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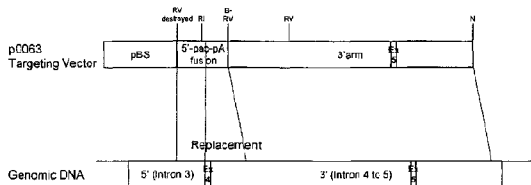
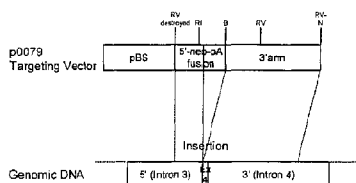
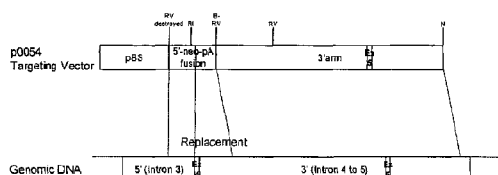
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(54) Title: ANIMAL TISSUE WITH CARBOHYDRATE ANTIGENS COMPATIBLE FOR HUMAN TRANSPLANTATION



(57) Abstract: This disclosure provides a system for generating animal tissue with carbohydrate antigens that are compatible for transplantation into human patients. The tissue is inactivated homozygously for expression of $\alpha(1,3)$ galactosyltransferase, and comprises a transgene for $\alpha(1,2)$ fucosyltransferase. As a result, cell-surface N-acetyl lactosamine is not converted to the Gal $\alpha(1,3)$ Gal xenoantigen. Instead, it is converted to Fuc $\alpha(1,2)$ Gal, which is H substance, a self-antigen in humans. The tissue may also contain A or B-transferase, which will cause H substance to be converted into other ABO blood group antigens for compatibility with patients of the same blood type. This invention improves transplant compatibility of the xenograft tissue by lessening the risk of reactions resulting from xenoantigen and unconverted N-acetyl lactosamine acceptor determinants.

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**ANIMAL TISSUE WITH CARBOHYDRATE ANTIGENS
COMPATIBLE FOR HUMAN TRANSPLANTATION**

TECHNICAL FIELD

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This invention relates generally to the fields of carbohydrate chemistry and animals engineered with a genetic knockout. It describes non-human mammals in which the enzyme $\alpha(1,3)$ galactosyl-transferase has been replaced with $\alpha(1,2)$ fucosyltransferase.

10

BACKGROUND

The acute shortage of human organs for transplantation provides a compelling need for the development of new sources of suitable tissue. An idea of considerable promise is to transplant patents with organs from non-human animals. The main challenge to overcome is rendering foreign tissue immunologically compatible with the patient being treated.

Tissue from most mammalian species would undergo hyperacute rejection when transplanted into humans. This is because human plasma contains natural antibodies against carbohydrate determinants of the animal tissue, thought to originate through prior immune stimulation by dietary antigen or mucosal microflora. Since the antibodies are pre-formed, rejection occurs within days of the transplant.

The main target for the natural antibodies mediating rejection is cell-surface oligosaccharides expressing the determinant Gal $\alpha(1,3)$ Gal (reviewed by Joziassse et al., Biochim. Biophys. Acta 1455:403, 1999). Humans, apes and Old World monkeys differ from other mammals in that they lack α -galactosyl epitopes in complex oligosaccharides. Other mammals express the Gal $\alpha(1,3)$ Gal epitope prominently on the surface of nucleated cells, including hepatic cells, renal cells, and vascular endothelium — which is especially problematic for xenotransplantation of whole organs.

The Gal $\alpha(1,3)$ Gal epitope is made by a specific enzyme, $\alpha(1,3)$ galactosyltransferase, abbreviated in this disclosure as $\alpha 1,3$ GT. The transferase uses UDP-galactose as a source of galactose, which it transfers specifically to an acceptor oligosaccharide, usually Gal $\beta(1,4)$ GlcNAc (N-acetyl lactosamine). In mammals that don't express the Gal $\alpha(1,3)$ Gal product, the $\alpha 1,3$ GT locus is inactivated (Gailili et al., Proc. Natl. Acad. Sci. USA 15:7401, 1991). There are frameshift and nonsense mutations within the locus, turning it into a non-functional, processed pseudogene (Laarsen et al., J. Biol. Chem. 265:7055, 1990; Joziassse et al., J. Biol. Chem. 266:6991, 1991).

