinase, which would remove the drug resistance marker. Cells selected for Cre expression are cloned and analyzed for the deletion of the drug resistance marker by PCR analysis. Following such confirmation, such cells are used for the next round of genetic targeting or for cloning.

Introduction of xenogenous nucleic acids

If desired, xenogenous nucleic acid molecules encoding a desired polypetide may be inserted into an endogenous gene as part of the introduced mutation. For example, genes encoding antibodies of a particular species may be introduced into an endogenous gene. Preferably, human artificial chromosomes are used for this purpose, such as those disclosed in PCT Publication Nos. WO97/07671 and WO00/10383, each hereby incorporated by reference. These human artificial chromosomes also are described in a corresponding issued Japanese Patent JP 30300092. The construction of artificial human chromosomes that contain and express human immunoglobulin genes is disclosed in Shen et al. (1997) Hum. Mol. Genet. 6:1375-1382; Kuroiwa et al. (2000) Nature Biotechnol. 18:1086-1090; and Loupert et al. (1998) Chromosome 107:255-259. Following

the stable insertion of the artificial chromosome, the cell line (e.g., a bovine fetal fibroblast) may be used as a donor cell for further gene targeting.

As an alternative to the use of human artificial chromosome, polynucleotides encoding genes of interest may also be integrated into the chromosome using a YAC vector, BAC vector, or cosmid vector. Such vectors may be introduced into cells (e.g., fetal fibroblasts cells) using known methods, such as electroporation, lipofection, fusion with a yeast spheroplast comprising a YAC vector, and the like. Desirably, vectors containing genes of interest may be targeted to the endogenous corresponding gene loci of the cells (e.g., fetal fibroblasts), resulting in the simultaneous introduction of the gene of interest and the mutation of the endogenous gene.

Integration of a nucleic acid encoding a gene of interest may also be carried out as described in the patents by Lonberg et al. (U.S. Patent Nos. 5,545,806, 5,569,825, 5,625,126, 5,633,425, 5,661,016, 5,750,172, 5,770,429, 5,789,650, 5,814,318 5,874,299, 5,877,397, and 6,300,129, all of which are hereby incorporated by reference). In the "knock-in" construct used for the insertion of gene of interest into a chromosome of a host mammal, one or more genes and an antibiotic resistance gene may be operably-linked to a promoter which is active in the cell type transfected with the construct. For example, a constitutively active, inducible, or tissue-specific promoter may be used to activate transcription of the integrated antibiotic resistance gene, allowing transfected cells to be selected based on their resulting antibiotic resistance. Alternatively, a knock-in construct in which the knock-in cassette containing the gene(s) of interest and the antibiotic resistance gene is not operably linked to a promoter may be used. In this case, cells in which the knock-in cassette integrates downstream of an endogenous promoter may be selected based on the resulting expression of the antibiotic resistance marker under the control of the endogenous promoter. These selected cells may be used in the embryo cloning procedures described herein to generate a

transgenic non-human mammal containing a gene of interest integrated into a host chromosome. Alternatively, an animal containing exogenous genes of interest may be mated with an animal in which the endogenous gene is inactivated.

Exemplary gene mutations

Any number of exemplary gene mutations may be introduced into mammals using the present methods. For example, the endogenous ungulate Ig J chain gene may be knocked out to prevent the potential antigenicity of the ungulate Ig J chain in the antibodies of the invention that are administered to humans. For the construction of the targeting vector, the cDNA sequence of the bovine Ig J chain region found in GenBank accession number U02301 may be used. This cDNA sequence may be used as a probe to isolate the genomic sequence of bovine Ig J chain from a BAC library such as RPC1-42 (BACPAC in Oakland, CA) or to isolate the genomic sequence of the J chain from any other ungulate. Additionally, the human J chain coding sequence may be introduced into the ungulates of the present invention for the functional expression of human IgA and IgM molecules. The cDNA sequence of human J chain is available as GenBank accession numbers AH002836, M12759, and M12378. This sequence may be inserted into an ungulate fetal fibroblast using standard methods, such as those described herein. For example, the human J chain nucleic acid in a HAC, YAC vector, BAC vector, cosmid vector, or knock-in construct may be integrated into an endogenous ungulate chromosome or maintained independently of endogenous ungulate chromosomes. The resulting transgenic ungulate cells may be used in the embryo cloning methods described herein to generate the desired ungulates that have a mutation that reduces or eliminates the expression of functional ungulate J chain and that contain a xenogenous nucleic acid that expresses human J chain.

In another example, if a non-human mammal, such as an ungulate, is

genetically engineered to produce a human antibody, it may be desirable to also reduce or eliminate the expression of the ungulate α-(1,3)-galactosyltransferase gene, a gene that encodes an enzyme that produces the galactosyl(α1,3)galactose epitope. Glycosylated human antibodies modified by this carbohydrate epitope are sometimes inactivated or eliminated when administered as therapeutics to humans by recipient antibodies reactive with the epitope. To eliminate this possible immune response, the sequence of bovine α-(1,3)-galactosyltransferase gene may be used to design a knockout construct to inactive this gene. The bovine sequence (GenBank accession number J04989; Joziasse et al. (1989) J. Biol. Chem. 264: 14290-14297) or the porcine α-(1,3)-galactosyltransferase sequence (disclosed in U.S. Patent Nos. 5,821,117 and 6,153,428) may be used to inactivate the genes in those species or to obtain the genomic α-(1,3)-galactosyltransferase sequence from a variety of other ungulates to generate mammals with reduced or eliminated expression of the epitope.

The ungulate PrP gene (encoding prion protein) may also be mutated or inactivated to reduce the potential risk of an infection such as bovine spongiform encephalopathy (BSE). Mutation of the bovine PrP gene is described below.

The additional mutations or the gene inactivation mentioned above may be incorporated into the ungulates of the present invention using various methodologies. Once a transgenic ungulate cell line is generated for each desired mutation, crossbreeding may be used to incorporate these additional mutations into the ungulates of the present invention. Alternatively, fetal fibroblast cells which have these additional mutations can be used as the starting material for the knockout of endogenous immunoglobulin genes and/or the introduction of xenogenous immunoglobulin genes. Also, as is described herein, fetal fibroblast cells having a knockout mutation in endogenous immunoglobulin genes and/or containing xenogenous immunoglobulin genes can be uses as a starting material for these additional mutations or inactivations.

Production of cloned non-human mammals

We have previously disclosed a variety of methods for cloning mammals (e.g., ungulates, such as bovines) that may be used to clone mammals with one or more mutations in genes encoding IgM heavy chain (see, e.g., U.S. Patent Application Publication No. 2002-0046722 and PCT Publication No. WO02/051997). In some of these methods, a permeabilized cell is incubated with a reprogramming media (e.g., a cell extract) to allow the addition or removal of factors from the cell, and then the plasma membrane of the permeabilized cell is resealed to enclose the desired factors and restore the membrane integrity of the cell. Some of these methods also involve the condensation of a donor nucleus (e.g., an isolated nucleus or a nucleus within a donor cell) into a chromatin mass to allow the release of nuclear components such as transcription factors that may promote the transcription of genes that are undesirable for the development of the nuclear transplant embryo into a viable offspring. If desired, the steps of any of these methods may be repeated one or more times or different reprogramming methods may be performed sequentially to increase the extent of reprogramming, resulting in greater viability of the cloned fetuses.

Other methods for the production of cloned mammals (e.g., bovines) and cloned transgenic non-human mammals are known in the art, described, for example, in U.S. Patent No. 5,995,577, assigned to University of Massachusetts, and in PCT Publication Nos. WO95/16670; WO96/07732; WO97/0669; and WO97/0668 (collectively, "the Roslin methods"). The Roslin methods differ from the University of Massachusetts techniques in that they use quiescent rather than proliferating donor cells. All of these patents are incorporated by reference herein in their entirety. These techniques are not limited to use for the production of transgenic bovines; the above techniques may be used for embryo cloning of other non-human mammals such as ungulates as well.

Following embryo cloning, production of desired animals may be affected either by mating the ungulates or by secondary gene targeting using the homologous targeting vector previously described.

Cre/Lox excision of the drug resistance marker

In one embodiment of the invention, the Cre/lox system is used to facilitate the efficient deletion of the marker following. Fetal fibroblasts carrying the targeting vector are transfected via electroporation with a Cre containing plasmid. A Cre plasmid that contains a GFPcre fusion gene (Gagneten et al. (1997) Nucleic Acids Res. 25:3326-3331) may be used. This allows the rapid selection of all clones that contain Cre protein. These cells are selected either by FACS sorting or by manual harvesting of green fluorescing cells via micromanipulation. Cells that are green are expected to carry actively transcribed Cre recombinase and hence delete the drug resistance marker. Cells selected for Cre expression are cloned and clones analyzed for the deletion of the drug resistance marker via PCR analysis. Those cells that are determined to have undergone excision are grown to small clones, split and one aliquot is tested in selective medium to ascertain with certainty that the drug resistance gene has been deleted. The other aliquot is used for the next round of targeted deletion.

Methods for breeding ungulates

In preferred embodiments of any of the above methods for generating ungulates or ungulate cells, an ungulate of the invention is mated with another ungulate to produce an embryo, fetus, or live offspring with two or more genetic modifications. Preferably, one or more cells are isolated from the embryo, fetus, or offspring, and one or more additional genetic modifications are introduced into the isolated cell(s).

Methods for producing antibodies

The invention also provides method for producing antibodies using an ungulate of the invention that expresses xenogenous antibodies (e.g., human antibodies). One such method involves administering one or more antigens of interest to an ungulate of the invention having nucleic acid encoding a xenogenous antibody gene locus. The nucleic acid segments in the gene locus undergo rearrangement resulting in the production of antibodies specific for the antigen. Antibodies are recovered from the ungulate. The antibodies may be monoclonal or polyclonal and are preferably reactive with an antigen of interest. Preferably, the antibodies are recovered from the serum or milk of the ungulate.

In a related aspect, the invention provides another method for producing antibodies that involves recovering xenogenous antibodies from an ungulate of the invention having nucleic acid encoding a xenogenous antibody gene locus. The nucleic acid segments in the gene locus undergo rearrangement resulting in the production of xenogenous antibodies. The antibodies may be monoclonal or polyclonal and are preferably reactive with an antigen of interest. Preferably, the antibodies are recovered from the serum or milk of the ungulate. Preferably, the ungulate antiserum or milk has polyclonal human immunoglobulins. Preferably, the antiserum or milk is from a bovine, ovine, porcine, or caprine. In another preferred embodiment, the immunoglobulins are directed against a desired antigen. In preferred embodiments, the antiserum is used as intravenous immunoglobulin (IVIG) for the treatment or prevention of disease in humans. In another preferred embodiment, an antigen of interest is administered to the ungulate, and immunoglobulins directed against the antigen are produced by the ungulate. Preferably, the nucleic acid segments in the xenogenous immunoglobulin gene locus rearrange, and xenogenous antibodies reactive with the antigen of interest are produced. Preferably, the antiserum and/or milk contains at least 2, 5, 10, 20, or 50 fold more xenogenous antibody than

endogenous antibody, or contains no endogenous antibody. If desired, hybridomas and monoclonal antibodies can be produced using xenogenous B-cells derived from the above-described transgenic ungulates (for example, transgenic bovines). It is also contemplated that xenogenous antibodies (e.g., human antibodies) isolated from ungulates may be subsequently chemically modified so that they are covalently linked to a toxin, therapeutically active compound, enzyme, cytokine, radiolabel, fluorescent label, or affinity tag. If desired, the fluorescent or radiolabel may be used for imaging of the antibody *in vitro* or *in vivo*.

Ungulates and donor cells

Ungulates include members of the orders Perissodactyla and Artiodactyla, such as any member of the genus Bos. Other preferred ungulates include sheep, big-horn sheep, goats, buffalos, antelopes, oxen, horses, donkeys, mule, deer, elk, caribou, water buffalo, camels, llama, alpaca, pigs, and elephants.

Preferred cells for gene targeting include differentiated cells such as epithelial cells, neural cells, epidermal cells, keratinocytes, hematopoietic cells, melanocytes, chondrocytes, B-lymphocytes, T-lymphocytes, erythrocytes, macrophages, monocytes, fibroblasts, and muscle cells; and undifferentiated cells such as embryonic cells (e.g., stem cells and embryonic germ cells). In another preferred embodiment, the cell is from the female reproductive system, such as a mammary gland, ovarian cumulus, granulosa, or oviductal cell. Preferred cells also include those from any organ, such as the bladder, brain, esophagus, fallopian tube, heart, intestines, gallbladder, kidney, liver, lung, ovaries, pancreas, prostate, spinal cord, spleen, stomach, testes, thymus, thyroid, trachea, ureter, urethra, and uterus. Preferably, the donor cell, donor nucleus, donor chromatin mass, or reconstituted oocyte is not tetraploid.

Transgenic ungulate cells

In one aspect, the invention provides an ungulate cell (e.g., bovine cell) having a mutation (e.g., a mutation after the initial ATC codon, such as a mutation that is within 10, 20, 50, or 100 nucleotides of this codon) in one or both alleles of at least two genes encoding IgM heavy chain. Preferably, the mutations reduce or substantially eliminate the expression of functional IgM protein. In preferred embodiments, expression of functional or total IgM protein is decreased by at least 10, 20, 40, 60, 80, 90, 95, or 100%. The mutations may be hemizygous or homozygous. In some embodiments, the mutations include an insertion of a positive selection marker (e.g., an antibiotic resistance gene) into the nucleic acid. Preferably, the positive selection marker is operably linked to a xenogenous promoter. For ungulates or ungulate cells with an antibiotic resistance gene inserted into both alleles of a gene encoding IgM heavy chain, each allele may contain the same or a different antibiotic resistance gene. In a preferred embodiment, a negative selection marker (e.g., DT-A or Tk) is operably linked to a xenogenous promoter and is present in a vector used to disrupt an endogenous allele. The mutation may or may not include the deletion of one or more nucleotides (e.g., contiguous nucleotides) in the gene.

In preferred embodiments of the above aspect, the ungulate (e.g., bovine) or ungulate cell (e.g., bovine cell) has one or more transgenes and expresses an mRNA or protein (e.g., antibody) encoded by the transgene(s). Preferred ungulates contain naturally arranged segments of human chromosomes (e.g., human chromosomal fragments) or artificial chromosomes that comprise artificially engineered human chromosome fragments (i.e., the fragments may be rearranged relative to the human genome). In some embodiments, the xenogenous nucleic acid is contained within a chromosome fragment. The nucleic acid may be integrated into a chromosome of the ungulate or maintained in the ungulate cell independently from the host chromosome. In various embodiments, the nucleic

acid is contained in a chromosome fragment, such as a ΔHAC, ΔΔHAC, or κHAC. In other embodiments, the xenogenous antibody is an antibody from another genus, such as a human antibody.

Preferred ungulates and ungulate cells have one or more nucleic acids having a xenogenous antibody gene locus (e.g., a nucleic acid encoding all or part of a xenogenous immunoglobulin (Ig) gene that undergoes rearrangement and expresses at least one xenogenous Ig molecule) in one or more B-cells. Preferably, the nucleic acid has unrearranged antibody light chain nucleic acid segments in which all of the nucleic acid segments encoding a V gene segment are separated from all of the nucleic acid segments encoding a J gene segment by one or more nucleotides. Other preferred nucleic acids have unrearranged antibody heavy chain nucleic acid segments in which either (i) all of the nucleic acid segments encoding a V gene segment are separated from all of the nucleic acid segments encoding a D gene segment by one or more nucleotides and/or (ii) all of the nucleic acid segments encoding a D gene segment are separated from all of the nucleic acid segments encoding a J gene segment by one or more nucleotides. Other preferred ungulates have one or more nucleic acids encoding all or part of a rearranged xenogenous immunoglobulin gene that expresses at least one xenogenous immunoglobulin.

In other preferred embodiments, the light chain and/or heavy chain of the xenogenous antibodies is encoded by a human nucleic acid. In preferred embodiments, the heavy chain is any class of heavy chain, such as μ, γ, δ, ε, or α, and the light chain is a lambda or kappa light chain. In other preferred embodiments, the nucleic acid encoding the xenogenous immunoglobulin chain or antibody is in its unrearranged form. In other preferred embodiments, more than one class of xenogenous antibody is produced by the ungulate. In various embodiments, more than one different xenogenous Ig or antibody is produced by the ungulate. The xenogenous antibody may be a polyclonal or monoclonal

antibody.

Preferably, the ungulate also has a mutation in one or both alleles of an endogenous nucleic acid encoding prion protein, alpha-(1,3)-galactosyltransferase and/or J chain. Preferably, the mutation reduces or eliminates the expression of the endogenous alpha-(1,3)-galactosyltransferase enzyme, galactosyl(α1,3)galactose epitope, and/or J chain. Preferably, the ungulate produces human IgA or IgM molecules containing human J chain. Preferred ungulate cells (e.g., bovine cells) include somatic cells, such as fetal fibroblasts or B-cells.

The process of producing a transgenic ungulate of the invention involves the mutation (e.g., by homologous recombination) of one or both alleles of at least two IgM heavy chain genes (e.g., bovine IgμU and IgμAY genes). Gene mutation may be effected by homologous recombination. In a preferred embodiment, fetal fibroblasts are targeted *in vitro* using a suitable homologous recombination vector. The use of fetal fibroblasts is preferred over some other somatic cells as these cells are readily propagated and genetically manipulated in tissue culture. However, the use of fetal fibroblasts is not essential to the invention, and other cells may be substituted therefor with equivalent results. Suitable somatic cells include fibroblasts, epithelial cells, endothelial cells, neural cells, keratinocytes, hematopoietic cells, melanocytes, chondrocytes, lymphocytes (B-cells and T-cells), macrophages, monocytes, mononuclear cells, cardiac muscle cells, other muscle cells, granulosa cells, cumulus cells, placental cells, and epidermal cells.

Targeted gene mutation requires constructing a DNA construct having regions of homology to the targeted IgM heavy chain allele such that the construct upon integration into an IgM heavy chain allele in the ungulate genome disrupts the expression thereof. Exemplary vectors for carrying out such targeted mutation of bovine IgμU and IgμAY are described in the examples that follow. Methods for constructing vectors that provide for homologous recombination at other

targeted sites are well known to those skilled in the art. Moreover, in the present instance, the construction of a suitable vector is within the level of skill in the art, given especially that the sequences of Igμ genes from other ungulates (e.g., sheep and goats) are known (see below). In order to facilitate homologous recombination, the vectors used to effect homologous recombination and inactivation of the IgM gene, respectively, comprise portions of DNA that exhibit substantial sequence identity to the ungulate IgM heavy and Ig light chain genes. Preferably, these sequences possess at least 98% sequence identity, more preferably, at least 99% sequence identity, and still more preferably are isogenic with the targeted gene loci to facilitate homologous recombination and targeted deletion or inactivation.

Typically, the construct includes a marker gene that allows for selection of desired homologous recombinants, for example, fibroblasts, wherein the IgM heavy chain gene has been disrupted by homologous recombination. Exemplary marker genes include antibiotic resistance markers, drug resistance markers, and green fluorescent protein, among others.

One neomycin resistance construct was assembled as follows. A construct designated "pSTneoB" (Katoh et al. (1987) Cell Struct. Funct. 12:575; Japanese Collection of Research Biologicals (JCRB) deposit number: VE039) was designed to contain a neomycin resistance gene under the control of an SV40 promoter and TK enhancer upstream of the coding region. Downstream of the coding region is an SV40 terminator sequence. The neo cassette was excised from "pSTneoB" as a *Xho*I fragment. After the ends of the fragment were converted to blunt ends using standard molecular biology techniques, the blunt ended fragment was cloned into the *EcoR*V site in the vector, pBS246 (Gibco/Life Technologies). This site is flanked by loxP sites. The new construct, designated "pLoxP-STNeoR", was used to generate the mu knockout DNA construct. The desired fragment of this construct is flanked by loxP sites and *Not*I sites, which were originally present in

the pBS246 cloning vector. The desired *Not*I fragment, which contains loxP-neo-loxP, was used for replacement of the immunoglobulin mu constant region exons. The SV40 promoter operably linked to the neomycin resistance gene activates the transcription of the neomycin resistance gene, allowing cells in which the desired *Not*I fragment has replaced the mu constant region exons to be selected based on their resulting antibiotic resistance.

After a cell line is obtained in which an IgM heavy chain allele has been effectively disrupted, it is used as a donor cell to produce a cloned ungulate fetus (for example, a cloned bovine fetus) and eventually a fetus or animal wherein one of the IgM heavy alleles is disrupted. Thereafter, a second round of gene targeted mutation can be effected using somatic cells (e.g., fibroblasts) derived from the fetus or animal to produce cells in which a second IgM heavy chain allele is disrupted.

The following examples are meant to illustrate the invention. They are not meant to limit the invention in any way.

EXAMPLES

Example 1: Targeting of the IgμU gene

We have developed a broadly applicable and rapid method for generating multiple gene targeting events. As discussed above, sequential application of a highly efficient targeting system and rejuvenation of cell lines by production of cloned fetuses (FIGURE 1) were employed. We chose to target the IgμU gene, which is transcriptionally silent in fibroblasts. This gene was characterized in a male Holstein fetal fibroblast cell line (#6939) to identify a polymorphic marker DNA sequence, outside the KO vector sequence, which could be used to distinguish the two alleles (FIGURE 2A; allele A and allele B as indicated). Fetal fibroblasts from cell line #6939 were electroporated with the first KO vector

(pBCμΔKOpuro; FIGURE 2A) to produce 446 wells resistant to puromycin. Wells were split on day 14 and half of the cells were used for screening by PCR (primer pairs; puroF2 x puroR2, FIGURE 2A) to identify wells containing correctly targeted cells. Initially, six wells were positive by PCR. To exclude false positive wells, all of the PCR products were subjected to bi-directional sequencing analysis with the puroF2 and puroR2 primers. Two wells (0.45%; #147, #384) were identified as being targeted correctly. Based on polymorphic differences identified by sequence analysis, the KO vector was integrated into allele A in well #384 and into allele B in well #147. The remaining cells from the two wells were used for embryonic cloning to generate fetuses and to rejuvenate the cell lines. Pregnancy rate at 40 days of gestation was 50% (15/30; two embryos per recipient) and at 60 days of gestation, six fetuses were collected and fibroblasts were re-established.