In humans, N-acetyl lactosamine acceptor oligosaccharides are processed differently. The enzyme $\alpha(1,2)$ fucosyltransferase builds the N-acetyl lactosamine into Fuca $\alpha(1,2)$ Gal $\beta(1,4)$ GlcNAc, which is blood group H substance. This in turn serves as an acceptor substance for blood group A GlcNAc-transferase, or blood group B Gal-transferase, forming A-substance or B-substance, respectively, depending on the blood type of the individual. Naturally occurring antibodies circulating in the blood are reactive against the alternative carbohydrate determinants that are not self-antigens.

Larsen et al. (Proc. Natl. Acad. Sci. USA 86:8227, 1989) isolated and characterized a cDNA encoding murine α 1,3GT. Joziassse et al. (J. Biol. Chem. 267:5534, 1992) detected four distinct mRNA transcripts, which predict four different isoforms of the α 1,3GT. The full-length mouse mRNA (including 5' untranslated mRNA) was reported to span at least 35-kb of genomic DNA, distributed over nine exons ranging from 36 base pairs to ~2600 base pairs in length. Numbering in the 5' to 3' direction, the coding region is distributed over Exons 4 to 9. The four transcripts are formed by alternative splicing of the pre-mRNA.

Joziassse et al. (J. Biol. Chem. 264:14290, 1989) isolated and characterized a cDNA encoding bovine cDNA. The coding sequence was predicted to be a membrane-bound protein with a large glycosylated COOH-terminal domain, a transmembrane domain, and a short NH₂ terminal domain.

The porcine α 1,3GT cDNA sequence has been reported from several different laboratories: Strahan et al. (Immunogenetics 41:101, 1995); U.S. Patent 5,821,117; U.S. Patent 5,849,991; and International Patent Application WO 95/28412. The genomic organization of porcine α 1,3GT was reported by Katayama et al. (Glycoconjugate J. 15:83, 1998). Again, the coding region spans six exons, conserving the arrangement present in the mouse genome, and extending over nearly 24-kb.

It has been reported that about 95% of the naturally occurring xenospecific antibody in humans recognize the Gal α (1,3)Gal epitope (McKensie et al., Transpl. Immunol. 2:81, 1994). Antibody in human serum binds specifically to pig endothelial cells in a manner that is inhibitable by Gal α (1,3)Gal, or by Gal α (1,6)Glc (melibiose). New age monkeys have the same naturally occurring antibody, and demonstrate hyperacute rejection of pig organ xenotransplants. The rejection reaction can be obviated in experimental animals by infusing the recipient with the free carbohydrate (Ye et al., Transplantation 58:330, 1994), or by adsorbing antibody from the circulation on a column of Gal α (1,3)Gal or melibiose (Cooper et al., Xenotransplantation 3:102, 1996).

It has been suggested that xenotransplants of pig tissue could provide a source of various tissue components — heart valves, pancreatic islet cells, and perhaps large organs such as livers and kidneys (Cowley, Newsweek, Jan 1/2000). If xenotransplants from non-primates into humans is ever to become viable, then techniques need to be developed to prevent Gal α (1,3)Gal mediated rejection. Possible genetic manipulation strategies are reviewed by Gustafsson et al. (Immunol. Rev. 141:59, 1994), Sandrin et al. (Frontiers Biosci. 2:e1-11, 1997), and Lavitrano et al. (Forum Genova 9:74, 1999).

One approach is to prevent the formation of Gal α (1,3)Gal by providing another transferase that competes with α 1,3GT for the N-acetyl lactosamine acceptor. International Patent Application WO 97/12035 (Nextran-Baxter) relates to transgenic animals that express at least one enzyme that masks or reduces the level of the xenoreactive antigens by competing with α 1,3GT. The enzymes proposed are α (1,2)fucosyltransferase (that makes H antigen in humans), α (2,6)sialyltransferase, and β (1,3)N-acetylglucosaminyltransferase. It is thought that once N-acetyl lactosamine has been converted by one of these transferases, it can no longer act as an acceptor for α 1,3GT. The xenotransplantation cells of Application WO 97/12035 have at least one enzyme that reduces Gal α (1,3)Gal expression, and also express a complement inhibitor such as CD59, decay accelerating factor (DAF), or membrane cofactor protein (MCP). Expression of human CD59 in transgenic pig organs enhances organ survival in an ex vivo xenogeneic perfusion model (Kroshus et al., Transplantation 61:1513, 1996).