Three of six fetuses (#2184-1, #2184-2 and #3287) were heterozygous KOs (IgμU-/+; FIGURE 2B) as confirmed by PCR (primer pairs; puroF2 x puroR2) and sequence analysis.

Non-targeted fetuses likely resulted from non-targeted cells that co-existed with the targeted cells in the wells. Both #2184-1 and #2184-2 were derived from well #384 where the KO vector was integrated into allele A, and fetus #3287 was from well #147 where the KO vector was integrated into allele B. Cloned IgμU-/+ embryos produced from all three regenerated cell lines were transferred to 153 recipients to produce 13 (8%) healthy IgμU -/+ calves, confirmed by PCR (FIGURE 2C) and sequence analysis.

All three $Ig\mu U^{-/+}$ cell lines (#2184-1 and #2184-2, targeted in allele A; #3287, targeted in allele B) were used for targeting with the second KO vector (pBCμΔKOneo; FIGURE 3A) in which the short homologous arm was replaced with a PCR-derived sequence amplified directly from allele A of the #6939 cell line. In #2184-1 and #2184-2 cell lines, a total of 1,211 wells, resistant to G418,

were screened by PCR (primer pairs; neoF3 x neoR3; FIGURE 3A) followed by sequence analysis. Five wells were positive and, in two, the vector was integrated into the intact allele B, producing homozygous KO (IgμU$^{-/-}$) cells, and in three wells the targeting vector in allele A was replaced. In #3287 cell line, 569 wells, resistant to G418, were screened by PCR (primer pairs; neoF3 x neoR3; FIGURE 3A) followed by sequence analysis. Seven wells were positive and, in six, the vector was integrated into the intact allele A producing IgμU$^{-/-}$ cells and, in one well, the targeting vector in allele B was replaced. Overall, the vector had a bias of 3:1 for allele A and was more efficient for homozygous targeting when used with cell line #3287 (6/569, 1.1% compared to 2/1211, 0.17%), as expected.

Two IgμU$^{-/-}$ wells (#76, #91), derived from cell line #3287, were selected for embryonic cloning to generate fetuses and rejuvenate the cell lines. Overall, the pregnancy rate for IgμU$^{-/-}$ fetuses at 40 to 50 days of gestation was 45% (40/89). At 45 days of gestation, five fetuses derived from well #76 and 15 fetuses from well #91 were collected and evaluated. All five from well #76 (FIGURE 3B) and three out of the 15 from well #91 were positive as determined by PCR (primer pairs; puroF2 x puroR2 and neoF3 x neoR3). PCR results were confirmed by sequence analyses and negative PCR (primer pairs; bCμf x bCμr; FIGURE 3A) for the wild-type alleles (FIGURE 3B). Confirmation of a functional KO was obtained by generation of 90 day fetuses from regenerated IgμU$^{-/-}$ fibroblasts and evaluation of IgμU gene expression in spleen cells. The absence of expression was confirmed by RT-PCR (primers pairs; bCμf x bCμr, FIGURE 3C). Cloned embryos were made from five IgμU$^{-/-}$ cell lines and were transferred to recipients for development to term.

Two calves from this group were born recently and were confirmed to be IgμU$^{-/-}$ by PCR (FIGURE 3D) and sequence analysis, verifying that sequential gene targeting and successive rounds of cell rejuvenation are compatible with full term development of healthy calves.

Example 2: Removal of selection markers using the Cre/LoxP system

Sequential targeting requires a strategy for antibiotic selection of a newly integrated targeting vector in a cell line that already contains one or multiple antibiotic selection markers. The simplest approach is to use a different selection marker gene for each targeting event. This approach however, limits the number of targeting events that may be performed in a cell line. Another approach is to remove the selection markers using a Cre-loxP recombination system, as has been done in murine embryonic stem cells (Abuin and Bradley (1996) Mol. Cell Biol. 16:1851-1856). In our regenerated IgμU targeted fibroblasts, the selection marker genes were not expressed, likely because the IgμU locus is silent in fibroblasts. Although selection marker removal was not necessary for further targeting in our $Ig\mu U^{-/-}$ fibroblasts, we evaluated the possibility of removing the selection markers by transfection with a Cre recombinase expression plasmid. Because the intention was for transient expression of Cre recombinase, a closed circular plasmid was used and antibiotic selection was restricted to the first three days of culture. Bovine $Ig\mu U^{-/-}$ cell line #4658 was used for transfection and 24 selected wells were evaluated by PCR for excision of the antibiotic selection genes from the targeted alleles (FIGURE 4A). Multiple wells showed evidence of excision of both puro and neo genes and one was chosen for fetal cloning and regeneration of cell lines. Pregnancy rate at 40 to 50 days of gestation was 35% (21/60).

Five fetuses were recovered and all had both selection markers removed (FIGURE 4B). The Cre recombinase plasmid integrated into the genome in all fetuses, except #1404. These results indicate that Cre-loxP recombination can be used to remove selection markers in somatic cells. Routine use in this system will require improvements to reduce integration frequency of Cre-expression plasmid.

Example 3: Targeting of the PrP gene

To evaluate the possibility of sequentially targeting a second gene, Cre-excised IgμU$^{-/-}$ (Cre/IgμU$^{-/-}$) fibroblasts (cell line #1404) were subjected to a third round of targeting to disrupt the PrP gene. This gene was first characterized to identify a polymorphic sequence, outside the KO vector sequence, to distinguish the two alleles (FIGURE 5A; allele C and allele D as indicated). Cells were transfected with the third KO vector (pBPrP(H)KOneo, FIGURE 5A) and 203 G418-resistant wells were screened by PCR.

Thirteen (6.4%) wells with cells showing a heterozygous KO PrP in Cre/IgμU$^{-/-}$ background (Cre/IgμU$^{-/-}$/PrP$^{-/+}$) were identified (primer pairs; neoF7 x neoR7; FIGURE 5A). Sequence analysis showed that the third KO vector was integrated into allele C of the PrP gene in all the positive wells. Some wells were used for cloning to generate 28 pregnancies at 45 days of gestation (71%; Table 1). Five fetuses were collected and all were positive for targeting at allele C of the PrP gene as indicated by PCR (FIGURE 5B; primer pairs; neoF7 x neoR7) and sequencing analyses.

Table 1: Embryo development, fetuses and calves with modified cells

Type of modification	No of cloned embryos produced (%)	No of recipients implanted	Pregnant at 40-45 d (%)	No of positive fetuses/number collected (%)	No of calves born (%)
IgμU$^{-/+}$	55/422 (19)	30	15 (50)	3/9 (33)	-
IgμU$^{-/+}$	153/3305 (15)	153	99 (65)	-	13 (8)
IgμU$^{-/-}$	438/2379 (26)	89	40 (45)	20/40 (50)	-
IgμU$^{-/-}$	333/4350 (11)	171	107 (63)	-	8 (6)
IgμU$^{-/+}$ Prp $^{-/+}$	112/739 (22)	39	28 (71)	5/19 (26)	-
IgμU$^{-/+}$ Prp $^{-/-}$	240/1673 (20)	38	26 (68)	33/33 (100)	-

Table 2: Triple Gene Targeting

Cell line (IgμU-/-)	No. of colonies screened	No. of triple targeted colonies (IgμU-/-/PrP-/+)	Frequency of triple targeting
#1404	203	13	6.4 %
#4658	181	11	6.0 %
#5112	187	10	5.3 %

No amplification was detected following negative IgμU PCR, as expected (FIGURE 4B; primer pairs; bCμf x bCμr). As shown in Table 2, targeting efficiency for PrP, a gene that is active in bovine fibroblasts, was substantially higher than for IgμU (6.4% vs. 0.63%, respectively), a gene that is not expressed in fibroblast cells.

To examine the feasibility of quadruple targeting to produce double homozygous KO fetuses and cell lines, the triple targeted cell line (#8443, Cre/IgμU$^{-/-}$/PrP$^{-/+}$) was transfected with a fourth KO vector for the remaining allele of the PrP gene. The vector was constructed by replacing the *neo* gene with a *puro* gene (pBPrP(H)KOpuro, FIGURE 6A) in the PrP targeting vector used for the first allele. As a result of selection and PCR screening (primer pairs; puroF14 x puroR14, FIGURE 6A), 17 (5.2%) wells were found to contain cells showing homozygous KO PrP in Cre/IgμU$^{-/-}$ background (Cre/IgμU$^{-/-}$/PrP$^{-/-}$). Sequence analysis indicated that the fourth KO vector was integrated into allele D of the PrP gene in all positive wells, except one, in which the targeted sequence in allele C was replaced. Cells from Cre/IgμU$^{-/-}$/PrP$^{-/-}$ positive wells were used for cloning to produce fetuses and at 45 days of gestation with a pregnancy rate of 71% (28/39). All 18 fetuses that were collected were Cre/IgμU$^{-/-}$/PrP$^{-/-}$ as indicated by positive PCR analysis using the targeting event-specific primer pairs, puroF14 x puroR14 and neoF7 x neoR7 (FIGURE 6B). Sequencing analyses confirmed integration of the third (neo) and fourth (puro) PrP targeting vectors into alleles C and D, respectively. Furthermore, we performed a negative PCR analysis to confirm the

absence of wild-type PrP alleles (primer pairs; BPrPex3F x BPrPex3R, FIGURE 6B) and IgμU alleles (primer pairs; bCμf x bCμr) and, as expected, all four KOs were confirmed. To evaluate PrP mRNA expression, fibroblasts from a $Ig\mu U^{-/-}$, a $Ig\mu U^{-/-}/PrP^{-/+}$ and $Cre/Ig\mu U^{-/-}/PrP^{-/-}$ cell lines were examined by RT-PCR. Functional disruption of PrP gene expression was confirmed (FIGURE 6C). Table 3 shows the frequency of quadruple targeting in the $Cre/Ig\mu U^{-/-}/PrP^{-/-}$ cell line #8018.

Table 3: Quadruple Gene Targeting

Cell line (IgμU-/-/PrP-/+)	No. of colonies screened	No. of quadruple targeted colonies (IgμU-/-/PrP-/-)	Frequency of quadruple targeting
#8018	325	3	0.92 %

Our results indicate that multiple rounds of gene targeting, both for active and silent genes, are readily accomplished in somatic cells using a cell rejuvenation approach. The system proved effective for targeting both transcriptionally silent and active genes, demonstrating broad application, and was compatible with development of healthy calves through at least two rounds of targeting. Furthermore, there is no indication that additional rounds of targeting compromised development of cloned embryos.

Methods

The results described in Examples 1-3 were obtained using the following methods.

Construction of KO vectors

A bovine genomic fragment around exon 2 of the IgμU constant region locus was obtained from non-isogenic Holstein genomic library by probing with a ^{32}P-labeled PCR fragment amplified with primer pair: 5'-TGGTCACTCCAAGT GAGTCG-3' (SEQ ID NO: 1) and 5'-TGGAGTGAAATCAGGTGAAGG-3' (SEQ ID NO: 2). One genomic clone was analyzed further by restriction mapping. The 7.2 kb of *Bgl*II-*Xho*I genomic fragment (5' homologous arm) and 2.0 kb of *Bam*HI-*Bgl*II fragment (3' homologous arm) around the exon 2 were subcloned into pBluescript II SK(-) (Stratagene), and then puro, STOP cassettes (pBS302, Stratagene) and DT-A (diphtheria toxin A) genes were inserted (pBCμΔKopuro). For construction of the second targeting vector, genomic PCR was performed from #6939 by using primer pair: 5'-GCAATAGCAAGTCCAGC CTCATCTG-3' (SEQ ID NO: 3) and 5'- CATCCTGTCTCTGGTGGTTTGAGG TC-3' (SEQ ID NO: 4). After digestion with *Bam*HI-*Bgl*II, the fragment replaced the 3' short arm of the pBCμΔKOpuro vector. By sequencing, the *Bam*HI-*Bgl*II fragment was confirmed to be amplified from "allele A".

The puro gene was replaced with a neo gene (pBCμΔKOneo vector). Bovine genomic fragment around exon 3 of the PrP locus was obtained by screening of the same Holstein genomic λ phage library with a ^{32}P-labeled DNA fragment amplified by PCR primer pair: 5'-GATTGAATGGTCTCCAGGATG CC-3' (SEQ ID NO: 5) and 5'-GACAAGCTTAATATCCGCAGG-3' (SEQ ID NO: 6). One genomic clone was analyzed further by restriction mapping. The 8.3 kb of *Bam* HI genomic fragment (3' homologous arm) and 1.2 kb of *Bam*HI-*Bgl*II

fragment (5' homologous arm) containing exon 3 were subcloned into pBluescript II SK(-), and then both *neo* and STOP cassettes were inserted at the *Bam*HI site, which is behind the initial ATG codon. The DT-A gene was also subcloned (pBPrP(H)KOneo vector). Similarly, another KO vector containing the puro gene was constructed (pBPrP(H)KOpuro vector).

Cell culture and transfection

Holstein fetal male fibroblasts were cultured as previously described (Kuroiwa et al. *supra*) and electroporated with 30 μg of each targeting vector at 550 V and 50 μF by using a GenePulser II (Bio-rad). After 48 hours, the cells were selected under 500 μg/ml of G418 or 1 μg/ml of puromycin for two weeks and the drug-resistant colonies were picked and transferred to replica plates; one for genomic DNA extraction (24-well plates) and the other for embryonic cloning (48-well plates).

Genomic PCR analyses

From the replica 24-well plates, fetus or ear biopsy genomic DNA from calves was extracted using a Puregene DNA extraction kit (GentraSystem). To identify each homologous recombination event that occurred at IgμU targeting, puroF2 (5'-GAGCTGCAAGAACTCTTCCTCACGC-3', SEQ ID NO: 7), puroR2 (5'-ATGTACCTCCCAGCTGAGACAGAGGG-3', SEQ ID NO: 8), neoF3 (5'-TTTGGTCCTGTAGTTTGCTAACACACCC-3', SEQ ID NO: 9) and neoR3 (5'-GGATCAGTGCCTATCACTCCAGGTTG-3', SEQ ID NO: 10) primer pairs were used. PCR was performed in 30 cycles comprising 98°C-10s, 68°C-8 min. For negative PCR, BCμf (5'-TGGTCACTCCAAGTGAGTCG-3', SEQ ID NO: 11) and BCμr (5'-TGGAGTGAAATCAGGTGAAGG-3', SEQ ID NO: 12) were used in 40 cycles of PCR composed of 98°C-10s, 62°C-30s, 72°C-1min. In the case of the PrP locus, neoF7 (5'-TGTCAAAGAGACACTCCTCATTTGTCT

TCC-3', SEQ ID NO: 13), neoR7 (5'- TCATAGCCGAATAGCCTCTCCACCC-3', SEQ ID NO: 14), puroF14 (5'-TTGCTCCACCTTCCGTCTTCTGTC-3', SEQ ID NO: 15) and puroR14 (5'-GTTGGCGCCTACCGGTGGATGTTTG-3', SEQ ID NO: 16) primer pairs were used. PCR was performed in 30 cycles comprising 98°C-10s, 68°C-5min. For negative PCR, BPrPexF (5'-CCACATAGGCAGTTG GATCC-3', SEQ ID NO: 17) and BPrPexR (5'-ATAAGAGGCCTGCTCATG GC-3', SEQ ID NO: 18) primer pairs were used in the 40 cycles of PCR composed of 98C-10s, 62C-30s, 72C-1min. To detect the Cre-mediated excision, PCR was carried out with CreExF (5'-CAATAGCAAGTCCAGCCTCATCTGC-3', SEQ ID NO: 19) and CreExR (5'-GTGGTTTCTTCGGTGGAAACAACG-3', SEQ ID NO: 20) primer pair in 40 cycles of PCR composed of 98°C-10s, 68°C-7min. All the PCR products were run on 0.8% agarose gels.

Sequencing of PCR products

To confirm whether homologous recombination correctly occurred at each targeting step, the PCR products amplified above were sequenced. The PCR products were purified through CHROMA SPIN-TE400 column (BD Biosciences Clontech) and sent to ACGT Inc. (Wheeling, IL) to sequence. Sequence was bi-directionally done both with forward and reverse primers which were used for PCR. The allele into which each KO vector was integrated was determined by polymorphisms in the sequence of the PCR products.

Permeabilized cell transfer

Cloned fetuses and calves were produced using a permeabilized cell transfer procedure as described previously (Sullivan et al. (2004) Biol. Reprod. 70:146-153). *In vitro* matured oocytes were enucleated at 20 h post maturation. Correctly targeted clones were permeabilized by incubation of about 50,000-100,000 cells in suspension with 31.2 U Streptolysin O (SLO; Sigma) in 100 µl

HBSS for 30 min in a 37°C H_2O bath.

Permeabilized cells were sedimented, washed and incubated with 40 µl mitotic extract containing an ATP generating system (1 mM ATP, 10 mM creatine phosphate and 25 µg/ml creatine kinase) for 30 min at 38°C. At the end of the incubation, the reaction mix was diluted, sedimented and washed.

These cells were fused to enucleated oocytes, activated at 28 h post maturation with 5 µM calcium ionophore for 4 min followed by 10 µg/ml cycloheximide and 2.5 µg/ml cytochalasin D for 5 h. After activation, embryos were washed and co-cultured with mouse fetal fibroblasts to the blastocyst stage in vitro. Grade 1 and 2 blastocysts were selected and transferred into synchronized recipients. All animal work was done following a protocol approved by the Transova Genetics Institutional Animal Care and Use Committee.

RT-PCR

RNA was extracted from spleen of wild-type (#6939) and IgµU-/- fetuses using an RNeasy mini kit (Qiagen) and first strand cDNA synthesis was done using the superscript first strand synthesis system for RT-PCR (Invitrogen). PCR was done using BCµf (5'-TGGTCACTCCAAGTGAGTCG-3', SEQ ID NO: 21) and BCµr (5'-TGGAGTGAAATCAGGTGAAGG-3', SEQ ID NO: 22) primers in 40 cycles of PCR composed of 98°C-10s, 62°C-30s, 72°C, 1min. RNA was also extracted from #4658 ($Ig\mu U^{-/-}$), #8443 ($Ig\mu U^{-/-}/PrP^{-/+}$) and double homozygous KO ($Ig\mu U^{-/-}/PrP^{-/-}$) fibroblasts and first strand cDNA synthesis was done as above. PCR was done by using PrPmF3 (5'-CAAAACCTGGAGGAGGATGG-3', SEQ ID NO: 23) and PrPmR3 (5'-ATAAGAGGCCTGCTCATGGC-3', SEQ ID NO: 24) primers in 40 cycles of 98°C-10s, 62°C-30s, 72°C-1min. For detection of bovine α-actin mRNA expression, bBAF (5'-ACATCCGCAAGGACCTCT AC-3', SEQ ID NO: 25) and bBAR (5'-AACCGACTGCTGTCACCTTC-3', SEQ

ID NO: 26) primers were used in the same PCR condition.

To exclude the possibility of genomic DNA contamination, another RT-PCR was performed without reverse-transcriptase. The PCR products were ran on 0.8% agarose gel.

Example 4: Identification of IgμAY

During our analysis of IgμU$^{-/-}$ fibroblasts we identified expression of a transcript that could be detectable by primers designed to amplify IgμU$^{-/-}$. To determine the identity of this transcript, total RNA was extracted by using RNeasy Mini kit (QIAGEN) from spleen of IgμU$^{-/-}$ fetuses collected at 90 days of gestation. One microliter of total RNA was subjected to first-strand cDNA synthesis (SuperScript First-Strand Synthesis System for RT-PCR, Invitrogen), followed by RT-PCR, which was carried out as below. The primer pair used was 5'-TGGTCACTCCAAGTGAGTCG-3' (BCμf; SEQ ID NO: 27) and 5'-TGGAGTGAAATCAG GTGAAGG-3' (BCμr; SEQ ID NO: 28). The PCR reaction mixtures contained 32.5 μl water, 5 μl of 10X Ex Taq buffer (TAKARA), 8 μl of dNTP mixture, 10 pmol forward primer, 10 pmol of reverse primer, 2 μl of the first-strand cDNA, and 0.5 μl of Ex Taq (TAKARA). Thirty five cycles of PCR were performed by incubating the reaction mixtures at the following conditions: 85°C for three minutes, 94°C for one minute, 98°C for 10 seconds, 62°C for 30 seconds and 72°C for 1 minute. After PCR, the reaction mixtures were analyzed by electrophoresis. There were no positive PCR products in the IgμU$^{-/-}$ fetuses. However, when another primer pair 5'-TCTCTGGTGACGGC AATAGC-3' (BCμf2; SEQ ID NO: 30) and 5'-CTTCGTGAGGAAGATGTC GG-3' (BCμr2; SEQ ID NO: 31) was used for RT-PCR, a positive PCR product was detected in the IgμU$^{-/-}$ fetuses (FIGURE 10). To determine the source of this discrepancy, each primer sequence was analyzed by BLAST. The results

indicated that the BCμf primer sequence was specific to the bovine Igμ sequence corresponding to U63637.2. On the other hand, BCμf2 and BCμr2 sequences matched U63637.2 and another sequence AY230207, which had been previously reported as being a polymorphic variant of U63637.2. We conclude that U63637.2 and AY230207 are not polymorphic variants of the same gene, but rather are different Igμ genes in bovine. We refer to U63637.2 Igμ gene as IgμU, and to AY230207 as IgμAY.

To determine whether both IgμAY and IgμU genes are expressed following VDJ rearrangement, mRNA was similarly extracted from spleens of #6939 (original cell line)-derived and $Ig\mu U^{-/-}$ fetuses. RT-PCR was performed using 5'-CCCTCCTCTTTGTGCTGTCA-3' (BL17; SEQ ID NO: 32) and 5'-GTTCA GGCCATCATAGGAGG-3' (mBCμ-R2; SEQ ID NO: 33) in thirty five cycles composed of conditions: 85°C for three minutes, 94°C for one minute, 98°C for 10 seconds, 62°C for 30 seconds and 72°C for 1 minute. The PCR products were purified through CHROMA SPIN TE-100 column (BD) and sent to ACGT Inc. to directly sequence them with mBCμ-R2 primer. According to sequence peak chart, IgμAY transcript was mainly expressed, in addition to a very small amount of that of IgμU in #6939 fetuses, revealing that both IgμAY and IgμU underwent VDJ rearrangement to be expressed and that both of them are functional in terms of transcription (FIGURE 11). However, in the IgμU KO fetuses, it is IgμAY that is expressed following VDJ rearrangement (FIGURE 11).

Example 5: Mutation of IgμAY

IgμAY KO vectors were generated as follows. To isolate genomic DNA around exon 2 of the IgμAY gene, a DNA probe was amplified by PCR using 5'-TCTCTGGTGACGGCAATAGC-3' (SEQ ID NO: 34) and 5'-CTTCGTGAGGA AGATGTCGG-3' (SEQ ID NO: 35) (BCμ-f2 and BCμ-r2). Using this probe, a

bovine (Holstein) genomic λ phage library derived from #4658 IgμU homozygous KO cell line was screened, and 83 positive λ phage clones were identified. These clones should contain both alleles of intact IgμAY gene and both alleles of targeted IgμU gene. To distinguish intact IgμAY clones from the targeted IgμU clones, λDNA isolated from each clone was subjected to PCR using primer pair BCμ-f2 and BCμ-r2. In the case of clones containing the targeted IgμU gene, the PCR product cannot be amplified because of presence of the KO cassette integrated at exon 2. On the other hand, the PCR product can be amplified from intact IgμAY locus; clones producing the PCR product should be ones including intact IgμAY gene, but clones not producing the PCR products should be ones including the targeted IgμU gene. Out of 83 λ phage clones, 26 produced the PCR products and these were confirmed to be clones containing intact IgμAY gene by sequence (primer AYU-F2; 5'-GGCTGACTCCCTACCTCCCCTACAC-3' (SEQ ID NO: 36). At least other 10 clones that did not produce the PCR products proved to contain the targeted IgμU gene, confirmed by sequence (primer AYU-F2). The foregoing demonstrated that there are at least two Igμ genes in bovine, and that one gene (which we refer to as IgμU) is disrupted in our KO cell line (#4658) but the other gene (IgμAY) is still intact (FIGURE 12).

To distinguish both alleles of IgμAY, we sequenced all the λ phage DNA using primer AYU5' (5'-CGGAGCCCCTGGAGATGAGC-3') (SEQ ID NO: 37). According to this sequencing, we found polymorphic sequences to differentiate the alleles of IgμAY gene, which we named the AY allele and ay allele (FIGURE 13). Out of the 26 clones, 5 clones contained AY allele and 21 clones contained ay allele. To construct AY- or ay-specific KO vectors, we chose #37 clone for AY and #49 for ay. Each of #37 and #49 was analyzed further by restriction mapping. The 9 kilobases of *Sal*I-*Bam*HI genomic fragment containing all of the Cμ*AY* exons was subcloned into pBluescript II SK(-) in which the *Kpn*I site is already

replaced with an *Srf*I site. Then, both the bsr and STOP cassettes were inserted at the *Bgl*II site, which is just located in exon 2 of Cμ. Both bsr and the STOP cassettes were in a sense strand-orientation related to the IgμAY gene. A diphtheria toxin gene (DT-A, Gibco) was then added to the *Not*I site in the pBluescript II SK(-). DT-A was inserted in forward orientation relative to the bsr gene in the targeting cassette to kill cells in which the targeting cassette was randomly integrated in the genome (pBCμAYKObsr vector; FIGURE 14). Similarly, another KO vector for ay allele containing hyg gene was constructed (pBCμayKOhyg vector; FIGURE 14).

Transfection of IgμU homozygous KO cell lines with IgμAY KO vectors was performed using the following standard electroporation protocol. The medium used to culture the bovine fetal fibroblasts contained 500 ml alpha MEM (Gibco, 12561-049), 50 ml fetal calf serum (Hy-Clone #ABL13080), 5 ml penicillin-streptomycin (SIGMA), and 1 ml 2-mercaptoethanol (Gibco/BRL #21985-023). On the day prior to transfection, cells were seeded on a T175 tissue culture flask with a confluency of 80-100 %, as determined by microscopic examination. On the day of transfection, about 10^7 bovine fibroblasts cells were trypsinized and washed once with alpha-MEM medium. After resuspension of the cells in 800 μl of alpha-MEM, 30 μg of the *Srf*I-digested KO vector (pBCμAYKObsr vector) dissolved in HEPES buffer saline (HBS) containing 1 mM spermidine was added to the cell suspension and mixed well by pipetting. The cell-DNA suspension was transferred into an electroporation cuvette and electroporated at 550 V and 50 μF. After that, the electroporated cells were plated onto thirty 48-well plates with the alpha-MEM medium supplemented with the serum. After a 48 hour-culture, the medium was replaced with medium containing 10 μg/ml of blasticidine, and the cells were cultured for 2-3 weeks to select blasticidine resistant cells. After selection, all colonies which reached close to

100% confluency were divided into two replica plates (24-well and 48-well plates): one for genomic DNA extraction, and the other plate for embryo cloning. Genomic DNA was extracted from the colonies to screen for the desired homologous recombination events by PCR.

Genomic DNA was independently extracted from each 24-well using the PUREGENE DNA isolation Kit (Gentra SYSTEMS) according to the manufacturer's protocol. Each genomic DNA sample was resuspended in 20 μl of 10 mM Tris-Cl (pH 8.0) and 1 mM EDTA (EDTA). Screening by PCR was performed using the following primer pair AYKObsrF2 (5'-GGTAGTGCAGT TTCGAATGGACAAAAGG-3'; SEQ ID NO: 38) and AYKObsrR2 (5'-TCAGG ATTTGCAGCACACAGGAGTG-3'; SEQ ID NO: 39). The sequence of one primer is located in the KO vector, and the sequence of the other primer is located just outside of the integrated vector in the targeted endogenous locus. Therefore, the expected PCR product is detected only when the KO vector is integrated into the targeted locus by homologous recombination. The PCR reaction mixtures contained 17.9 μl water, 3 μl of 10X LA PCR buffer II (Mg^{2+} plus), 4.8 μl of dNTP mixture, 10 pmol of forward primer, 10 pmol of reverse primer, 2 μl of genomic DNA, and 0.3 μl of LA Taq. Forty cycles of PCR were performed by incubating the reaction mixtures under the following conditions: 85°C for three minutes, 94°C for one minute, 98°C for 10 seconds, and 68°C for 8 minutes. After PCR, the reaction mixtures were analyzed by electrophoresis. Out of 322 screened clones, 22 clones generated the expected PCR products. As a result of sequencing of the PCR products, the KO vector designed to target AY allele was exclusively integrated into the AY allele in all the clones.

pBCμayKOhyg vector also was transfected to IgμU homozygous KO cell lines, except that the vector was digested with *Sal*I before electroporation. As a result of screening of 453 hygromycin-resistant colonies, 29 clones were identified

as a positive by PCR using the following primer pair ayKOhygF2 (5'- TGGTTGG CTTGTATGGAGCAGCAGAC-3'; SEQ ID NO: 40) and ayKOhygR2 (5'-TAGG ATATGCAGCACACAGGAGTGTGG-3'; SEQ ID NO: 41). Sequencing of the PCR products demonstrated that the KO vector designed to target ay allele was exclusively integrated into the ay allele. Judging from the above results, it can be said that both the AY and ay KO vectors specifically target each allele in an allele-specific manner and produce correct targeted clones at a frequency of 7-8%. Chromatin transfer was performed as follows. *In vitro*-matured oocytes were enucleated at 20 hpm. Bovine IgμU knockout fibroblasts were trypsinized and washed in Ca/Mg Hank's Balanced Salt Solution (HBSS) and permeabilized by incubation of 50, 000 - 100,000 cells in 31.25 units Streptolysin O (SLO; Sigma, St. Louis, MO) in 100 μl for 30 minutes in a 37°C H_2O bath. Cell samples were incubated with propidium iodide and observed by fluorescent microscopy to monitor permeabilization based on uptake of the dye. Permeabilized fibroblasts were washed, pelleted, and incubated in 40 μl of mitotic extract prepared from MDBK cells containing an ATP-generating system (1 mM ATP, 10 mM creatine phosphate, and 25 μg/ml creatine kinase) for 30 minutes in a 37°C H_2O bath. Cell samples were stained with Hoechst 33342 and observed by florescent microscopy to monitor chromatin condensation. At the end of incubation, the reaction mix was diluted with 500 μl cell culture media (alpha MEM with 10% FBS). These cells were pelleted and resuspended in TL HEPES and used for chromatin transfer in enucleated oocytes. Twelve fetuses were determined to be hemizygous IgμAY KO fetuses in which the bsrKO vector is integrated into AY allele of the IgμAY gene. Likewise, eleven fetuses were determined to be hemizygous IgμAY KO fetuses in which the hygKO vector is integrated into ay allele of the IgμAY gene. These fetuses were also $Ig\mu U^{-/-}$. One of the $Ig\mu AY^{-/+}/Ig\mu U^{-/-}$ cell lines (A227,

targeted in AY allele) was used for a second targeting experiment with pBCμayKOhyg. As a result of screening of 197 hygromycin-resistant colonies, 18 clones were identified as a positive by PCR using the primer pair (ayKOhygF2 and ayKOhygR2). Sequencing of the PCR products demonstrated that the KO vector designed to target *ay* allele was exclusively integrated into the *ay* allele, producing double homozygous knockout ($Ig\mu AY^{-/-}/Ig\mu U^{-/-}$) cells.

We produced $Ig\mu AY^{-/-}/Ig\mu U^{-/-}$ fetuses using the methods described herein. To examine whether these fetuses were B cell-deficient, we collected $Ig\mu AY^{-/-}/Ig\mu U^{-/-}$ fetuses at 180 days of gestation. From spleen, total RNA was extracted by using RNeasy Mini kit (QIAGEN). One microliter of total RNA was subjected to first-strand cDNA synthesis (SuperScript First-Strand Synthesis System for RT-PCR, Invitrogen), followed by RT-PCR. One RT-PCR reaction was carried out as below. The primer pair used (5'- CCCTCCTCTTTGTGCTGTCA-3' (BL17; SEQ ID NO: 42) and 5'- GTTCAGGCCATCATAGGAGG-3' (mBCμR2; SEQ ID NO: 43)) is compatible both with IgμAY and IgμU amplification. The PCR reaction mixtures contained 32.5 μl water, 5 μl of 10X Ex Taq buffer (TAKARA), 8 μl of dNTP mixture, 10 pmol forward primer, 10 pmol of reverse primer, 2 μl of the first-strand cDNA, and 0.5 μl of Ex Taq (TAKARA). Thirty five cycles of PCR were performed by incubating the reaction mixtures at the following conditions: 85°C for three minutes, 94°C for one minute, 98°C for 10 seconds, 62°C for 30 seconds and 72°C for 1 minute. After PCR, the reaction mixtures were analyzed by electrophoresis. There were no positive PCR products in the $Ig\mu AY^{-/-}/Ig\mu U^{-/-}$ fetuses. After PCR, the reaction mixtures were analyzed by electrophoresis. No expression of IgμAY or IgμU could be detected. We also performed flow cytometry analysis to detect the presence of IgM heavy chain protein. No such protein could be detected.

To determine if the IgμU gene can, by itself, support B cell development, we generated IgμAY knockouts similarly as described above by using pbCμayKOhyg and pbCμAYKObsr vectors. From one IgμAY$^{-/-}$ cell line, we generated 180 day fetuses and performed RT-PCR on spleen tissue in an attempt to detect VDJ-rearranged IgμU transcripts. Sequence of the RT-PCR products showed VDJ-rearranged sequence of IgμU transcripts, exclusively, and disruption of IgμAY gene expression was confirmed. We confirmed, from sequence analysis, the presence of possible V_H and D_H segments associated with J_H-$C\mu_L$ segments, which comprise the corresponding FR1, CDR1, FR2, CDR2, FR3 and CDR3 regions. Furthermore, direct sequencing of the RT-PCR products clearly showed diversified sequence in the CDR3 region of IgμU transcripts as well as that of IgμAY. The sequence of IgμU cDNA appears to encode functional IgM-like polypeptides, which are slightly different from IgμAY, especially in FR4 and the constant region. These data demonstrate that the IgμU gene can be expressed independently of IgμAY expression, following functional VDJ-rearrangement, which included diversification of CDR3.

To investigate whether functional expression of IgμU could sufficiently execute B cell development independently of the classical pathway through IgμAY, we performed flow cytometry analysis in IgμAY$^{-/-}$ 180 day fetuses. We recognized significant populations of developing B cells (IgM$^+$B220$^+$), comparable to controls. This result indicates that IgμU protein can be displayed on the B cell surface. Next, to investigate generation of immature B cells (IgM$^+$Igλ$^+$), staining was done with both an anti-bovine IgM polyclonal antibody and an anti-bovine Igλ monoclonal antibody. Double positive B cells were detected, suggesting the presence of immature B cells where IgμU heavy chain couples with Igλ light chain to form B cell-receptors. Furthermore, we detected

generation of mature B cells, recognized by anti-CD21 antibody, and confirmed gene expression of VDJ-rearranged bIgD gene by RT-PCR. This observation suggests that IgμU can support mature B cell development and elicit the expression of a VDJ-C_δ gene. Moreover, we detected the occurrence of class switching and expression of VDJ-rearranged bIgG, even though the γ constant region segment has not been identified as part of the IgμU gene cluster. Interestingly, the extent of diversification of CDR3 in the bIgG transcripts, from $Ig\mu AY^{-/-}$ fetuses, was significantly less than that detected in $Ig\mu U^{-/-}$ fetuses. However, CDR3 in IgμU transcripts in $Ig\mu AY^{-/-}$ fetuses was diversified at a level comparable to that of IgμAY transcripts in $Ig\mu U^{-/-}$ fetuses. This result suggests that IgμU may be less efficient at generation of well-diversified IgG compared to the classical IgμAY locus, probably because the IgμU locus does not link, in cis, with the bIgG constant region. These data strongly suggest that IgμU can largely substitute for lack of IgμAY function in driving B cell development, but likely recruits γ constant region segments from the IgμAY locus by some trans-class switch mechanism.

From these results, it is demonstrated that there are two functional IgM loci, IgμAY and IgμU, in bovine and that double homozygous knockouts $Ig\mu AY^{-/-}$ /$Ig\mu U^{-/-}$ are useful for B cell-deficiency.

Example 6: Production of bovines that are deficient in prion protein expression

The present invention features the production of bovines that are deficient in prion protein expression. Although $PrP^{-/-}$ mice are viable and appear healthy, in cattle the effect of knocking out the gene had not previously been determined. In an attempt to generate $PrP^{-/-}$ cattle, we exploited a sequential gene targeting system for bovine fibroblasts described above. Male Holstein fibroblasts (cell line 4685,

which is also $Ig\mu U^{-/-}$) were transfected with a knockout vector (pBPrP(H)KOneo), and correctly targeted cells were cloned to generate $PrP^{-/+}$ fetuses at 40 days of gestation that were used to establish $PrP^{-/+}$ cell lines. Fetal cell lines were evaluated to confirm correct targeting by PCR genotyping. The $PrP^{-/+}$ cell line was then transfected with a second knockout vector (pBPrP(H)KOpuro) followed by selection and cloning to generate $PrP^{-/-}$ fetuses and cell lines. Correct targeting in cell lines was verified by PCR genotyping. Wild type, $PrP^{+/+}/Ig\mu U^{-/-}$ (cell line 5112), heterozygous knockout, $PrP^{-/+}Ig\mu U^{-/-}$ (cell line 8018), and homozygous knockout, $PrP^{-/-}/Ig\mu U^{-/-}$ (cell line 1718) fibroblasts were used for embryonic cloning to produce calves (Table 4). In our first cloning series we obtained one calf each from $PrP^{+/+}/Ig\mu U^{-/-}$ (calf 347; 5.9%), $PrP^{-/+}/Ig\mu U^{-/-}$ (calf 341; 4.3%) and $PrP^{-/-}/Ig\mu U^{-/-}$ (calf 342; 3.2%) cell lines (FIGURE 15).

Table 4: Production of first series of cloned calves from $PrP^{-/-}$ fibroblast cell lines

Genotype	Cell line ID	No. of recipients implanted	No. of calves born (%)	No. of calves survived (%)
$PrP^{+/+}/Ig\mu U^{-/-}$	5112	17	1 (5.9)	1 (5.9)
$PrP^{-/+}/Ig\mu U^{-/-}$	8018	23	2 (8.6)	1 (4.3)
$PrP^{-/-}/Ig\mu U^{-/-}$	1718	31	2 (6.4)	1 (3.2)

In order to verify the genotype of calves 341 and 342, we collected a small biopsy of skin from each calf and established fibroblast cell lines (FIGURE 16A). No differences were observed in morphology or growth rate of fibroblasts from the two knockout genotypes and control fibroblasts. Genotyping was done by PCR using primer pairs that were specific for each targeted PrP gene allele (primer pairs; neoF7 x neoR7 and puroF14 x puroR14; FIGURE 16B) followed by sequence analysis for confirmation. Additionally, we performed a second PCR (Kuroiwa et al., *supra*) to confirm the absence of the wild type PrP alleles (primer

pairs; BPrPex3F x BPrPex3R, FIGURE 16C). The results confirmed that calf 341 was a heterozygous PrP knockout and calf 342 was a homozygous PrP knockout. In addition, we performed a similar PCR analysis to confirm the absence of wild-type IgμU alleles using primer pairs: bCμf x bCμr (FIGURE 16D) in calves 341 and 342, and, as expected, all four targeting events ($PrP^{-/-}/Ig\mu U^{-/-}$) were confirmed. This represented the first generation of a double homozygous knockout ($PrP^{-/-}/Ig\mu U^{-/-}$) calf, demonstrating that four rounds of gene targeting in primary somatic cells followed by embryonic cloning are compatible with generation of live calves.

To demonstrate functional inactivation of the PrP gene in calf 342, we extracted mRNA and total protein from the fibroblasts. As controls, we analyzed $PrP^{+/+}/Ig\mu U^{-/-}$ calf 347 and $PrP^{-/+}/Ig\mu U^{-/-}$ calf 341. For mRNA expression analysis, we performed RT-PCR (primer pairs; PrPmF3 x PrPmR3, FIGURE 17A) and then confirmed the disruption of PrP mRNA expression in $PrP^{-/-}/Ig\mu U^{-/-}$ calf 342, while clear expression was detected in $PrP^{+/+}/Ig\mu U^{-/-}$ calf 347 and $PrP^{-/+}/Ig\mu U^{-/-}$ calf 341. For protein expression analysis, we performed a western blot using a mouse anti-bovine PrP monoclonal antibody. We detected appropriate bands for $PrP^{+/+}/Ig\mu U^{-/-}$ calf 347 and $PrP^{-/+}/Ig\mu U^{-/-}$ calf 341 but no band was observed for $PrP^{-/-}/Ig\mu U^{-/-}$ calf 342 or negative control mouse fibroblasts (FIGURE 17B). In addition, we collected brain samples from two $PrP^{-/-}$ calves that died within the first week after birth and performed western blot analysis on the samples. No PrP-positive bands were detected from the brain samples (FIGURE 18). This result was also confirmed with a different mouse anti-bovine PrP monoclonal antibody (6H4, Prionics) in a separate laboratory. These data clearly demonstrate that the PrP gene is functionally inactivated in the $PrP^{-/-}/Ig\mu U^{-/-}$ and $PrP^{-/-}$ calves.

All calves have been evaluated by either a licensed veterinarian or trained animal care technician. At one week and one month, calves 341 and 342, along with controls, were given physicals which included evaluation of the following parameters: body weight, body temperature, heart rate, heart sound, jugular vein distension, respiratory rate, respiratory sound, cough, presence of nasal discharge or eye abnormalities, appetite, general behavior (alert and active, sluggish, hyperactive), gait, posture, joints, hooves, feces (diarrhea, constipation), genitalia, and umbilical cord (dry, enlarged, inflamed, infected). In addition, blood samples were taken for standard hematology and serum chemistry. To date all parameters for calves 341 and 342 have been normal for these age groups.

For added verification, a second set of cloned $PrP^{-/-}$ embryos, which were only $PrP^{-/-}$ without any other genetic modification, were produced and transferred into 51 recipients. From these transfers, seven calves (14%) have been born (Table 5). These calves were verified by PCR genotyping and western blot analysis of peripheral blood lymphocytes (PBLs) isolated from blood biopsy (FIGURE 19) to be $PrP^{-/-}$. Physical examination of the calves at one week did not reveal any obvious abnormalities, and there have been no apparent differences in phenotype between the $PrP^{-/-}/Ig\mu U^{-/-}$ and $PrP^{-/-}$ calves to date.