Another approach is to disassemble Gal α (1,3)Gal after it is formed. International Patent Application WO 95/33828 (Diacrin) suggests modifying cells for xenogeneic transplants by treating tissue with an α -glycosidase. Osman et al. (Proc. Natl. Acad. Sci. USA 23:4677, 1997) reported that combined transgenic expression of both α -glycosidase and α (1,2)fucosyltransferase leads to optimal reduction in Gal α (1,3)Gal epitope. Splenocytes from mice overexpressing human α -glycosidase showed only a 15-25% reduction in binding of natural human anti-Gal α (1,3)Gal antibodies. Mice overexpressing human α (1,2)fucosyltransferase as a transgene showed a reduction of Gal α (1,3)Gal epitopes by ~90%. Doubly transfected COS cells expressing both the glycosidase and the transferase showed negligible cell surface staining with anti-Gal α (1,3)Gal, and were not susceptible to lysis by human serum containing antibody and complement.

A further alternative is to prevent Gal α (1,3)Gal expression in the first place. Strahan et al. (Xenotransplantation 2:143, 1995) reported the use of antisense oligonucleotides for inhibiting pig α 1,3GT, leading to a partial reduction in expression of the major target for human natural antibodies on pig vascular endothelial cells. Hayashi et al. (Transplant Proc. 29:2213, 1997) reported adenovirus-mediated gene transfer of antisense ribozyme for α 1,3GT and α (1,2)fucosyltransferase genes in xenotransplantation.

U.S. Patent 5,849,991 (Bresatch) describes DNA constructs based on the mouse α 1,3GT sequence. They are designed to disrupt expression of functional α 1,3GT by undergoing homologous recombination across Exon 4, 7, 8, or 9. The constructs contain a selectable marker such as *neo^R*, *hyg^R* or thymidine kinase. It is proposed that such constructs be introduced into mouse embryonic stem (ES) cells, and recovering cells in which at least one α 1,3GT gene is inactivated. Experiments are reported which produced mice that are homozygous for inactivated α 1,3GT, resulting in lack of expression of Gal α (1,3)Gal epitope, as determined by specific antibody.

U.S. Patent 5,821,117 (Austin Research Inst.) report cDNA sequence data for porcine α 1,3GT. This was used to probe a pig genomic DNA library, and two lambda phage clones were obtained that contain different regions of the porcine transferase gene. International Patent Application WO 95/28412 (Biotransplant) also reports cDNA sequence data for porcine α 1,3GT. It is proposed that genomic DNA fragments be isolated from an isogenic DNA library, and used to develop a gene-targeting cassette including a positive or negative selectable marker.

International Patent Application WO 99/21415 (Stem Cell Sciences, Biotransplant) reports construction of a DNA library from miniature swine. A vector is obtained comprising a *pgk-neo* cassette, and fragments of the α 1,3GT gene. This is used for homologous recombination to eliminate α 1,3GT activity in porcine embryonic fibroblasts. Costa et al., Alexion Pharmaceuticals (Xenotransplantation 6:6, 1999) report experiments with transgenic mice expressing the human complement inhibitor CD59. In α 1,3GT knockout mice, the CD59 gene helped prevent human serum-mediated cytolysis. It had a similar effect in mice expressing α (1,2)fucosyltransferase. Combination of all three modifications provided no additional protective effect.

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There have been no reports of the use of $\alpha 1, 3$ GT inactivated tissue suitable for xenotransplantation into humans. In view of the paucity of available organs for human transplantation, there is a pressing need to develop further options.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed before the priority date of each claim of this application.

SUMMARY

This disclosure provides technology for generating animal tissue with carbohydrate antigens that are compatible for transplantation into human patients. The tissue is inactivated homozygously for expression of $\alpha(1,3)$ galactosyltransferase, and comprises a transgene for $\alpha(1,2)$ fucosyltransferase. As a result, cell-surface N-acetyl

lactosamine is not converted to the $\text{Gal}\alpha(1,3)\text{Gal}$ antigen - but converted to $\text{Fuca}(1,2)\text{Gal}$, which is H substance, a self-antigen in humans.