Table 5: Production of first series of cloned calves from $PrP^{-/-}$ fibroblast cell lines

Genotype	Cell line ID	No. of recipients implanted	No. of calves born (%)	No. of calves survived (%)
$PrP^{-/-}$	5211	30	7 (23)	5 (16)
$PrP^{-/-}$	5232	21	3 (14)	2 (10)

Methods

The results described in Example 6 were obtained using the following methods.

Embryonic cloning

Cloned calves were produced using a chromatin transfer procedure. *In vitro* matured oocytes were enucleated at 20 h post maturation. Correctly targeted clones were permeabilized by incubation of about 50,000-100,000 cells in suspension with 31.2 U Streptolysin O (SLO; Sigma) in 100 µl HBSS for 30 min in a 37°C H_2O bath. Permeabilized cells were sedimented, washed, and incubated with 40 µl mitotic extract containing an ATP generating system (1 mM ATP, 10 mM creatine phosphate and 25 µg/ml creatine kinase) for 30 min at 38°C. At the end of the incubation, the reaction mix was diluted, sedimented, and washed. These cells were fused to enucleated oocytes, activated at 28 h post maturation with 5 µM calcium ionophore for 4 min, followed by 10 µg/ml cycloheximide and 2.5 µg cytochalasin D for 5 h. After activation, CT embryos were washed and co-cultured with mouse fetal fibroblasts to blastocyst stage in vitro. Grade 1 and 2 blastocysts were selected and transferred into synchronized recipients. All animal work described in this section was done following a protocol approved by the Transova Genetics institutional animal care and use committee.

Cell culture and transfection

Holstein fetal male fibroblasts were cultured and electroporated with 30 µg of each knockout vector at 550 V and 50 µF using a GenePulser II (Bio-Rad). After 48 hours, the cells were selected using 500 µg/ml G418 or 1 µg/ml puromycin for two weeks, and the drug-resistant colonies were picked and transferred to replica plates; one for genomic DNA extraction (24-well plates) and the other for embryonic cloning (48-well plates).

Genomic PCR analyses

Genomic DNA was extracted from fibroblasts originated from either ear biopsy or blood of $PrP^{-/-}$ homozygous KO calves using a PUREGENE DNA isolation Kit (Gentra SYSTEMS) and the manufacture's protocol. Each genomic DNA sample was resuspended in 50-100 μl of 10 mM Tris-Cl (pH 8.0) and 1 mM EDTA (EDTA). Confirmation by PCR was performed using the following primer pairs neoF7; 5'- TGTCAAAGAGACACTCCTCATTTGTCTTCC-3' (SEQ ID NO: 44) and neoR7; 5'- TCATAGCCGAATAGCCTCTCCACCC-3' (SEQ ID NO: 45) to detect the first targeted allele, and puroF14; 5'-TTGCTCCACCTTCC GTCTTCTGTC-3' (SEQ ID NO: 46) and puroR14; 5'- GTTGGCGCCTACCGG TGGATGTG-3' (SEQ ID NO: 47) to detect the second targeted allele. The PCR reaction mixtures contained 17.9 μl water, 3 μl of 10X LA PCR buffer II (Mg^{2+} plus), 4.8 μl of dNTP mixture, 10 pmol forward primer, 10 pmol of reverse primer, 2 μl of genomic DNA, and 0.3 μl of LA Taq. Thirty cycles of PCR were performed by incubating the reaction mixtures at the following conditions: 85°C for three minutes, 94°C for one minute, 98°C for 10 seconds, and 68°C for 5 minutes. In addition, another PCR was performed to confirm the absence of (i) wild type PrP alleles using the following primer pairs: BPrPex3F; 5'- CCACATA GGCAGTTGGATCC-3' (SEQ ID NO: 48) and BPrPex3R; 5'- ATAAGAGGCC TGCTCATGGC-3' (SEQ ID NO: 49) and (ii) IgμU alleles (for $PrP^{-/-}$ $Ig\mu U^{-/-}$ calves only) using the following primer pairs: BCμf; 5'-TGGTCACTCCAAGT GAGTCG-3' (SEQ ID NO: 50) and BCμr; 5'-TGGAGTGAAATCAGGTGAA GG-3' (SEQ ID NO: 51). The PCR reaction mixtures contained 17.9 μl water, 3 μl of 10X Ex Taq buffer , 4.8 μl of dNTP mixture, 10 pmol forward primer, 10 pmol of reverse primer, 2 μl of genomic DNA, and 0.3 μl of Ex Taq. Thirty cycles of PCR were performed by incubating the reaction mixtures at the

following conditions: 85°C for three minutes, 94°C for one minute, 98°C for 10 seconds, 60-62°C for 30 seconds, and 72°C for 1 minute. Following PCR, the reaction mixtures were analyzed by electrophoresis.

RT-PCR

From biopsy samples, total RNA was extracted using an RNeasy Mini kit (QIAGEN). One microliter of total RNA was subjected to first-strand cDNA synthesis (SuperScript First-Strand Synthesis System for RT-PCR, Invitrogen), followed by RT-PCR. The RT-PCR was carried out using the primer pair: 5'- AA GAAGCGACCAAAACCTGG-3' (SEQ ID NO: 52) and 5'- GTAACGGTGCAT GTTTTCACG-3' (SEQ ID NO: 53). The PCR reaction mixtures contained 32.5 µl water, 5 µl of 10X Ex Taq buffer (TAKARA), 8 µl of dNTP mixture, 10 pmol forward primer, 10 pmol of reverse primer, 2 µl of the first-strand cDNA, and 0.5 µl of Ex Taq (TAKARA). Thirty five cycles of PCR were performed by incubating the reaction mixtures at the following conditions: 85°C for three minutes, 94°C for one minute, 98°C for 10 seconds, 62°C for 30 seconds and 72°C for 1 minute. For detection of bovine β-actin mRNA expression, primers bBAF (5'-ACATCCGCAAGGACCTCTAC-3'; SEQ ID NO: 54) and bBAR (5'-AACC GACTGCTGTCACCTTC-3'; SEQ ID NO: 55) were used under the same PCR conditions. To exclude the possibility of genomic DNA contamination, another set of RT-PCR reactions without reverse-transcriptase was also performed. Following PCR, the reaction mixtures were analyzed by electrophoresis. There were no positive PCR products in any biopsy sample prepared from a $PrP^{-/-}$ or $PrP^{-/-}$ $Ig\mu U^{-/-}$ KO calf. This result verified the complete absence of prion gene expression in the calves.

Western blotting

From biopsy or blood samples of live calves or brain samples of dead calves, total protein was extracted and the protein content was quantified using a Bio-Rad protein assay reagent. Western blot analysis was carried out by running approximately 75μg of protein sample on a 12% SDS PAGE gel under non reducing conditions. The proteins were then transferred to a nitrocellulose membrane, and the membrane was stained using an anti-bovine prion protein monoclonal antibody (F 89/160.1.5; Alexis Biochemicals) as a primary antibody and secondarily stained with peroxidase-labeled affinity purified antibody directed to mouse IgG(H+L). The stained membrane was developed using an ECL plus western blotting detection system (Amersham Bioscience) and exposed to a Biomax light film by film developer. As a positive control, recombinant bovine PrP protein (Alexis Biochemicals) was used. As a negative control, protein extracts from murine fibroblasts were analyzed. As an internal positive control, the same blot was similarly stained with an anti-CDC2 monoclonal antibody. No prion protein band was detected in any biopsy sample prepared from a $PrP^{-/-}$ or $PrP^{-/-}$ $Ig\mu U^{-/-}$ KO calf. This result verified the complete absence of prion protein expression in the calves.

Example 7: Production of bovines that are deficient in prion protein and IgM heavy chain expression

Transfection of $Ig\mu U^{-/-}PrP^{-/-}$ cell line 8454 with pBCμAYKObsr vector was performed using the following standard electroporation protocol. The medium used to culture the bovine fetal fibroblasts contained 500 ml Alpha MEM (Gibco, 12561-049), 50 ml fetal calf serum (Hy-Clone #ABL13080), 5 ml penicillin-streptomycin (SIGMA), and 1 ml 2-mercaptoethanol (Gibco/BRL #21985-023). On the day prior to transfection, cells were seeded on a T175 tissue

culture flask with a confluency of 80-100 %, as determined by microscopic examination. On the day of transfection, about 10^7 bovine fibroblasts cells were trypsinized and washed once with alpha-MEM medium. After resuspension of the cells in 800 µl of alpha-MEM, 30 µg of the *Srf*I-digested KO vector (pBCµAYKObsr vector) dissolved in HEPES-buffered saline (HBS) containing 1 mM spermidine was added to the cell suspension and mixed well by pipetting. The cell-DNA suspension was transferred into an electroporation cuvette and electroporated at 550 V and 50 µF. After that, the electroporated cells were plated onto thirty 48-well plates with the alpha-MEM medium supplemented with the serum. After a 48 hour-culture, the medium was replaced with medium containing 10 µg/ml of blasticidine, and the cells were cultured for 2-3 weeks to select blasticidine resistant cells. After selection, all colonies which reached close to 100% confluency were divided into two replica plates (24-well and 48-well plates): one for genomic DNA extraction, and the other plate for nuclear transfer. Genomic DNA was extracted from the colonies to screen for the desired homologous recombination events by PCR.

As described above, the genomic DNA was independently extracted from each 24-well using the PUREGENE DNA isolation Kit (Gentra SYSTEMS) according to the manufacture's protocol. Each genomic DNA sample was resuspended in 20 µl of 10 mM Tris-Cl (pH8.0) and 1 mM EDTA (EDTA). Screening by PCR was performed using the following primer pair AYKObsrF2 (5'-GGTAGTGCAGTTTCGAATGGACAAAAGG-3'; SEQ ID NO: 56) and AYKObsrR2 (5'-TCAGGATTTGCAGCACACAGGAGTG-3'; SEQ ID NO: 57). The sequence of one primer is located in the KO vector, and the sequence of the other primer is located just outside of the integrated vector in the targeted endogenous locus. Therefore, the expected PCR product is detected only when the

KO vector is integrated into the targeted locus by homologous recombination. The PCR reaction mixtures contained 17.9 µl water, 3 µl of 10X LA PCR buffer II (Mg^{2+} plus), 4.8 µl of dNTP mixture, 10 pmol of forward primer, 10 pmol of reverse primer, 2 µl of genomic DNA, and 0.3 µl of LA Taq. Forty cycles of PCR were performed by incubating the reaction mixtures under the following conditions: 85°C for three minutes, 94°C for one minute, 98°C for 10 seconds, and 68°C for 8 minutes. After PCR, the reaction mixtures were analyzed by electrophoresis. Out of 198 screened clones, 14 clones generated the expected PCR products. As a result of sequencing of the PCR products, the KO vector designed to target *AY* allele was exclusively integrated into the *AY* allele in all the clones.

The above knockout ($Ig\mu AY^{-/+}Ig\mu U^{-/-}PrP^{-/-}$) cells or the nuclei from these cells were used in any of the nuclear transfer methods described herein to generate an $Ig\mu AY^{-/+}Ig\mu U^{-/-}PrP^{-/-}$ knockout ungulate (e.g., a knockout bovine calf). If desired, cells from a knockout fetus or live ungulate can be used as described below to generate homozygous prion knockout cells. In one particular method, fibroblasts (e.g., bovine primary fetal fibroblasts) were synchronized in mitosis with 1 µg/ml nocodazole for 18 hours, harvested by mitotic shake-off, and washed twice in phosphate buffered saline and once in cell lysis buffer (20 mM Hepes, pH 8.2, 5 mM $MgCl_2$, 10 mM EDTA, 1 mM DTT and protease inhibitors). Sedimented cells were resuspended in one volume of ice-cold cell lysis buffer, allowed to swell on ice for one hour, and Dounce-homogenized using a tight-fitting glass pestle. The lysate was centrifuged at 15,000 x *g* for 15 minutes at 4°C, and the supernatant (mitotic extract) was aliquoted, frozen in liquid nitrogen, and stored at -80°C. Fresh or frozen extracts were used. *In vitro*-matured oocytes were enucleated at 20 hpm. Transfected bovine fetal fibroblasts from selected colonies were washed in Ca^{2+}/Mg^{2+}-free Hank's Balanced Salt Solution (HBSS) and permeabilized by incubation of cells in suspension with 31.2 U Streptolysin O

(SLO; Sigma) in 100 µl HBSS for 30 minutes in an approximately 37°C water bath. Permeabilization was assessed by uptake of the membrane impermeant DNA stain, propidium iodide (0.1 µg/ml). Permeabilized fibroblasts were sedimented, washed, and incubated in 40 µl mitotic extract containing an ATP-generating system (1 mM ATP, 10 mM creatine phosphate, and 25 µg/ml creatine kinase) for 30-45 minutes at approximately 37°C. Aliquots were labeled with 0.1 µg/ml Hoechst 33342 to monitor chromatin condensation. At the end of incubation, the reaction mixture was diluted with 500 µl Alpha MEM/10% fetal bovine serum (Hyclone). Cells were fused to enucleated oocytes; oocytes were activated at 28 hpm, and embryos cultured to the blastocyst stage *in vitro*. Two embryos were transferred per recipient female. Pregnancies were monitored by ultrasonography, and C-sections were performed on recipients to recover fetuses for cell line production.

pBCµayKOhyg vector was transfected to the above $Ig\mu AY^{-/+}Ig\mu U^{-/-}PrP^{-/-}$ cell lines, except that the vector was digested with *Sal*I before electroporation. As a result of screening of 119 hygromycin-resistant colonies, four clones were identified as being positive by PCR using primer pair ayKOhygF2 (5'-TGGTTGG CTTGTATGGAGCAGCAGAC-3'; SEQ ID NO: 58) and ayKOhygR2 (5'-TAGG ATATGCAGCACACAGGAGTGTGG-3'; SEQ ID NO: 59). Sequencing result of the PCR products demonstrated that the KO vector designed to target *ay* allele was exclusively integrated into the ay allele. From the above results, we conclude that both the AY and ay KO vectors can specifically target each allele in an allele-specific manner and produce correct targeted clones.

Using the methods described herein, we performed chromatin transfer from the identified $Ig\mu AY^{-/-}Ig\mu U^{-/-}PrP^{-/-}$ colonies and finally generated four $Ig\mu AY^{-/-}Ig\mu U^{-/-}PrP^{-/-}$ fetuses, from which we established four cell lines. Moreover, we obtained one $Ig\mu AY^{-/-}Ig\mu U^{-/-}PrP^{-/-}$ calf. Genotyping was performed as follows. The genomic DNA was extracted from fibroblasts

originated from ear biopsy or from blood of the IgμAY$^{-/-}$IgμU$^{-/-}$PrP$^{-/-}$ triple homozygous KO calf using the PUREGENE DNA isolation Kit (Gentra SYSTEMS) according to the manufacture's protocol. Each genomic DNA sample was resuspended in 50-100 μl of 10 mM Tris-Cl (pH8.0) and 1 mM EDTA (EDTA). Confirmation by PCR was performed using the following primer pair 5'-TCTCTGGTGACGGCAATAGC-3' (BCμf2; SEQ ID NO: 60) and 5'-CTTCGTGAGGAAGATGTCGG-3' (BCμr2; SEQ ID NO: 61), which is compatible both with IgμAY and IgμU amplification, to confirm the absence of wild-type alleles of the IgμAY and IgμU genes, and another primer pair BPrPex3F (5'-CCACATAGGCAGTTGGATCC-3' (SEQ ID NO: 629) and BPrPex3R (5'-ATAAGAGGCCTGCTCATGGC-3' (SEQ ID NO: 63) to confirm the absence of wild-type alleles of the PrP gene. The PCR reaction mixtures contained 17.9 μl water, 3 μl of 10X Ex Taq buffer, 4.8 μl of dNTP mixture, 10 pmol forward primer, 10 pmol of reverse primer, 2 μl of genomic DNA, and 0.3 μl of Ex Taq. Thirty cycles of PCR were performed by incubating the reaction mixtures at the following conditions: 85°C for three minutes, 94°C for one minute, 98°C for 10 seconds, 60-62°C for 30 seconds and 72°C for 1 min. After PCR, the reaction mixtures were analyzed by electrophoresis. The results demonstrated that the calf born was IgμAY$^{-/-}$IgμU$^{-/-}$PrP$^{-/-}$.

Example 8: Verification of the absence of prion protein expression in the IgμAY$^{-/-}$IgμU$^{-/-}$PrP$^{-/-}$ triple homozygous KO calf

From blood samples taken from the IgμAY$^{-/-}$IgμU$^{-/-}$PrP$^{-/-}$ triple homozygous KO calf, total protein was extracted and the protein content was quantified by using Bio-Rad protein assay reagent. Western blot analysis was carried out by running approximately 75μg of protein sample on a 12% SDS page gel at non-reducing condition. The proteins were transferred to nitrocellulose

membrane and the membrane was stained with anti-bovine Prion protein monoclonal antibody (F 89/160.1.5 from Alexis Biochemicals) as a primary antibody, followed by a peroxidase-labeled affinity purified secondary antibody to mouse IgG(H+L). The stained membrane was developed by ECL plus western blotting detection system (Amersham Bioscience) and exposed to Biomax light film by film developer. As a positive control, recombinant bovine PrP protein (Alexis Biochemicals) was used. As a negative control, protein extract from murine fibroblasts was used.

In the biopsy sample prepared from the IgμAY$^{-/-}$IgμU$^{-/-}$PrP$^{-/-}$ triple homozygous KO calf, no band was stained with antibody. This result verifies the complete absence of prion protein expression in the calf.

Example 9: Verification of the absence of IgM protein expression in the IgμAY$^{-/-}$IgμU$^{-/-}$PrP$^{-/-}$ triple homozygous KO calf

To confirm that the IgμAY$^{-/-}$IgμU$^{-/-}$PrP$^{-/-}$ triple homozygous KO calf lacked IgM protein, we performed flow cytometry analysis as described below. Peripheral blood was collected from the newborn calf by jugular venipunture into heparinized tubes. Whole white blood cells (leukocytes) were isolated from heparinized blood by red blood cell lysing using RBC-lysis buffer (Sigma, St. Louis, MO) and washing twice with sterile Hanks Buffered Salt Solution (HBSS) (Sigma, St. Louis, MO). Cells were resuspended in FACS staining medium (Phosphate Buffered Saline containing 4% horse serum or goat serum, 2 mM EDTA, and 0.2% sodium azide) and incubated for 30 minutes at room temperature for blocking non-specific binding. Sheep anti-bovine IgM-FITC (Bethyl Laboratories, Mongomery, TX) or donkey anti-sheep/bovine Ig-biotin antibody (Amersham Biosciences, Piscataway, NJ) followed by Strepavidin-FITC or Sterpavidin-PE secondary antibody (Caltag Laboratories, Burlingame, CA) was used to label bovine surface IgM (sIgM) on the B cells. To label surface B220

marker on developing bovine B cells, mouse anti-bovine B220 (CD45R) antibody clone GS5A (VMRD, Pullman, WA) followed by anti-mouse IgG1-PE secondary antibody (Caltag Laboratories, Burlingame, CA) was used. Mouse anti-bovine CD21 Clone MCA1424 and mouse anti-ruminant CD43 Clone 1096 from Serotec Inc. (Raleigh, NC) antibodies followed by anti-mouse IgG1-PE secondary antibody (Caltag Laboratories, Burlingame, CA) were used to label surface CD21 and CD43 markers on bovine B cells. Fifty microliter cell suspension containing 10^6 cells (WBCs) in FACS staining medium were used for staining with each surface marker antibody alone as well as in combinations in V-bottom microtiter wells or tubes. Cells were incubated with primary antibodies at room temperature in dark for 20-30 minutes and washed twice with FACS wash buffer (Phosphate Buffered Saline containing 2mM EDTA and 0.2% sodium azide) by centrifugation. Cells were resuspended again in 50 µl of FACS staining medium and incubated with appropriate fluorescent labeled secondary antibodies for 15-20 minutes at room temperature in dark. Finally cells were washed twice with FACS wash buffer and fixed with 2-4% formaldehyde in PBS. Surface labeled fixed cells were then analyzed and data acquired in FACScan flow cytometer (BD Biosciences, San Diego, CA). The list mode file data were finally analyzed using WinMDI software for single color or dual color profiles. The FACS result demonstrated complete absence of any developing B cell in the triple homozygous KO calf.

Example 10: HAC transfer into Igμ$AY^{-/-}$/Igμ$U^{-/-}$ cell lines

One use of animals having reduced IgM levels is the generation of xenogenous antibodies. One method of producing xenogenous antibody is to produce an animal having one or more human artificial chromosomes expressing antibody heavy chain and/or light chain. To this end, ΔΔHAC (λHAC) and κHAC were transferred from DT40 cell hybrids to Chinese hamster ovary (CHO) cells

using microcell-mediated chromosome transfer (MMCT) (Kuroiwa et al. (2000) Nature Biotech. 18:1086-1090). The CHO clone containing λHAC ("ΔΔC10 clone") was cultured in F12 (Gibco) medium supplemented with 10% FBS (Gibco), 1 mg/ml of G418, and 0.2 mg/ml of hygromycin B at 37°C and 5% CO_2. The ΔΔC10 clone was expanded into twelve T25 flasks. When the confluency reached 80-90%, colcemid (Sigma) was added to the medium at a final concentration of 0.1 μg/ml. After three days, the medium was exchanged with DMEM (Gibco) supplemented with 10 μg/ml of cytochalacin B (Sigma). The flasks were centrifuged for 60 minutes at 8,000 rpm to collect microcells. The microcells were purified through 8, 5, and 3-μm filters (Costar) and then resuspended in DMEM medium. The microcells were used for fusion with bovine fibroblasts as described below.

Bovine fetal fibroblasts were cultured in α-MEM (Gibco) medium supplemented with 10% FBS (Gibco) at 37°C and 5% CO_2. The fibroblasts were expanded in a T175 flask. When the confluency reached 70-80%, the cells were detached from the flask with 0.05% trypsin. The fibroblast cells were washed twice with DMEM medium and then overlayed on the microcell suspension. After the microcell-fibroblast suspension was centrifuged for five minutes at 1,500 rpm, PEG1500 (Roche) was added to the pellet according to the manufacturer's protocol to enable fusion of the microcells with the bovine fibroblasts. After fusion, the fused cells were plated into six 24-well plates and cultured in α-MEM medium supplemented with 10% FBS for 24 hours. The medium was then exchanged with medium containing 0.8 mg/ml of G418. After growth in the presence of the G418 antibiotic for about two weeks, the G418 resistant, fused cells were selected. These G418-resistant clones were used for chromatin transfer, as described herein.

Example 11: Expression of human IgM, IgG, and Igλ in HAC/ IgμAY$^{-/-}$/ IgμU$^{-/-}$ fetuses at 180 days of gestation

To examine whether HAC/IgμAY$^{-/-}$/IgμU$^{-/-}$ fetuses could express human immunoglobulin such as IgM, IgG, Igλ and Igκ, we collected HAC/IgμAY$^{-/-}$/ IgμU$^{-/-}$ fetuses at 180 days of gestation. Total RNA was extracted from the spleen by using RNeasy Mini kit (QIAGEN). One microliter of total RNA was subjected to first-strand cDNA synthesis (SuperScript First-Strand Synthesis System for RT-PCR, Invitrogen), followed by RT-PCR. To detect human IgM expression, RT-PCR reaction was carried out using primer pair; 5'- AGGCCAGCATCTGCGAG GAT-3' (CH3-F3; SEQ ID NO: 64) and 5'- GTGGCAGCAAGTAGACATCG-3' (CH4-R2; SEQ ID NO: 65). For human IgG expression, the following primers were used: 5'-CAGGTGCAGCTGGTGCAGTCTGG-3' (SEQ ID NO: 66, 5'- CAGGTCACCTTGAAGGAGTCTGG-3' (SEQ ID NO: 67), 5'-GAGGTGCA GCTGGTGGAGTCTGG-3' (SEQ ID NO: 68), 5'-CAGGTGCAGCTGCAGGAG TCGGG-3' (SEQ ID NO: 69), 5'-GAGGTGCAGCTGGTGCAGTCTGG-3', 5'-CAGGTACAGCTGCAGCAGTCAGG-3' (SEQ ID NO: 70), and 5'-CAGGTGCA GCTGGTGCAGTCTGG-3' (SEQ ID NO: 71) (VH All Mix) in combination with 5'- CACCACGCTGCTGAGGGAGTAGAGT-3' (hCg1R2; SEQ ID NO: 72). For human Igλ expression, the following primer pair was used: 5'-TCCTCTGAGGAGCTTCAAGC-3' (hCL-F2; SEQ ID NO: 73) and 5'-AGGGTTTATTGAGT GCAGGG-3' (hCL-R2; SEQ ID NO: 74). The PCR reaction mixtures contained 32.5 μl water, 5 μl of 10X Ex Taq buffer (TAKARA), 8 μl of dNTP mixture, 10 pmol forward primer, 10 pmol of reverse primer, 2 μl of the first-strand cDNA, and 0.5 μl of Ex Taq (TAKARA). Thirty five cycles of PCR were performed by incubating the reaction mixtures at the following

conditions: 85°C for three minutes, 94°C for one minute, 98°C for 10 seconds, 60-62°C for 30 seconds and 72°C for 1 minute. After PCR, the reaction mixtures were analyzed by electrophoresis. From a λHAC/IgμAY$^{-/-}$/IgμU$^{-/-}$ fetus, human IgM, IgG, and Igλ expression was detected by RT-PCR.

To confirm cell surface expression of human immunoglobulin at the protein level, we also performed flow cytometry analysis as described above. Goat anti-human IgM-FITC (Bethyl Laboratories, Mongomery, TX), goat anti-human IgM-FITC (Serotec Inc., Raleigh, NC), or goat anti-human IgM-PE (Serotec Inc., Raleigh, NC) antibody was used to label the human sIgM expressed on the peripheral blood B cells of ≥180 day HAC fetuses. To detect the light chains expressed on the B cells, goat anti-human lambda-FITC antibody (Bethyl Laboratories) or goat anti-human lambda-PE antibody (Serotec Inc) was used. For dual color analysis, a FITC-labeled anti-human IgM antibody and PE-labeled light chain antibody combination was used. We detected cell populations that were positive with the anti-human IgM and light chain antibodies. Furthermore, we performed sandwich ELISA analysis to detect secreted human IgG in λHAC/IgμAY$^{-/-}$/IgμU$^{-/-}$ calves using an affinity purified capture antibody and an appropriate HRP-enzyme labeled detection antibody. Details of the capture antibody and detection antibody for each assay are given in Table 6 below.

Table 6

Assay	Standards (Calibrator)	Capture antibody	Detection antibody
Human IgG ELISA	Human Reference Serum (Bethyl laboratories)	Goat anti-human IgG, affinity purified or goat anti-human IgG-Fc specific, affinity purified (Bethyl laboratories)	Goat anti-human IgG-HRP conjugated (Bethyl laboratories)

The capture antibody diluted in coating buffer (0.05 M sodium carbonate, pH 9.6) was coated on the microtiter plates (Nunc ImmunoMaxiSorp Elisa plates) by

incubating at room temperature for 1.5 hours. After coating the capture antibody, the plates were washed with phosphate buffered saline (PBS)/Tween 20 buffer 3-5 times using an automated plate washer. Appropriate standards (Calibrators) were added in serial dilutions for quantification using a standard curve.

Positive controls and negative controls were included in all assays for QC check. Serum samples from λHAC/IgμAY$^{-/-}$/IgμU$^{-/-}$ calves were added then into duplicate wells in four serial dilutions and incubated for 1 hour at room temperature. After serum immunoglubulins were captured, the plates are washed again with PBS-Tween buffer 3-5 times using automated plate washer. HRP-enzyme labeled appropriate detection antibody was added into all the wells and incubated for 1 hour at room temperature. At the end of incubation, the plates were washed again with PBS/Tween buffer 3-5 times using automated plate washer. The bound antibodies were detected by adding TMB-Substrate solution (KPL Inc, Gaithersburg, MA) and incubating for 10-20 minutes at room temperature. The reaction was stopped by addition of 10% phosphoric acid. The plates were then read on a microtiter plate reader using KC4 software. Data were analyzed by KC4 software and values were determined by interpolation on a four-parameter standard curve. In blood sample collected at 14 days after birth, 7.1 μg/ml of human IgG was detected by ELISA.

Other Embodiments

All publications and patents cited in this specification are incorporated herein by reference as if each individual publication or patent were specifically and individually indicated to be incorporated by reference. Although the foregoing invention has been described in some detail by way of illustration and

example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

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What is claimed is:

Claims

1. A transgenic ungulate whose genome comprises a mutation of two genes encoding IgM heavy chain.

2. The transgenic ungulate of claim 1, wherein at least one mutation is a hemizygous mutation.

3. The transgenic ungulate of claim 2, wherein both mutations are hemizygous mutations.

4. The transgenic ungulate of claim 1, wherein at least one mutation is a homozygous mutation.

5. The transgenic ungulate of claim 4, wherein both mutations are homozygous mutations.

6. The transgenic ungulate of claim 5, wherein said ungulate lacks functional IgM heavy chain.

7. The transgenic ungulate of claim 1, wherein at least one mutation is by the insertion of an exogenous sequence.

8. A transgenic ungulate producing less than 10% of endogenous IgM heavy chain, relative to a control ungulate, and whose genome comprises a mutation of a gene encoding IgM heavy chain.

9. The transgenic ungulate of claim 8, wherein said mutation substantially eliminates the expression of functional IgM heavy chain.

10. The transgenic ungulate of claim 9, wherein said ungulate is a bovine and said gene is IgμU.

11. The transgenic ungulate of claim 9, wherein said ungulate is a bovine and said gene is IgμAY.

12. The transgenic ungulate of claim 1, said ungulate comprising a homozygous mutation of PrP.

13. The transgenic ungulate of claim 1, said ungulate further comprising a nucleic acid encoding all or part of a xenogenous immunoglobulin gene that undergoes rearrangement and expresses a xenogenous immunoglobulin.

14. The transgenic ungulate of claim 13, wherein said xenogenous immunoglobulin is a human immunglobulin.

15. The transgenic ungulate of claim 13, wherein said nucleic acid is contained within a chromosome fragment.

16. The transgenic ungulate of claim 15, wherein said chromosome fragment is a ΔHAC, ΔΔHAC, or κHAC.

17. The transgenic ungulate of claim 1, said ungulate further comprising stably engrafted human hematopoietic stem cells.

18. The transgenic ungulate of claim 1, said ungulate further comprising stably engrafted canine, feline, murine or non-human primate hematopoietic stem cells.

19. A transgenic ungulate somatic cell whose genome comprises a mutation of two genes encoding IgM heavy chain.

20. The cell of claim 19, wherein at least one mutation is a hemizygous mutation.

21. The cell of claim 19, wherein said cell is a bovine cell and said two genes are IgμU and IgμAY.

22. A transgenic bovine somatic cell whose genome comprises a hemizygous mutation of IgμAY.

23. A transgenic bovine somatic cell whose genome comprises a homozygous mutation of IgμAY.

24. The cell of claim 23, said cell further comprising a nucleic acid encoding all or part of a xenogenous immunoglobulin gene that undergoes rearrangement and expresses a xenogenous immunoglobulin.

25 The cell of claim 24, wherein said xenogenous immunoglobulin is a human immunglobulin.

26. The cell of claim 24, wherein said nucleic acid is contained within a chromosome fragment.

27. The cell of claim 26, wherein said chromosome fragment is a ΔHAC, ΔΔHAC, or κHAC.

28. The cell of claim 19, wherein said cell is selected from fibroblasts, epithelial cells, endothelial cells, neural cells, keratinocytes, hematopoietic cells, melanocytes, chondrocytes, B-cells, T-cells, macrophages, monocytes, mononuclear cells, cardiac muscle cells, other muscle cells, placental cells, and epidermal cells.

29. The cell of claim 19, wherein said cell further comprises a homozygous mutation of PrP.

30. A method of producing xenogenous antibodies, said method comprising the steps of:

(a) providing a transgenic ungulate of claim 19, said ungulate further comprising a nucleic acid encoding all or part of a xenogenous immunoglobulin gene that undergoes rearrangement and expresses a xenogenous immunoglobulin;

(b) administering one or more antigens of interest to said ungulate; and

(c) recovering xenogenous antibodies from said ungulate.

31. The method of claim 30, wherein said xenogenous antibodies comprise human antibodies.

32. A method of making xenogenous antibodies in an ungulate, said method comprising the steps of:

(a) providing a transgenic ungulate of claim 19, said ungulate further comprising engrafted xenogenous hematopoietic stem cells; and

(b) recovering xenogenous antibodies from said ungulate.

33. The method of claim 32, wherein said xenogenous antibodies comprise human antibodies.

34. A method of expanding xenogenous hematopoietic stem cells, said method comprising the steps of:

(a) providing a transgenic ungulate of claim 19, said ungulate further comprising engrafted xenogenous hematopoietic stem cells; and

(b) allowing said xenogenous hematopoietic stem cells to expand in said transgenic ungulate.

35. The method of claim 34, further comprising (c) recovering said expanded hematopoietic stem cells of step (b) from said transgenic ungulate.

36. A method for maintaining a desired tissue or organ in vivo, said method comprising the steps of:

(a) providing a transgenic ungulate of claim 19;

(b) engrafting desired allogeneic or xenogeneic tissue or organ in said transgenic ungulate; and

(c) maintaining said tissue or organ in said ungulate.

37. The method of claim 36, wherein said tissue comprises skin, heart, lung, pancreatic, liver or kidney tissue.

38. A bovine or bovine fetus comprising a non-naturally occurring mutation in one or both alleles of an endogenous prion nucleic acid.

39. The bovine or bovine fetus of claim 38, wherein said mutation reduces the expression of functional prion protein.

40. The bovine or bovine fetus of claim 38, wherein said mutation substantially eliminates the expression of functional prion protein.

41. The bovine or bovine fetus of claim 38, wherein said mutation is hemizygous.

42. The bovine or bovine fetus of claim 38, wherein said mutation is homozygous.

43. The bovine or bovine fetus of claim 38, further comprising a mutation that reduces the expression of an endogenous antibody.

44. The bovine or bovine fetus of claim 43, wherein said mutation reduces the expression of functional IgM heavy chain.

45. The bovine or bovine fetus of claim 43, wherein said mutation substantially eliminates the expression of functional IgM heavy chain.

46. The bovine or bovine fetus of claim 38, wherein said bovine fetus is at least 30 days post-fertilization.

47. The bovine or bovine fetus of claim 38, wherein said bovine is a newborn.

48. The bovine or bovine fetus of claim 38, wherein said bovine is at least 1 week in age.

49. The bovine or bovine fetus of claim 48, wherein said bovine is at least 2 months of age.

50. A product produced from a bovine or bovine fetus of claim 38.

51. The product of claim 50, wherein said product is milk, gelatin, collagen, or serum.

52. The product of claim 50, wherein said product is a recombinant protein.

53. A method of producing a product that substantially lacks prion protein, said method comprising manufacturing said product in, or obtaining said product from, a bovine or bovine fetus comprising a non-naturally occurring mutation in one or both alleles of an endogenous prion nucleic acid.

54. The method of claim 53, wherein said product is milk, gelatin, collagen, or serum.

55. The method of claim 53, wherein said product is a recombinant protein.

56. A method of producing a cell comprising two genetic modifications, said method comprising the steps of:

(a) providing a non-human mammalian somatic cell having a first genetic modification;

(b) inserting said cell or a progeny thereof, a chromatin mass from said cell or progeny thereof, or a nucleus from said cell or progeny thereof into a nucleated or enucleated oocyte;

(c) transferring said oocyte obtained from step (b) or an embryo formed from said oocyte into a recipient;

(d) isolating a cell from said embryo, or from a fetus or juvenile produced therefrom, wherein said cell contains said first genetic modification; and

(e) introducing a second genetic modification into the genome of said cell or a progeny thereof, thereby producing a cell comprising two genetic modifications.

57. The method of claim 56, wherein said first genetic modification or said second genetic modification comprises a mutation.

58. The method of claim 57, wherein said first genetic modification comprises a first mutation and said second genetic modification comprises a second mutation.

59. The method of claim 58, wherein said first mutation is in a first allele of an endogenous gene and said second mutation is in a second allele of an endogenous gene.

60. The method of claim 58, wherein said first mutation is in a first allele of a first endogenous gene and said second mutation is in a first allele of a second endogenous gene.

61. The method of claim 56, wherein said first genetic modification or said second genetic modification comprises an artificial chromosome.

62. The method of claim 61, wherein said artificial chromosome encodes an antibody.

63. The method of claim 56, further comprising, beginning with the cell obtained in step (e) or a progeny thereof, repeating said method one or more times, thereby introducing additional genetic modifications.

64. The method of claim 56, wherein said somatic cell in step (a) is a fetal cell or adult cell.

65. The method of claim 64, wherein said fetal or adult cell is selected from the group consisting of a fibroblast, epithelial cell, neural cell, epidermal cell, keratinocyte, hematopoietic cell, melanocyte, chondrocyte, B-lymphocyte, T-lymphocyte, erythrocyte, macrophage, monocyte, or muscle cell.

66. A method of producing a non-human mammal comprising two genetic modifications, said method comprising the steps of:

(a) providing the cell obtained by the method of claim 56 or a progeny thereof;

(b) inserting said cell or a progeny thereof, a chromatic mass from said cell or said progeny, or a nucleus from said cell or said progeny into a nucleated or enucleated oocyte; and

(c) transferring said oocyte or an embryo formed from said oocyte into a recipient, under conditions that allow said oocyte or said embryo to develop into a mammal, thereby producing a mammal comprising two genetic modifications.

67. The method of claim 66, wherein said mammal is an ungulate

68. The method of claim 67, wherein said ungulate is a bovine.

69. A method of producing a cell comprising two genetic modifications, said method comprising the steps of:

(a) providing a non-human mammalian somatic cell having a first genetic modification;

(b) permeabilizing said somatic mammalian cell under conditions that allow chromatin condensation;

(c) inserting said permeabilized cell into a nucleated or enucleated oocyte;

(d) transferring said oocyte obtained from step (c) or an embryo formed from said oocyte into a recipient;

(e) isolating a cell from said embryo, or from a fetus or juvenile produced therefrom, wherein said cell contains said genetic modification; and

(f) introducing a second genetic modification into the genome of said cell or a progeny thereof, thereby producing a cell comprising two genetic modifications.

70. The method of claim 69, wherein said first genetic modification or said second genetic modification comprises a mutation.

71. The method of claim 70, wherein said first genetic modification comprises a first mutation and said second genetic modification comprises a second mutation.

72. The method of claim 71, wherein said first mutation is in a first allele of an endogenous gene and said second mutation is in a second allele of an endogenous gene.

73. The method of claim 71, wherein said first mutation is in a first allele of a first endogenous gene and said second mutation is in a first allele of a second endogenous gene.

74. The method of claim 69, wherein said first genetic modification or said second genetic modification comprises an artificial chromosome.

75. The method of claim 74, wherein said artificial chromosome encodes an antibody.

76. The method of claim 69, further comprising, beginning with the cell obtained in step (e) or a progeny thereof, repeating said method one or more times, thereby introducing additional genetic modifications.

77. The method of claim 69, wherein said somatic cell in step (a) is a fetal cell or adult cell.

78. The method of claim 77, wherein said fetal or adult cell is selected from the group consisting of a fibroblast, epithelial cell, neural cell, epidermal cell, keratinocyte, hematopoietic cell, melanocyte, chondrocyte, B-lymphocyte, T-lymphocyte, erythrocyte, macrophage, monocyte, or muscle cell.

79. A method of producing a non-human mammal comprising two genetic modifications, said method comprising the steps of:

(a) providing the cell obtained by the method of claim 69 or a progeny thereof;

(b) inserting said cell or a progeny thereof, a chromatic mass from said cell or said progeny, or a nucleus from said cell or said progeny into a nucleated or enucleated oocyte; and

(c) transferring said oocyte or an embryo formed from said oocyte into a recipient, under conditions that allow said oocyte or said embryo to develop into a mammal, thereby producing a mammal comprising two genetic modifications.