One embodiment of this invention is a mammalian cell that is homozygous for inactivation of the $\alpha 1,3$ GT gene (which means that the $\alpha 1,3$ GT enzyme is not produced from either allele, whether or not the manner of inactivation is the same on both alleles). The cell also expresses a $\alpha 1,2$ FT transgene, either by integration at a random site, or by replacing at least part of the encoding sequence in the $\alpha 1,3$ GT gene with an $\alpha 1,2$ FT encoding sequence (this means that the $\alpha 1,3$ GT encoding sequence or a portion of it is no longer expressed, whether or not it is still present in the genome). In some cases, the $\alpha 1,2$ FT encoding sequence is placed under control of the endogenous $\alpha 1,3$ GT promoter.

In a related embodiment, the cells of this invention are adapted to express glycosyl transferase enzymes capable of synthesizing either the A determinant, the B determinant, or both, of the human ABO blood group.

Another embodiment of this invention is tissue from a mammal that has been genetically modified to be devoid of antibody-detectable $\text{Gal}\alpha(1,3)\text{Gal}$ determinants that it would otherwise express. The tissue also expresses at least one ABO blood group substance, such as H substance, A substance, B substance, or any combination thereof, and may express other antigens such as Secretor substance or CD59. Another embodiment of this invention is tissue from a mammal that has been genetically

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modified to expresses $\alpha 1,2$ FT de novo, and to suppress expression of endogenous $\alpha 1,3$ GT.

A further embodiment of this invention is a non-human mammal that is homozygous for inactivation of the $\alpha 1,3$ GT gene. The mammal also expresses a $\alpha 1,2$ FT transgene, either randomly integrated into the genome, or replacing at least part of the encoding sequence in the $\alpha 1,3$ GT gene. In a related embodiment, the mammal is transgenic for the ABO blood group A-transferase, B-transferase, or both. The genetically modified cells, tissues and animals of this invention can be from any vertebrate or mammalian species, of which sheep and pigs are exemplary.

Another embodiment of the invention is a process for making genetically modified cells by nuclear transfer. At least one allele of the $\alpha 1,3$ GT gene in a donor cell is inactivated or replaced by any effective mechanism, as described later in this disclosure. The donor cell comprises a $\alpha 1,2$ FT transgene, and optionally has increased telomerase activity to facilitate multiple targeting events or other genetic manipulations in a single generation. Following genetic manipulation, the nucleus of the donor cell is transferred to a recipient cell. Certain cells produced by this method can be used to create an embryo, which can be engrafted the cell into the uterus of a surrogate host to produce a birthed animal. Tissue of this invention can be harvested from the embryo, the birthed animal, or its progeny.

This invention also provides a system for selecting a cell that has undergone genetic alteration by homologous recombination from amongst a population of cells that do not have the alteration. The successfully targeted cell is identified and separated according to surface glycosylation that has changed as a result of the homologous recombination. The recombination event may inactivate an endogenous gene, or introduce a transgene, either of which may be a carbohydrate modulating enzyme, such as a glycosyltransferase or specific glycosidase in any combination. Altering carbohydrate modulating enzymes can be done for its own sake, or as a means for tracking inactivation of other endogenous genes or insertion of other genetic elements. Exemplary reagents and separation methods for use in this system are provided later in the disclosure. This cell selection system has important advantages for producing genetically altered cell types for a variety of purposes.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

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These and other embodiments of the invention will be apparent from the description that follows.

DRAWINGS

5 **Figure 1** is a map showing location of introns in the sheep $\alpha 1,3$ GT gene. Designs are shown for targeting vectors that inactivate the sheep $\alpha 1,3$ GT gene by homologous recombination. Each targeting vector comprises a selectable marker (*neo*), flanked on one side by an intron sequence of 1-2-kb, and on the other side by an intron sequence of 7-10-kb. A number of vectors have been obtained that target Exon 4, Exon 8, or Exon 9.

10 **Figure 2** is a set of drawings that compare the details of the targeting vectors p0054, p0079, and p0063, respectively, alongside the $\alpha 1,3$ GT gene. These vectors are designed to replace the coding region in Exon 4 (which contain the translation start site) with the selectable marker *neo* or *pac*.

15 **Figure 3** is a photocopy of a gel showing PCR analysis of sheep fetal fibroblasts targeted with the p0054 vector. Using two primers for intron sequences that flank Exon 4 (upper panel), the expected product is 2.8-kb for native $\alpha 1,3$ GT, and 2.2-kb after homologous recombination. Using a primer for the selectable marker (lower panel), amplification product is expected only after recombination. The results show that one of the samples is from a fibroblast that has successfully been targeted-replacing Exon 4 with the selectable marker. Since Exon 4 contains the translation initiation site, this would inactivate the $\alpha 1,3$ GT gene.