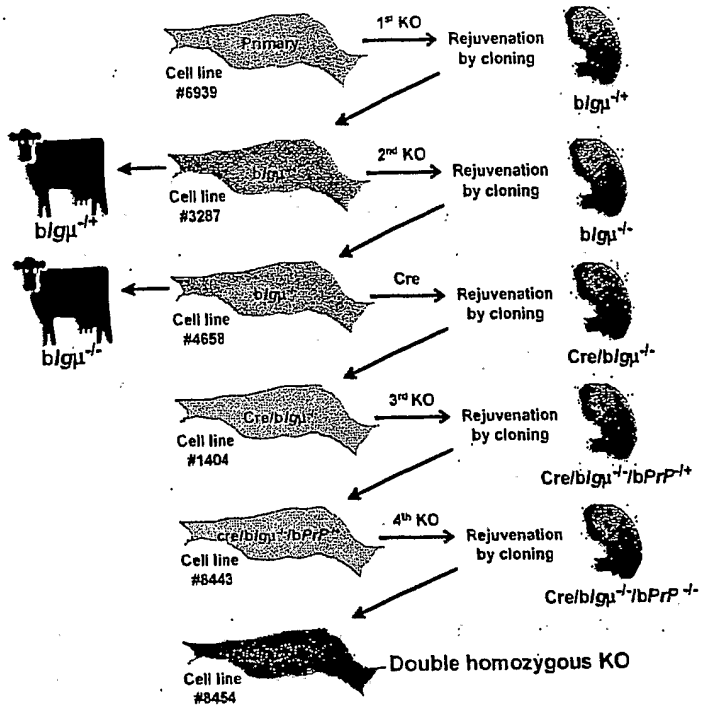
80. The method of claim 79, wherein said mammal is an ungulate

81. The method of claim 80, wherein said ungulate is a bovine.

82. A non-human mammal produced using the cell obtained from the method of claim 56.

83. The mammal of claim 84, wherein said mammal is an ungulate.

84. The mammal of claim 83, wherein said ungulate is a bovine.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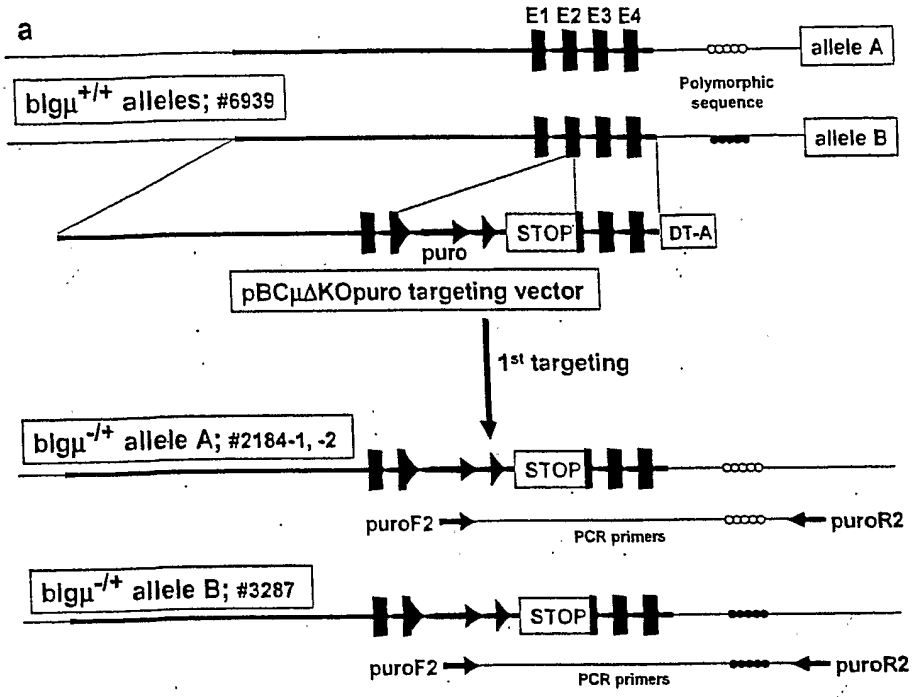
Rejuvenation by cloning
bIgμ-/+
bIgμ-/+
Cell line #3287
2nd KO
Rejuvenation by cloning
bIgμ-/-
bIgμ-/-
Cell line #4658
Cre
Rejuvenation by cloning
Cre/bIgμ-/-
Cre/bIgμ-/-
Cell line #1404
3rd KO
Rejuvenation by cloning
Cre/bIgμ-/-/bPrP-/+
Cell line #8443
4th KO
Rejuvenation by cloning
Cre/bIgμ-/-/bPrP -/-
Cell line #8454
Double homozygous KO

Fig. 1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puro
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puroF2
PCR primers
puroR2
blgμ-/+ allele B; #3287
STOP
puroF2
PCR primers
puroR2

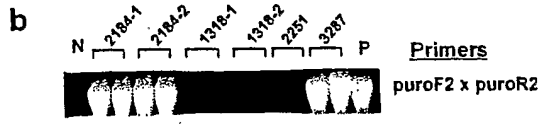

b
N
2184-1
2184-2
1318-1
1318-2
2251
3287
P
Primers
puroF2 x puroR2

Fig. 2

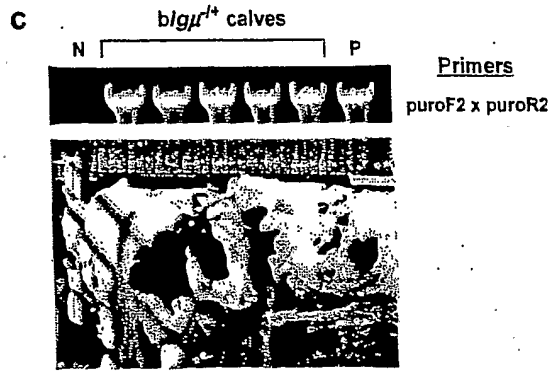
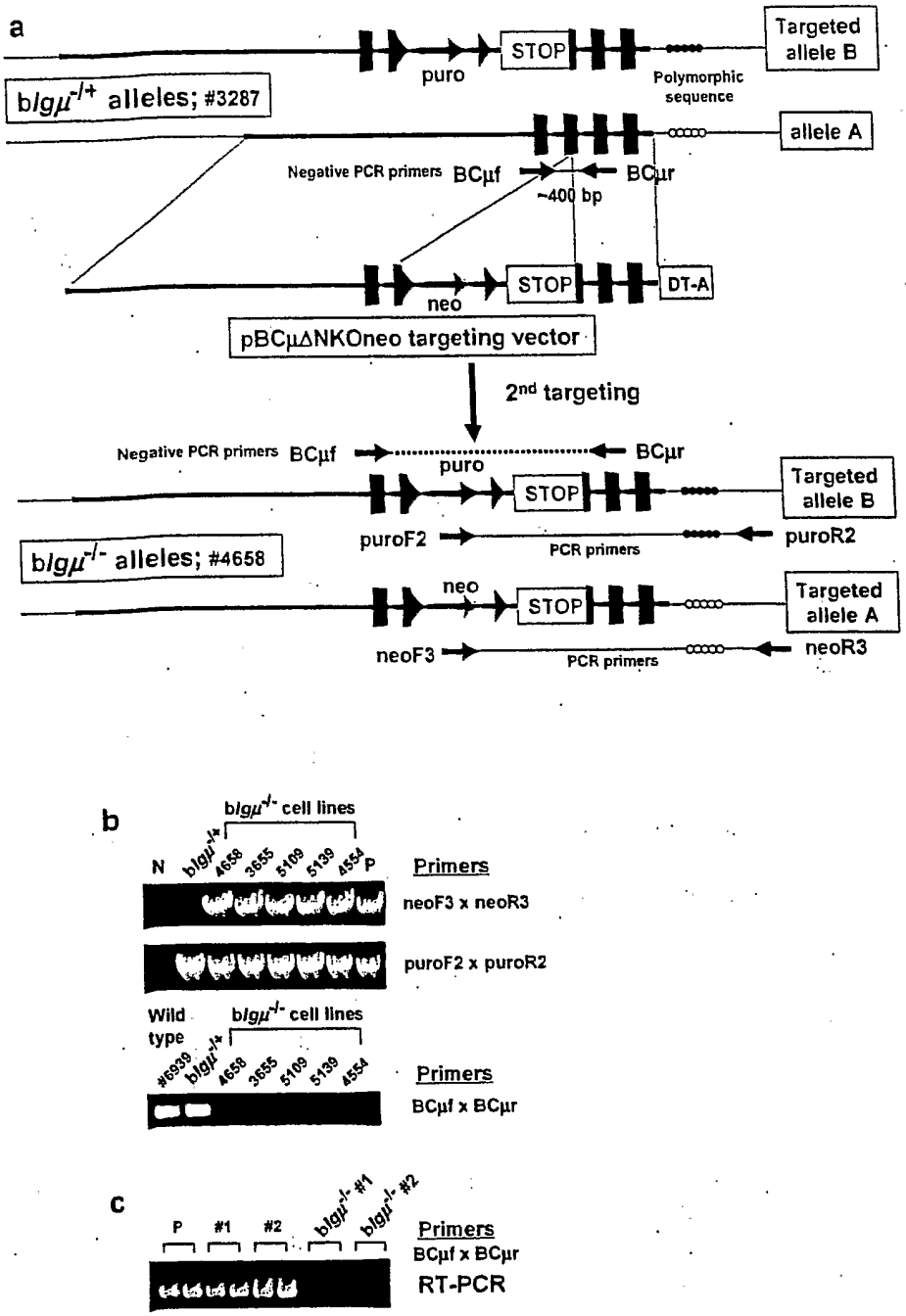


Fig. 2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STOP
puro
Polymorphic sequence
blgμ-/+ alleles; #3287
allele A
Negative PCR primers BCμf
BCμr
~400 bp
STOP
DT-A
neo
pBCμΔNKOneo targeting vector
2nd targeting
Negative PCR primers BCμf
BCμr
puro
Targeted allele B
STOP
puroF2
PCR primers
puroR2
blgμ-/- alleles; #4658
neo
STOP
Targeted allele A
neoF3
PCR primers
neoR3
b
blgμ-/- cell lines
N
blgμ-/+
4658
3655
5109
5139
4554
P
Primers
neoF3 x neoR3
puroF2 x puroR2
Wild type
blgμ-/- cell lines
#6939
blgμ-/+
4658
3655
5109
5139
4554
Primers
BCμf x BCμr
c
P
#1
#2
blgμ-/- #1
blgμ-/- #2
Primers
BCμf x BCμr
RT-PCR

Fig. 3

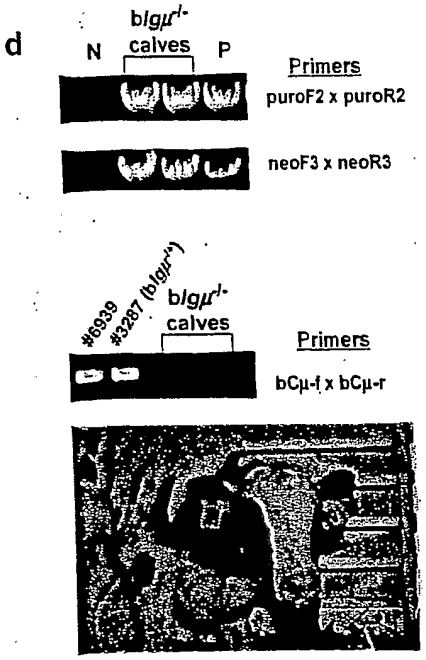
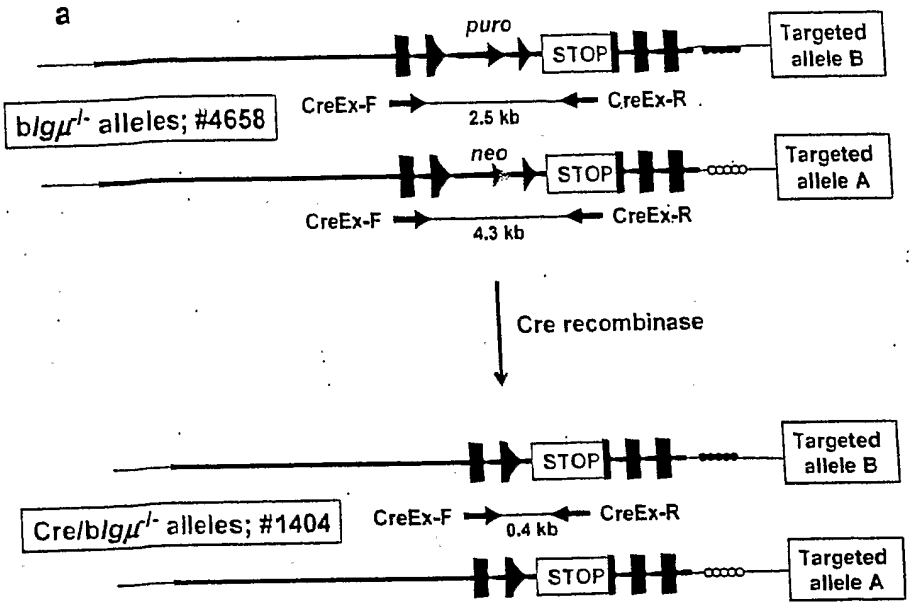

d
bIgμ-/-
calves
N
P
Primers
puroF2 x puroR2
neoF3 x neoR3
#6939
#3287 (bIgμ+/-)
bIgμ-/-
calves
Primers
bCμ-f x bCμ-r

Fig. 3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blgμ-/- alleles; #4658
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neo
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Targeted allele A
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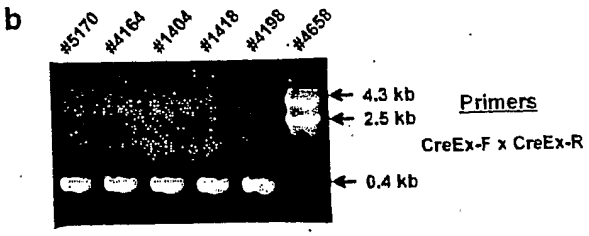
Targeted allele A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#1404
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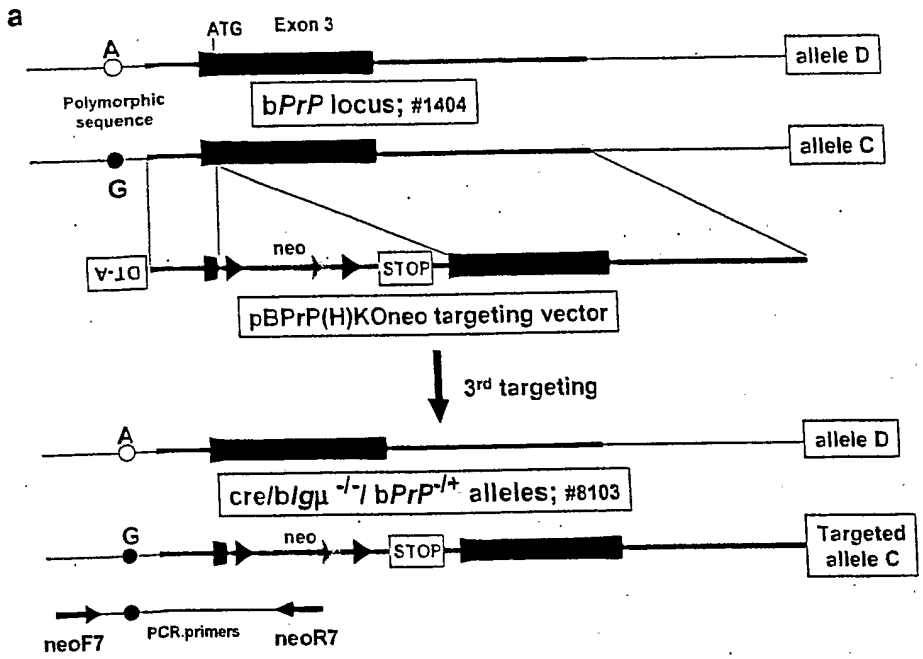
4.3 kb
2.5 kb
Primers
CreEx-F x CreEx-R
0.4 kb

Fig. 4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A
allele D
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allele C
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neo
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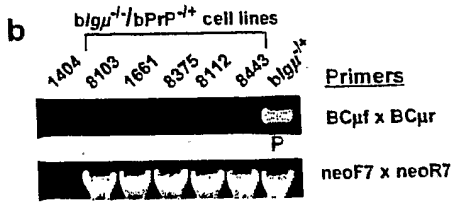
DT-A
pBPrP(H)KOneo targeting vector
3rd targeting
A
allele D
cre/blgμ -/-/ bPrP-/+ alleles; #8103
G
neo
STOP
Targeted allele C
neoF7
PCR.primers
neoR7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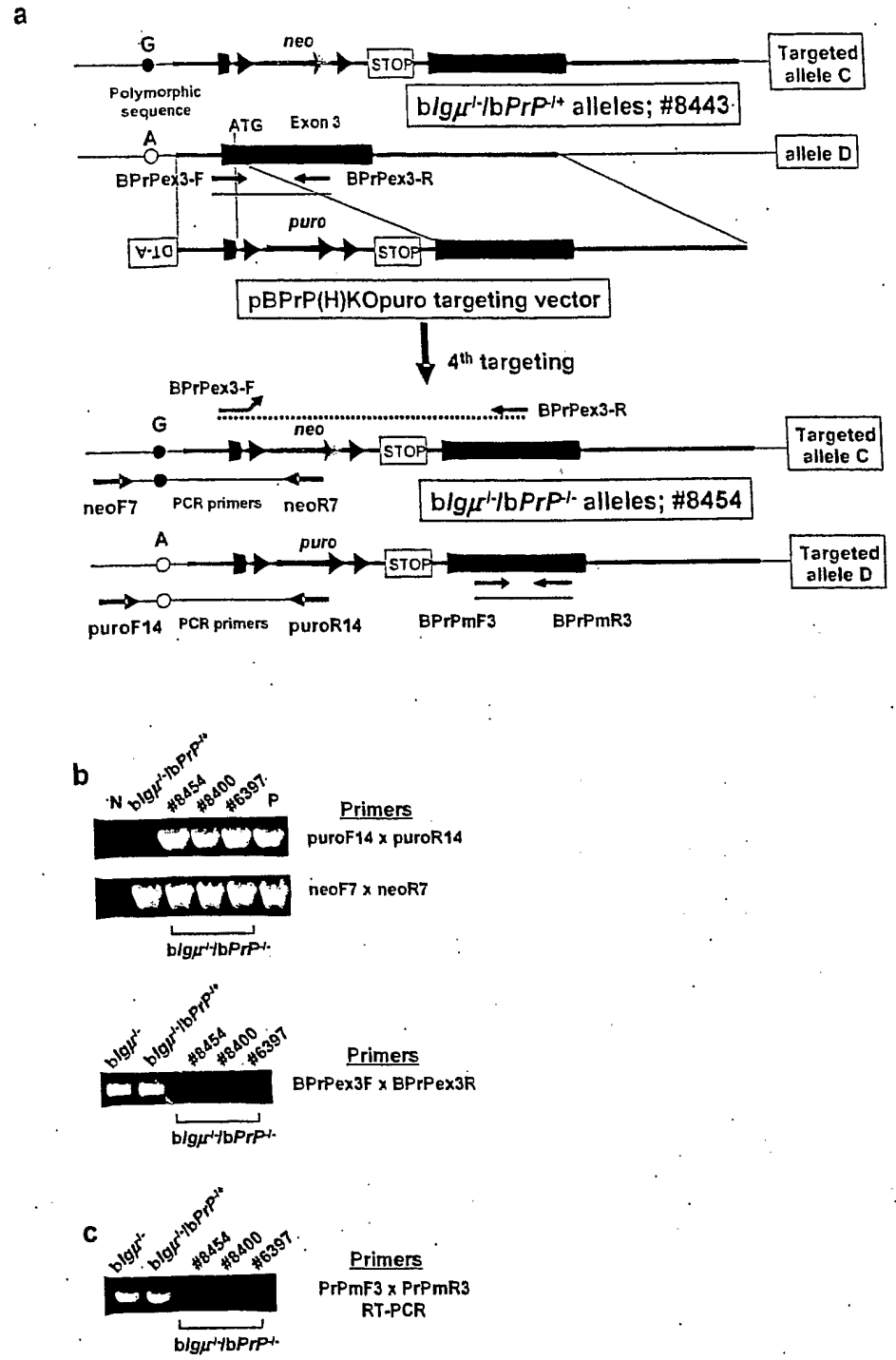
blgμ-/-/bPrP-/+ cell lines
1404
8103
1661
8375
8112
8443
blgμ-/+
Primers
BCμf x BCμr
P
neoF7 x neoR7

Fig. 5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neo
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Targeted allele C
Polymorphic sequence
bIgμ-/-bPrP-/+ alleles; #8443
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Exon 3
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allele D
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BPrPex3-R
puro
DT-A
STOP
pBPrP(H)KOpuro targeting vector
4th targeting
BPrPex3-F
BPrPex3-R
G
neo
STOP
Targeted allele C
neoF7
PCR primers
neoR7
bIgμ-/-bPrP-/- alleles; #8454
A
puro
STOP
Targeted allele D
puroF14
PCR primers
puroR14
BPrPmF3
BPrPmR3
b
N bIgμ-/-bPrP-/+
#8454
#8400
#6397
P
Primers
puroF14 x puroR14
neoF7 x neoR7
bIgμ-/-bPrP-/-
bIgμ-/-
bIgμ-/-bPrP-/+
#8454
#8400
#6397
Primers
BPrPex3F x BPrPex3R
bIgμ-/-bPrP-/-
c
bIgμ-/-
bIgμ-/-bPrP-/+
#8454
#8400
#6397
Primers
PrPmF3 x PrPmR3
RT-PCR
bIgμ-/-bPrP-/-

Fig. 6

Fig. 7 (pg 1 of 2)

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Fig. 7 (pg 2 of 2)

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Fig. 8 (pg 1 of 2)

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Fig. 8 (pg 2 of 2)

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ATAAA

Fig. 9

GAGAGTCTTCCCCCTGGTGTCCTGCGTGAGCTCGCCATCCGATGAGAGCACGGTGGCCCTGGGCTGCCTG
GCCCGGGACTTCGTGCCCAATTCAGTCAGCTTCTCCTGGAAGTTCAACAACAGCACAGTCAGCAGCGAGA
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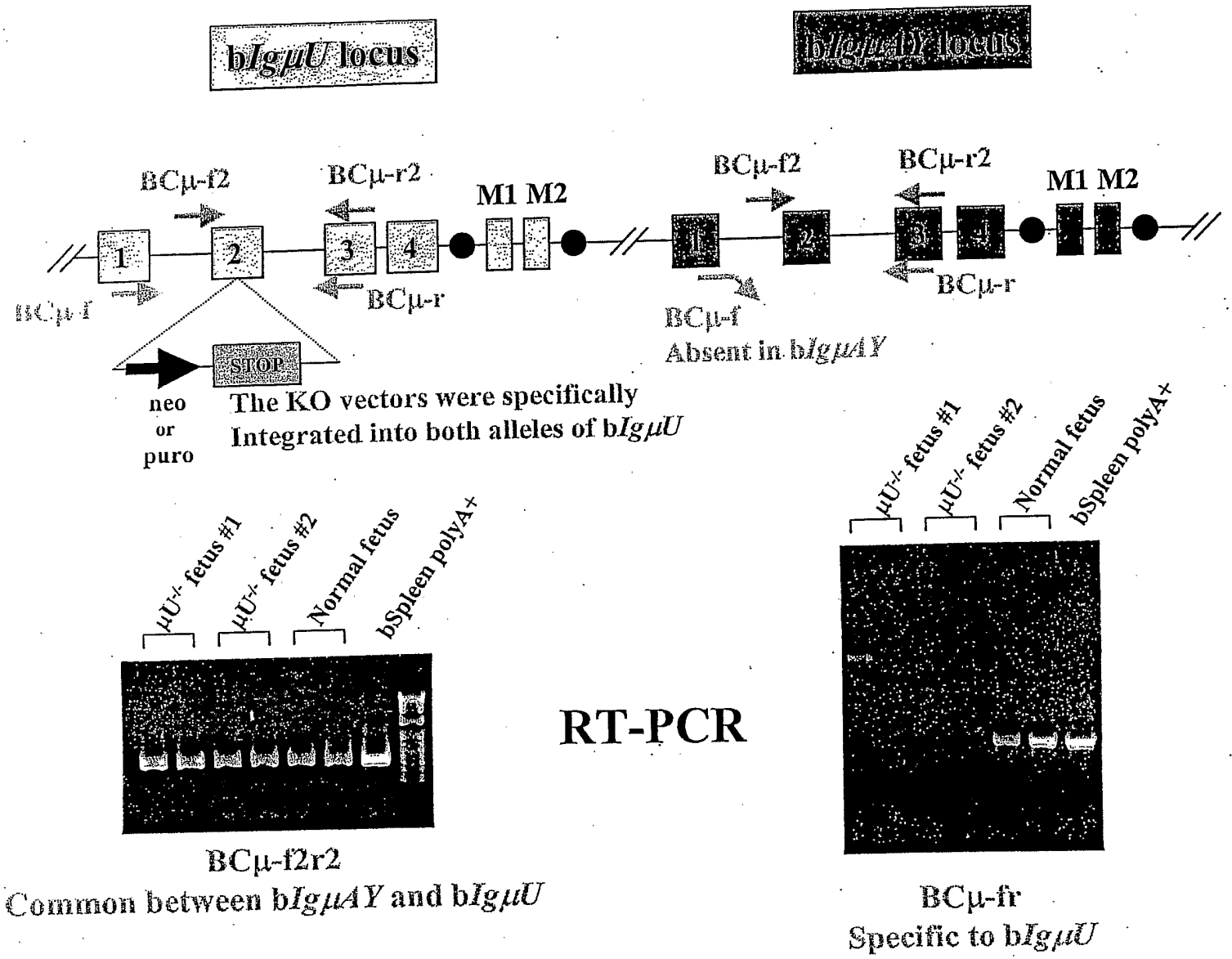
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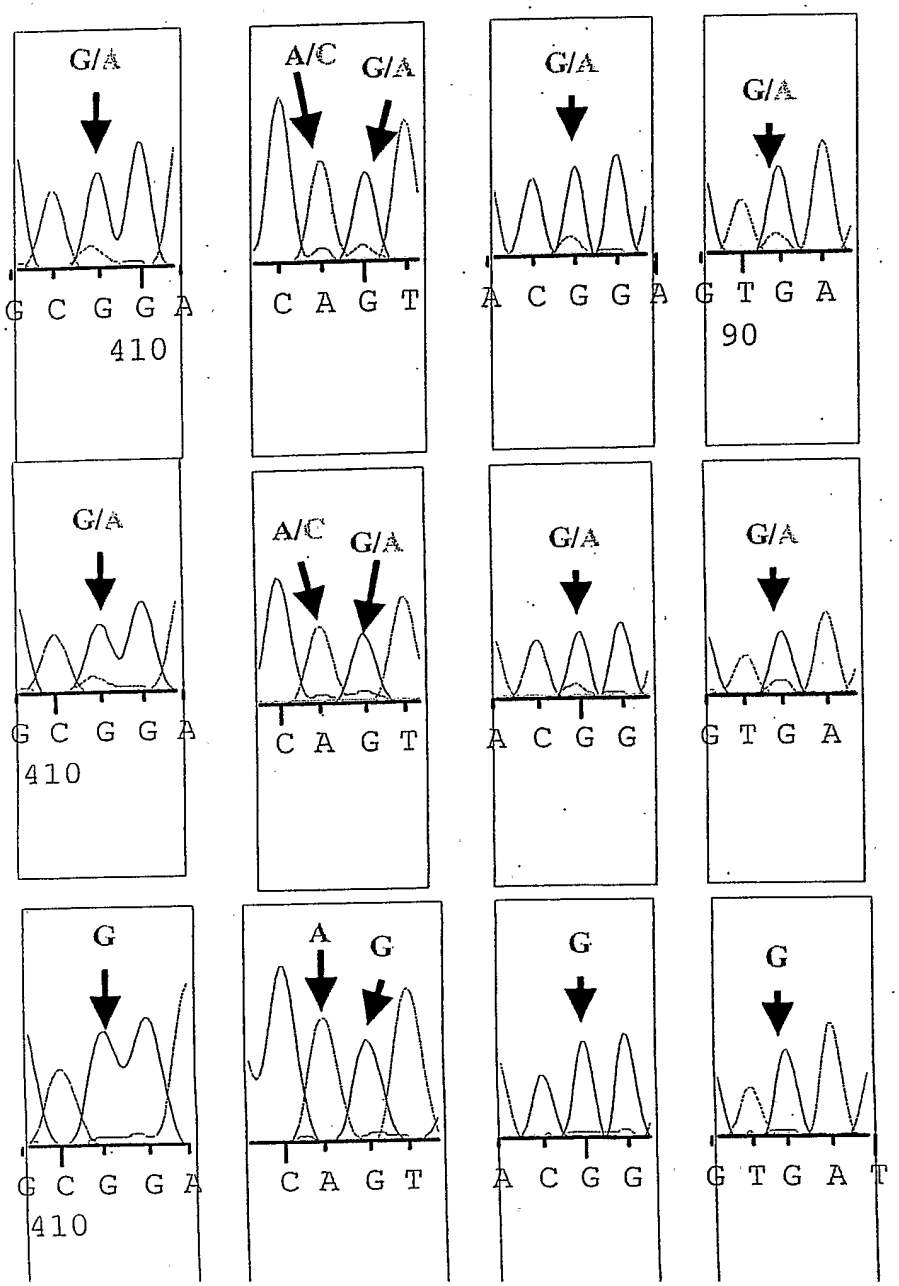
bIgμAY locus
BCμ-f2
BCμ-r2
M1 M2
BCμ-f2
BCμ-r2
M1 M2
1
2
3
4
1
2
3
4
BCμ-f
BCμ-r
BCμ-f
Absent in bIgμAY
BCμ-r
STOP
neo
or
puro
The KO vectors were specifically
Integrated into both alleles of bIgμU
μU-/- fetus #1
μU-/- fetus #2
Normal fetus
bSpleen polyA+
μU-/- fetus #1
μU-/- fetus #2
Normal fetus
bSpleen polyA+
RT-PCR
BCμ-f2r2
Common between bIgμAY and bIgμU
BCμ-fr
Specific to bIgμU

Fig. 10



#6939 fetus 1

On top of *IgμAY*, a very small amount of *IgμU* is expressed, following VDJC rearrangement.

#6939 fetus 2

On top of *IgμAY*, a very small amount of *IgμU* is expressed, following VDJC rearrangement.

b*IgμU* KO fetus

Only *IgμAY* is expressed

Fig. 11

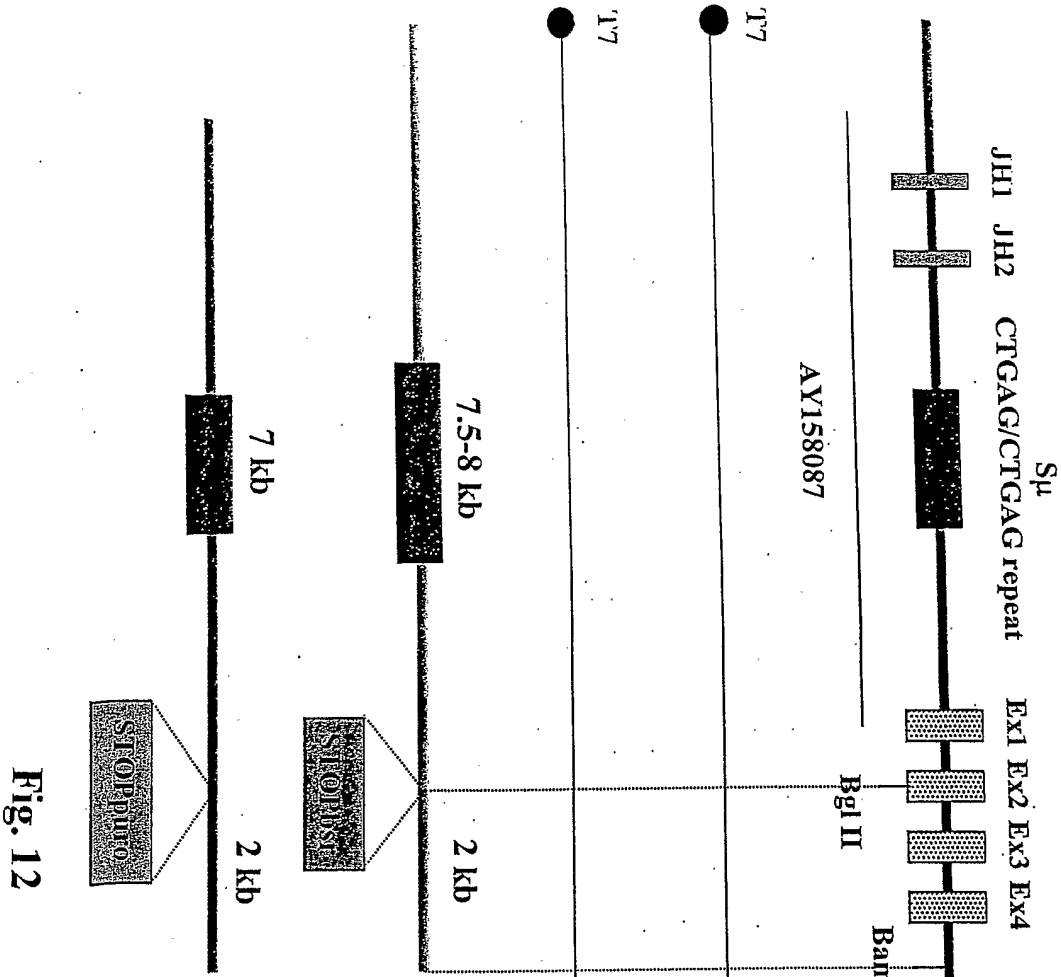
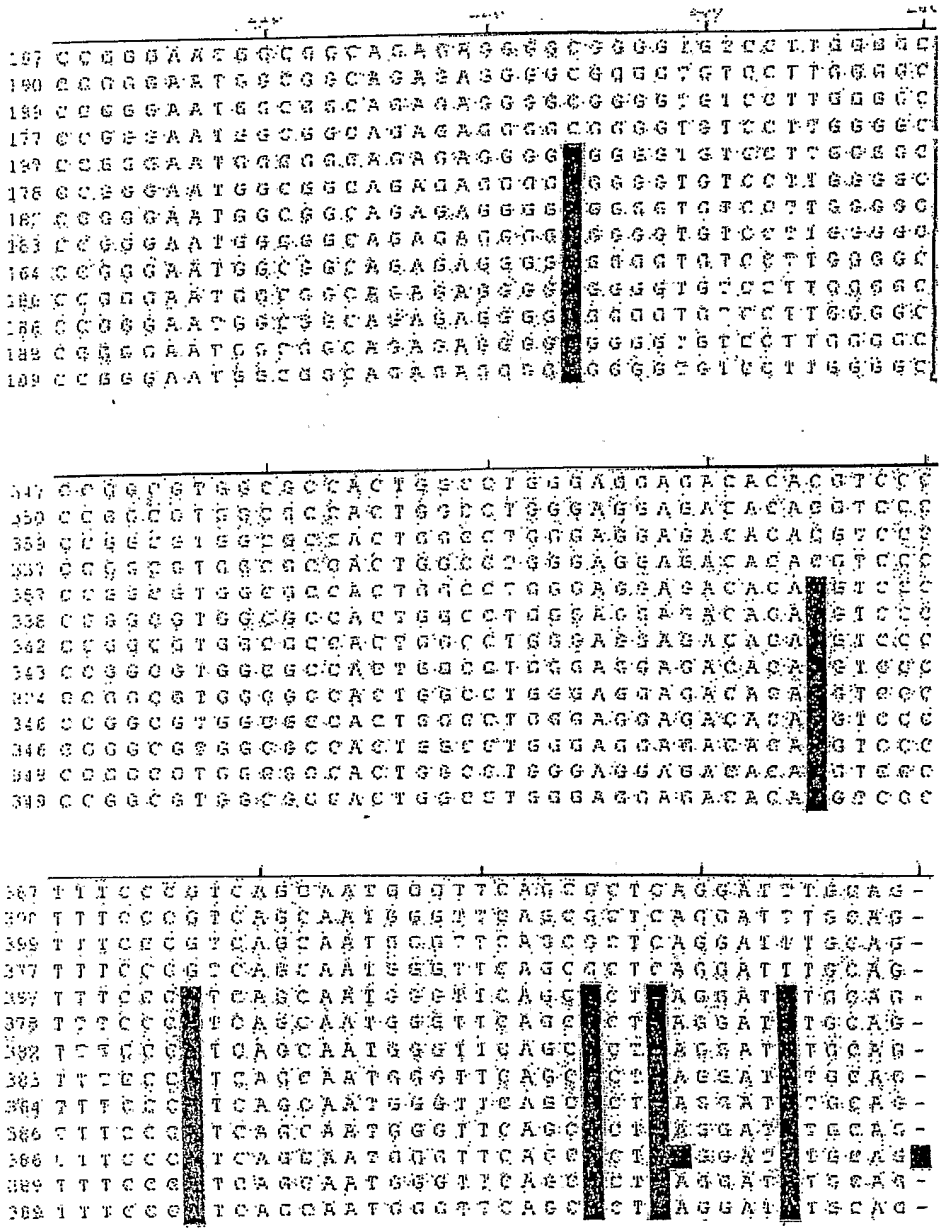

Sμ
JH1 JH2 CTGAG/CTGAG repeat Ex1 Ex2 Ex3 Ex4 M1 M2 Bov B
AY158087
Bgl II
Bam HI
T7
T3
#19, 37, 98, 101 (bIgμAY)
T7
T3
#49, 50, 66, 106 (bIgμav)
7.5-8 kb
2 kb
STOPpuro
7 kb
2 kb
STOPpuro
KO vectors for bIgμAY
KO vectors for bIgμU

Fig. 12



We identified two alleles Of b*Igμ*A*Y*, *AY* and *ay*, Based on polymorphic Sequence.

Fig. 13

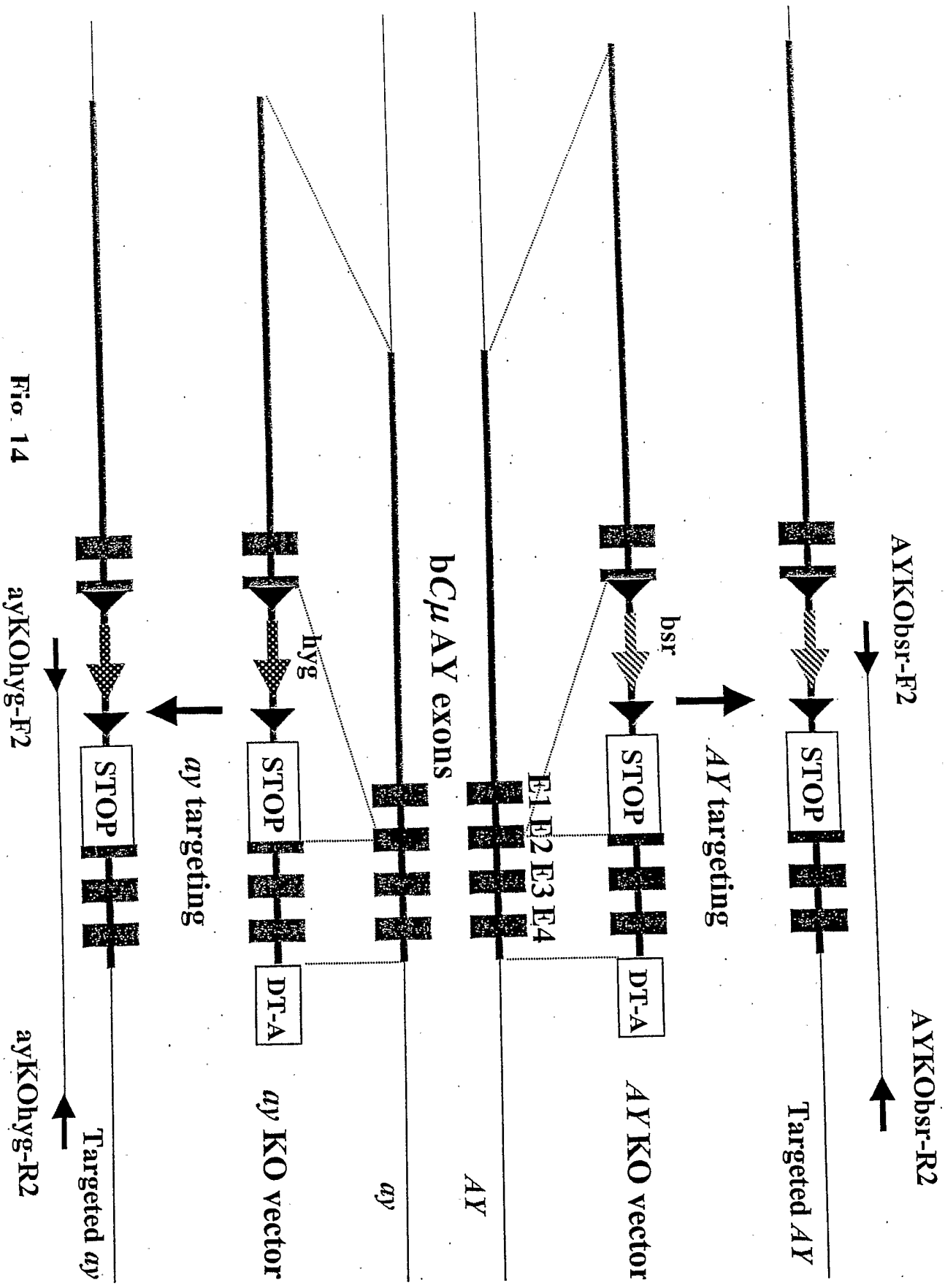

AYKObsr-F2
STOP
AYKObsr-R2
Targeted AY
bsr
AY targeting
STOP
DT-A
AY KO vector
E1 E2 E3 E4
AY
bCu AY exons
ay
hyg
STOP
DT-A
ay KO vector
ay targeting
STOP
ayKOhyg-F2
ayKOhyg-R2
Targeted ay

Fig. 14

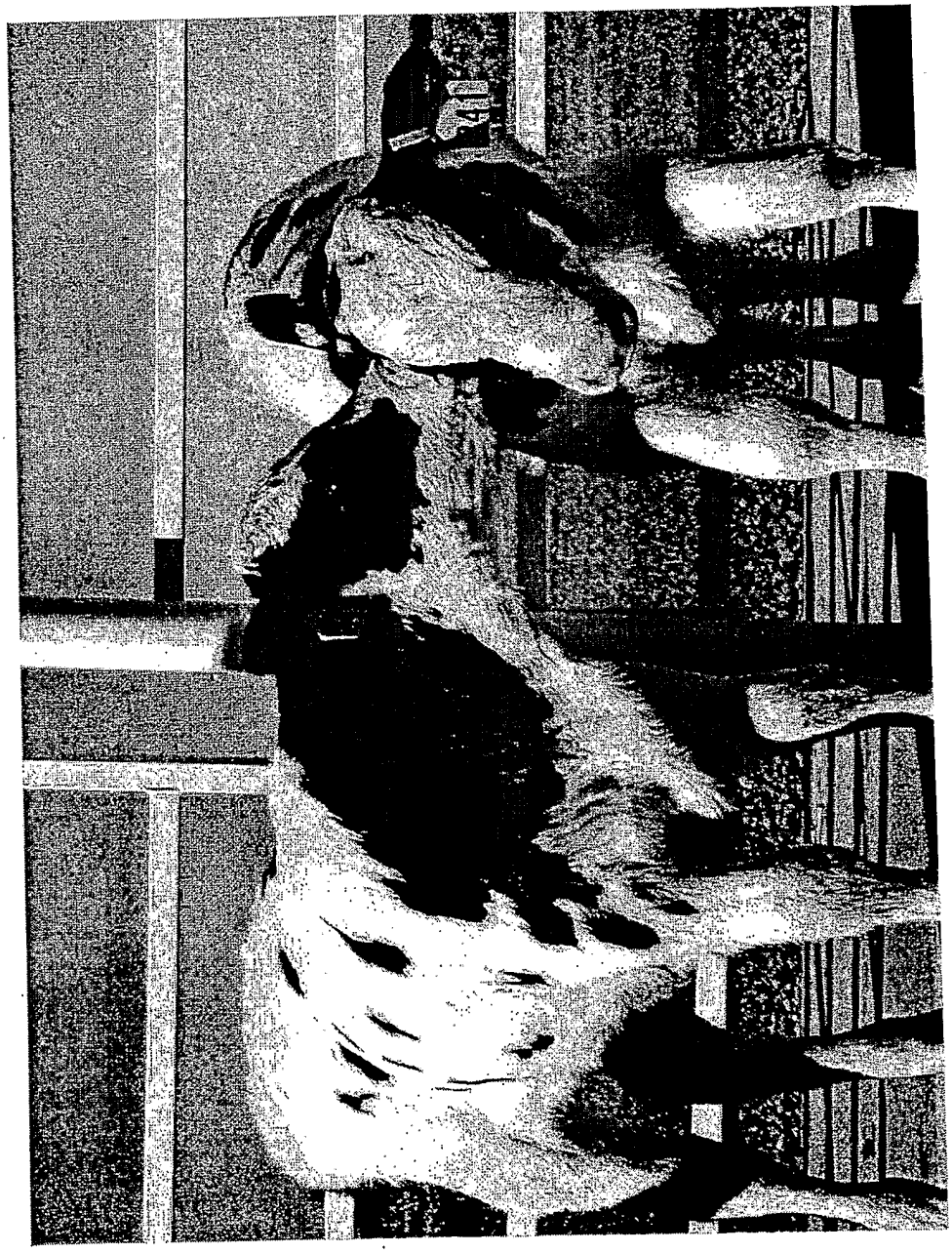
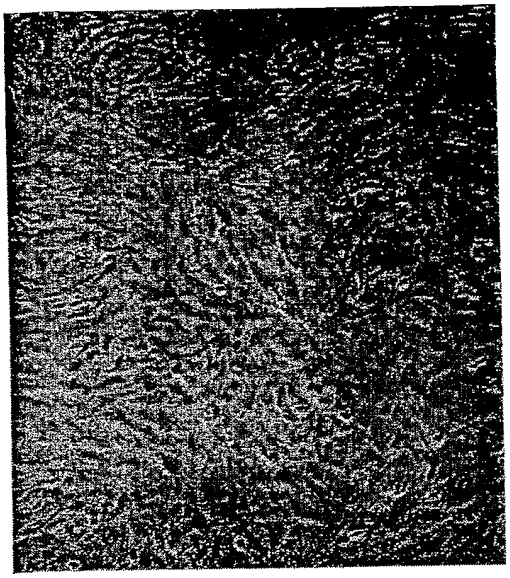
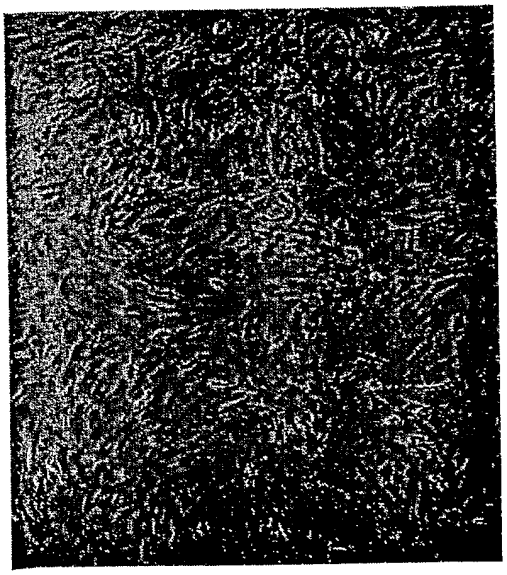