20 **Figure 4** is a map of plasmid pGRN145, which causes cells to express telomerase reverse transcriptase (abbreviated here as hTERT), the limiting component of telomerase activity in most mammalian cells. Transcription is under control of the myeloproliferative sarcoma virus (MPSV) promoter.

25 **Figure 5** is a graph of the growth profile for primary sheep fibroblasts transduced to express telomerase reverse transcriptase. Each line is a single clone, except BW6F23, which is the parental (untransfected) fibroblast line. • = telomerase-expressing clones; π = telomerase-negative clones; $\square$ = clones that were telomerase-negative initially, but became positive later.

Figure 6 is a family of maps showing targeting vectors that replace part of the $\alpha 1,3$ GT encoding region with the full-length $\alpha 1,2$ FT sequence. The uppermost map is a modification of the p0054 vector for inactivating the sheep $\alpha 1,3$ GT. The human $\alpha 1,2$ FT sequence is flanked by sequence of the $\alpha 1,3$ GT gene that flanks the targeted region in Exon 4. The map in the center is a targeting vector for inactivating the pig $\alpha 1,3$ GT gene, replacing the beginning of the encoding region with the drug resistance marker *neo*. The map at the bottom is an adaptation of the pig vector, with the human $\alpha 1,2$ FT encoding sequence between the flanking $\alpha 1,3$ GT sequences. Sheep or pig tissue having the $\alpha 1,3$ GT inactivated on both alleles and replaced on at least one allele with $\alpha 1,2$ FT will not form the Gal $\alpha(1,3)$ Gal xenogeneic antigen. In its place, they form Fuca $\alpha(1,2)$ Gal, which is H substance, a self-antigen in humans.

Figure 7 is a reproduction of fluorescence micrographs, showing that expression of the human $\alpha 1,2$ FT gene in sheep fibroblasts does indeed cause cell-surface expression of H substance carbohydrate. DAPI and *Ulex Europaeus* (UEA) lectin staining of human 293 cells (a positive control) is compared with telomerized sheep cells of the B9 and C9 line — both of which have been lipofected with a vector containing the human $\alpha 1,2$ FT gene. As a result of transfection, the sheep cell lines now bind the UEA lectin, showing that the $\alpha 1,2$ FT has synthesized H substance.

DETAILED DESCRIPTION

The cell-surface carbohydrate antigen Gal $\alpha(1,3)$ Gal is the linchpin for hyperacute rejection of tissue xenografts from animals into humans. For this reason, considerable effort has been made to obtain animal tissue lacking $\alpha(1,3)$ galactosyltransferase ($\alpha 1,3$ GT), which is responsible for the synthesis of this determinant.

It is a hypothesis of this invention that simply inactivating $\alpha 1,3$ GT expression may be suboptimal for tissue used in xenotransplantation.

There are two reasons. First, the acceptor substance for the $\alpha 1,3$ GT enzyme is Gal $\beta(1,4)$ GlcNAc (N-acetyl lactosamine). In the absence of $\alpha 1,3$ GT, the galactose residue is presented at the terminus of the oligosaccharide branch, making it available for galectins of a corresponding specificity. The galectins constitute a family of animal lectins that have been implicated in cell adhesion and migration, tumor cell recognition, augmentation of immune defense, cytokine production, cytotoxicity, and apoptosis (H.-J. Gabius, Eur. J. Biochem 243:543, 1997; G.A. Rabinovitch, Cell Death Differ. :711, 1999). Galectin-1 and galectin-3 are expressed pathologically in colon cancer, melanoma, fibrosarcoma, lymphoma, leukemia, HTLV-1 infected T cells, and a variety of carcinomas (Perillo et al., J. Mol Med. 76:402, 1998). It is hypothesized that a graft with unusual density of terminal galactose residues may act as a magnet for inflammatory cells, or for tumor metastases.

The second reason is that N-acetyl lactosamine is itself an epitope against which humans have naturally occurring antibodies. The antibodies of this specificity are not generally a problem, because they are cold agglutinins not reactive at body temperature. However, their presence indicates that the human immune system is immunologically primed. Display of N-acetyl lactosamine in the context of other foreign antigens on pig tissue may be sufficiently immunogenic to generate antibodies against the epitope that would mediate delayed rejection.