Fig. 15



PRNP$^{-/+}$*IGHM*$^{-/-}$
Calf fibroblasts



PRNP$^{-/-}$*IGHM*$^{-/-}$
Calf fibroblasts

Fig. 16A

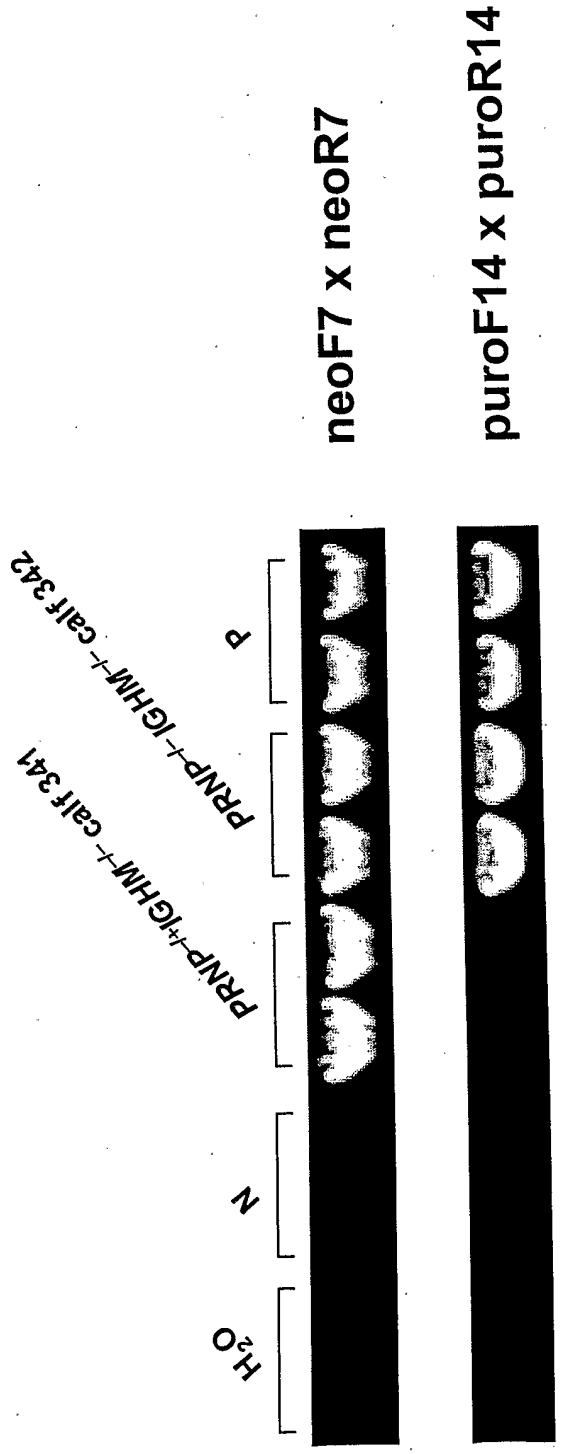

neoF7 x neoR7
puroF14 x puroR14
H2O
N
PRNP+/+IGHM+/- calf 341
PRNP+/-IGHM+/- calf 342
P

Fig. 16B

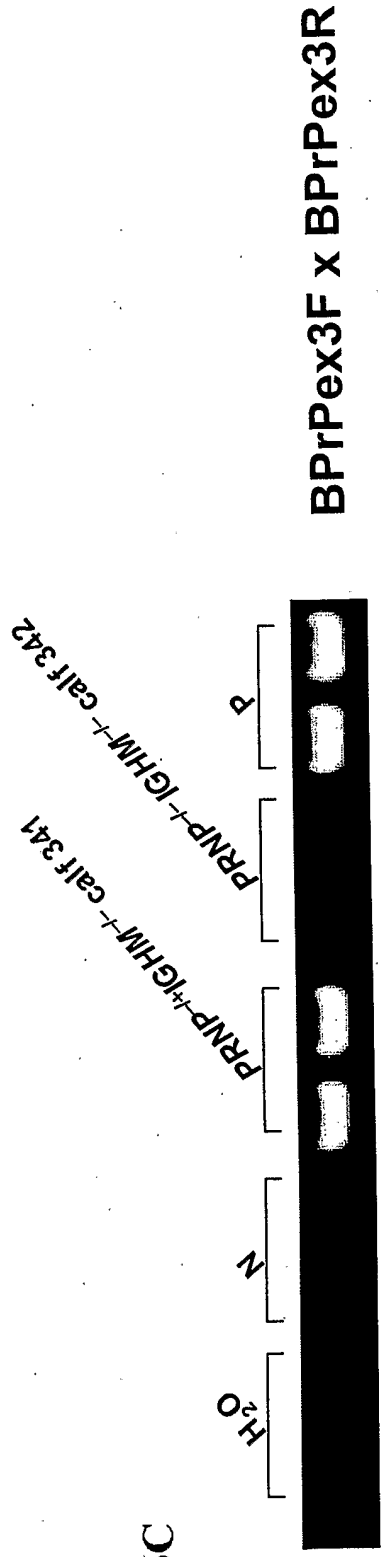


Fig. 16C

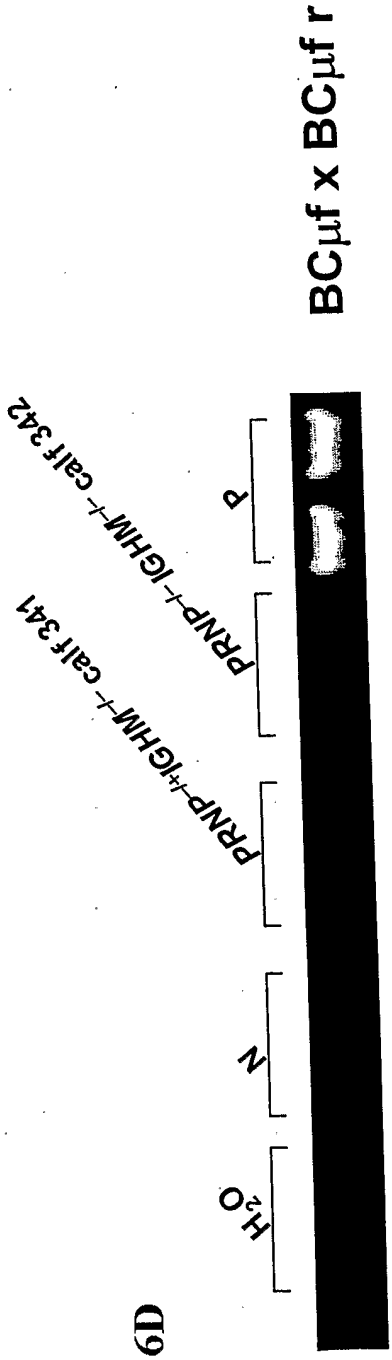


Fig. 16D

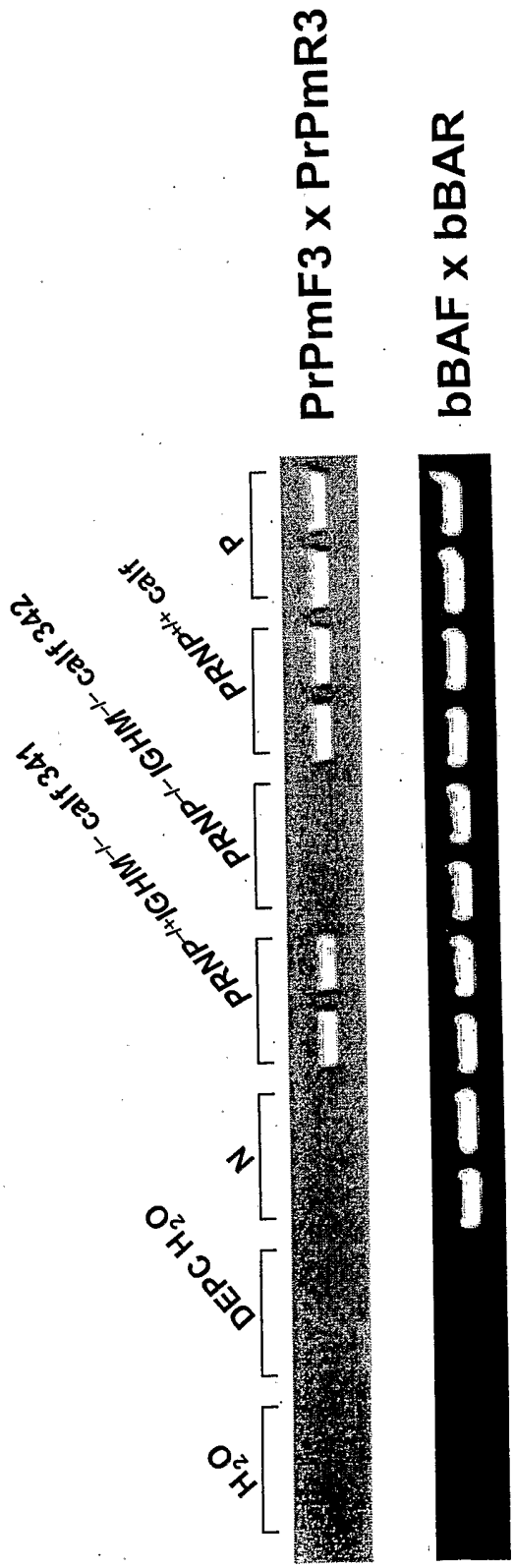

PrPmF3 x PrPmR3
bBAF x bBAR
H2O
DEPC H2O
N
PRNP+/+IGHM+/- calf 341
PRNP+/+IGHM+/- calf 342
PRNP+/+ calf
P

Fig. 17A

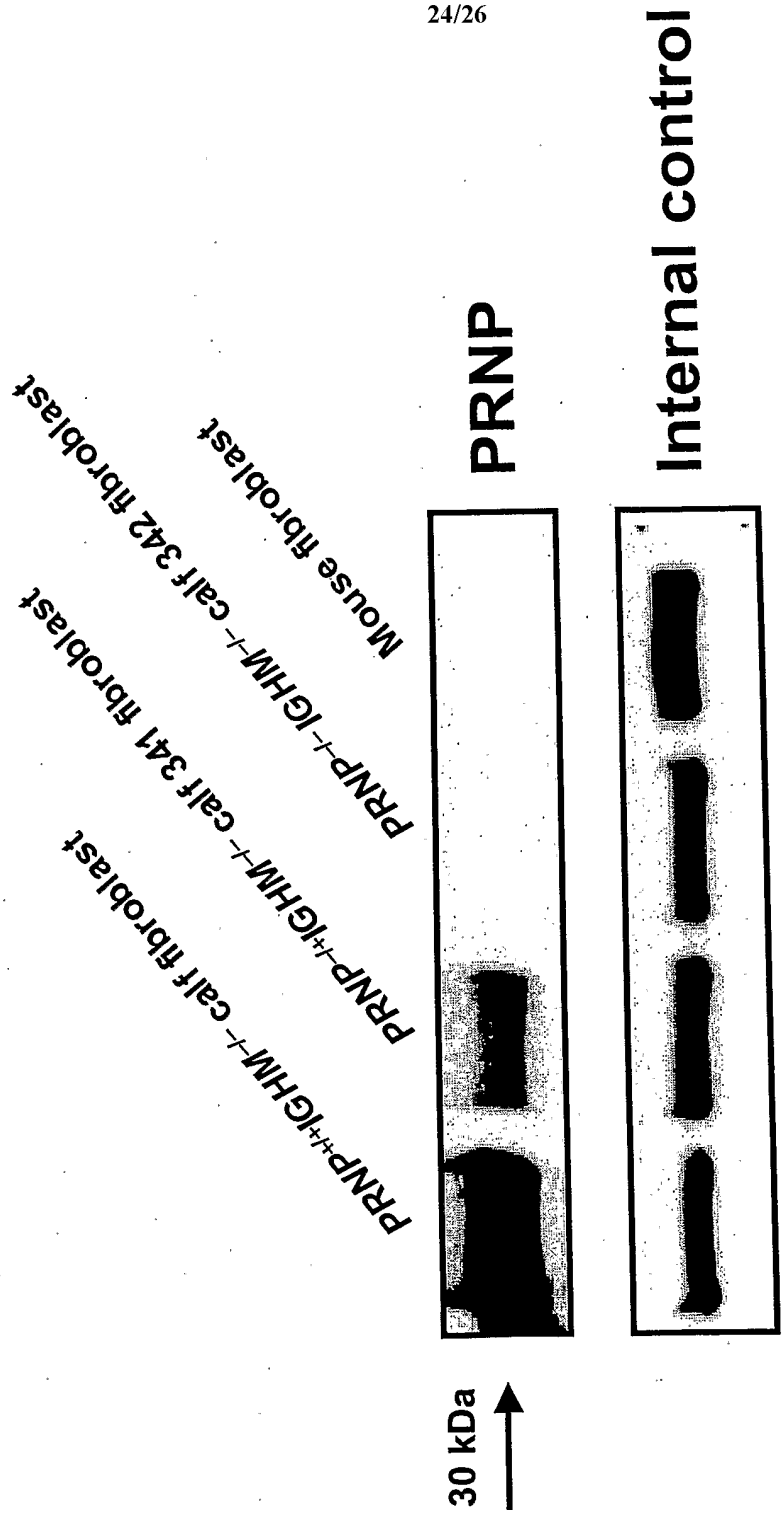

PRNP
Internal control
30 kDa
PRNP+/+IGHM+/- calf fibroblast
PRNP+/+IGHM+/- calf 341 fibroblast
PRNP+/-IGHM+/- calf 342 fibroblast
Mouse fibroblast

Fig. 17B

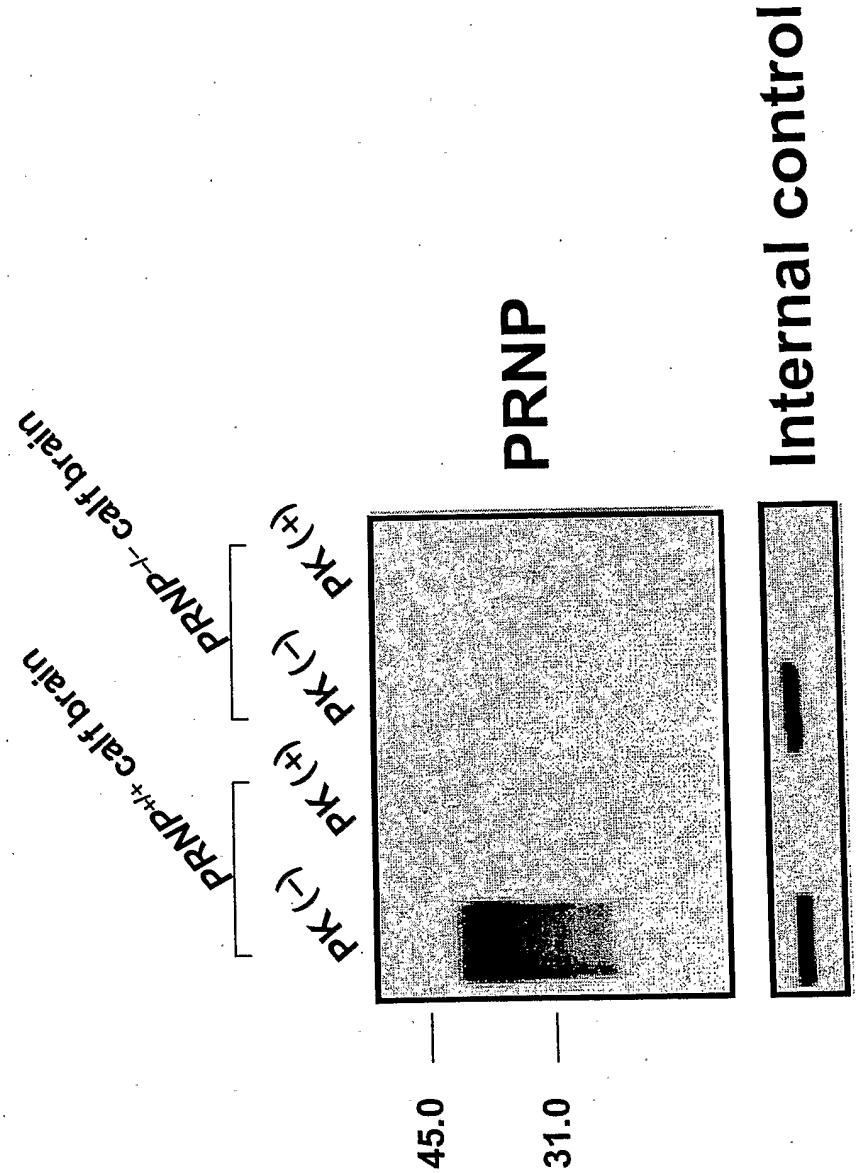

PRNP
Internal control
PRNP+/+ calf brain
PRNP+/- calf brain
PK (-)
PK (+)
PK (-)
PK (+)
45.0
31.0

Fig. 18

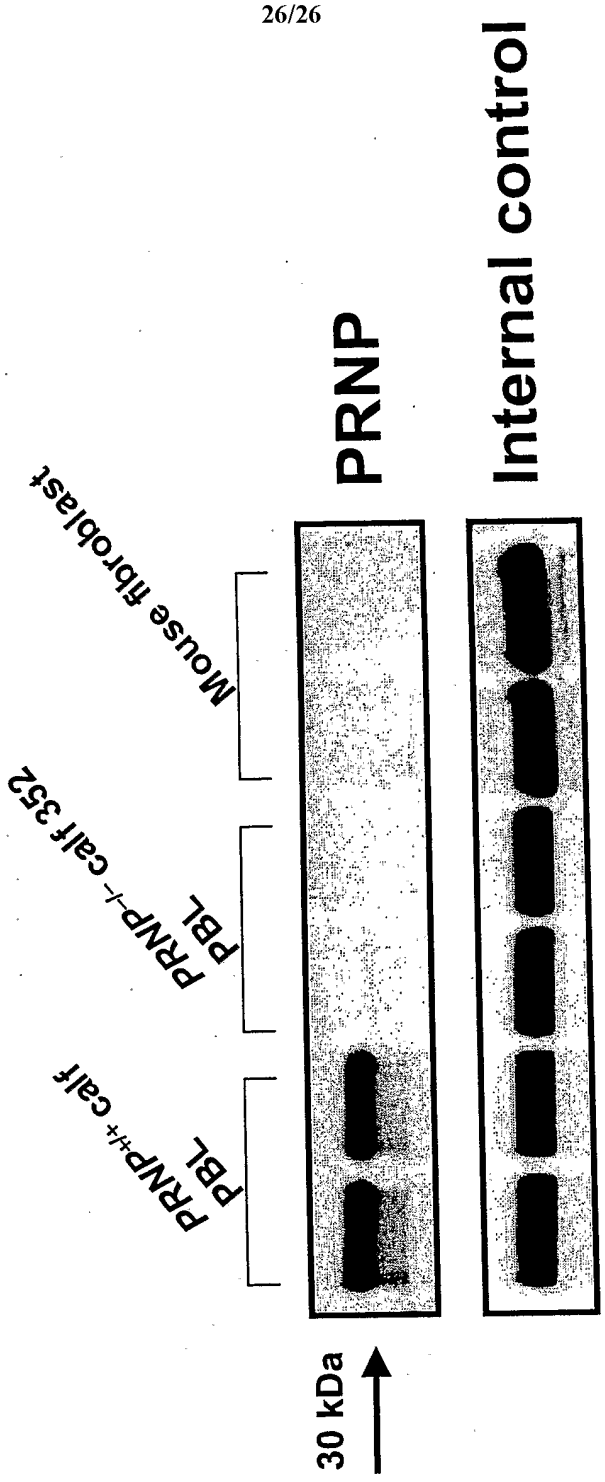

PRNP
Internal control
Mouse fibroblast
PBL PRNP-/- calf 352
PBL PRNP+/+ calf
30 kDa

Fig. 19