This invention provides a better alternative: *replacing* $\alpha 1,3$ GT activity with $\alpha(1,2)$ fucosyltransferase ($\alpha 1,2$ FT) activity. The transplant tissue is homozygously $\alpha 1,3$ GT inactivated, and contains at least one copy of a $\alpha 1,2$ FT encoding region. The $\alpha 1,3$ GT product Gal $\alpha(1,3)$ Gal is not synthesized. Instead, a normal proportion of the acceptor substrate is fucosylated to H-substance —
5 rendering it inert to pathogenic activity of galectins and naturally occurring antibody in humans, since H substance is a self-antigen. The transplant tissue will have the equivalent histo blood group type O — which in terms of the ABO blood group major cross-match is the universal donor blood type.

The carbohydrate phenotype can be matched even more closely to that of the patient being treated. H substance synthesized on the tissue is in turn the precursor substance for the A and B blood
10 group transferases. If the tissue contains an expressible copy of the gene for blood group A GlcNAc-transferase, or blood group B Gal-transferase, then it will convert a proportion of the precursor to A substance or B substance respectively. By having the appropriate glycosyltransferase enzymes present in the tissue, then animal tissue for xenotransplantation can be obtained from non-catharine animals that have tissue type corresponding to blood group A, B, AB, and O.

15 This way, a xenograft can be matched exactly according to the ABO type of the patient being treated. In liver transplants, an ABO minor mismatch (such as a type O graft in a type A patient) can cause a graft-versus-host reaction, due to passenger lymphocytes in the transplanted tissue. The compositions and techniques provided in this disclosure make it possible to minimize immunological reaction either way due to mismatch of either the Gal $\alpha(1,3)$ Gal antigen, or the ABO blood group antigens.

20 A series of complex genetic modifications of this nature has not previously been achieved in a large animal species. The engineering strategy provided in this description allows the skilled artisan to make all the genetic modifications required to a particular cell line as a matter of routine experimental optimization.

A particularly efficient manner of achieving $\alpha 1,2$ FT gene replacement animals is to do the
25 manipulations on a cell line (such as primary fetal fibroblasts) suitable for nuclear transfer and cloning into an animal. The genetic modifications and selection procedures typically involve considerable proliferation of the cells. It has been discovered that increasing telomerase activity in the cells helps preserve telomere length throughout the modifications, keeping the cells in a condition suitable for nuclear transfer. In addition, telomerizing the cell appears to increase the frequency of successfully
30 targeted clones, and the effectiveness of nuclear transfer. It has been discovered that telomerase reverse transcriptase can be used from a different vertebrate species in order to achieve this result.

An established cell line of animal origin is first transfected with human telomerase reverse transcriptase (hTERT) to extend its replicative capacity, and provide these other beneficial effects. It is then targeted sequentially on each allele to inactivate or replace the $\alpha 1,3$ GT gene, selecting the cells for
35 correct targeting events. Once all genetic manipulations are complete, the nucleus of the modified cell is transferred to a suitable recipient, and an embryo is formed according to established cloning techniques. Variations and alternatives to this strategy are also effective, as described in the following sections.

Definitions

For purposes of this disclosure, the term Gal α (1,3)Gal (abbreviated GAL) refers to an oligosaccharide determinant present on endothelial cells and other cells of most non-primate mammals, for which humans have a naturally occurring antibody. The usual structure is Gal α (1,3)Gal β (1,4)GlcNAc, although other forms of Gal α (1,3)Gal specifically detectable by the naturally occurring anti Gal α (1,3)Gal in human serum of B blood type are included. Gal α (1,3)Gal is distinct from the Gal α (1,3)[Fuc α (1,2)]-Gal β (1,4)GlcNAc determinant characteristic of the human B blood type antigen.

An "antibody detectable" determinant refers to a determinant that is present in an amount and is sufficiently accessible so that it can be detected by an antibody specific for the determinant in an appropriate immunoassay — such as an agglutination reaction, optionally developed with an antiglobulin reagent, or by immunohistochemistry.

The term " α (1,3)galactosyltransferase" and the abbreviation " α 1,3GT" refer to the enzyme present in non-primate mammals that catalyzes the formation of the Gal α (1,3)Gal determinant by attaching Gal in the α (1,3) position to the Gal β (1,4)GlcNAc acceptor. α 1,3GT has the Enzyme Commission designation EC 2.4.1.124. α 1,3GT is not naturally expressed in humans, and the term does not include the galactosyltransferase that forms the human B blood group antigen.

The term " α (1,2)fucosyltransferase" and the abbreviation " α 1,2FT" refer to the enzyme present in primate mammals that catalyzes the formation of the Fuc α (1,2)Gal determinant (blood group H substance, a.k.a. ABO precursor substance) by attaching fucose in the α (1,2) position to the acceptor substrate N-acetyl lactosamine. α 1,2FT has the Enzyme Commission designation EC 2.4.1.69. It is also known as FUT1, to distinguish it from Secretor blood group α (1,2)fucosyltransferase (FUT2), and other fucosyltransferases.

An "acceptor" substance for α 1,3GT or α 1,2FT is a carbohydrate structure that can act as a substrate and become further glycosylated as a result of transferase activity. Acceptors for α 1,3GT include both Gal β (1,3)GlcNAc and Gal β (1,4)GlcNAc (Basu et al., J. Biol. Chem. 248:1700, 1973; Blake et al., J. Biol. Chem. 256:5387, 1981).

A-transferase is said to be "detectably expressed" by a cell at the mRNA level when mRNA encoding the transferase can be measured in the cell by some suitable technique, such as Northern analysis or PCR-reverse transcriptase. It may also be expressed at the protein level, as detected by a specific antibody or demonstration of the characteristic enzymatic activity. Scientists skilled in the art will recognize that some cells (such as mature red blood cells) do not express any glycosyltransferases, even though they display certain oligosaccharide determinants. Inhibition of α 1,3GT expression is only meaningful in cells capable of expressing other glycosyltransferase enzymes.

A gene is said to be "inactivated" when it is rendered incapable of transcribing a functional protein. For example, an inactivated gene may be missing necessary transcription or translation control elements, it may be lacking an essential part of the protein encoding region, or the encoding region may be placed out of phase. In another example, the gene may be interrupted by an inserted sequence, or mutated in such a way as to interfere with transcription or translation of the gene product. In a third

example, the inactivated gene may produce a translation product that has been altered in such a way that it lacks important enzymatic activity of the native gene product. A gene is also "inactivated" when the normal encoding region is switched with an encoding region for a different gene product with a different biological function.

5 In the descriptions of genetic modification and inactivation in this disclosure, it is understood that changes to the genome of a cell are inherited by progeny of the cell, unless further genetic manipulation occurs. Thus, it is possible to select the modified cells, let them proliferate, and then make a subsequent modification to the progeny. A sequence of genetic modifications made to cell and its ancestors are considered equivalent to making all the modifications to the same cell, unless explicitly directed
10 otherwise.

A cell is said to be "transfected", "genetically transformed", or "genetically altered", when the cell has been introduced with a recombinant polynucleotide, or is the progeny of such a cell. The alteration may (but need not) be integrated into the genome of the cell. Non-limiting examples include the following: 1. A cell containing a vector with a sequence encoding a protein of interest, capable of causing
15 the protein to be expressed by the cell on a transient or inheritable fashion. 2. A cell containing a genetic construct for targeting an endogenous gene (whether or not the gene has been successfully targeted). 3. A cell containing a genetic modification introduced by recombinant means.

A cell is described as "telomerized" if it has been treated to increase the expression of telomerase reverse transcriptase (TERT) and/or functional telomerase activity by any suitable means
20 beyond the level usually expressed by cells of the same type in the same environment. Methods for telomerizing cells are illustrated in a later section of this disclosure. The term also applies to progeny of the originally treated cell that have inherited the ability to express telomerase at an elevated level.

The terms "polynucleotide" and "oligonucleotide" are used interchangeably to refer to a polymeric form of nucleotides of any length. Included are genes and gene fragments, mRNA, cDNA, plasmids,
25 vectors, synthetic nucleic acids, targeting constructs, nucleic acid probes, and primers.

A "control element" or "control sequence" is a nucleotide sequence involved in an interaction of molecules that contributes to the functional regulation of a polynucleotide, such as replication, duplication, transcription, splicing, or translation. Transcriptional control elements include promoters and enhancers.

30 The term "embryo" as it is used in this disclosure refers to an organism developing in the uterus of a species of interest at any time after fertilization or intrauterine transfer, not limited to a particular developmental period. The terms "engrafting" or "transplanting", in reference to embryo manipulation, refer to any process known in the art for artificially introducing one or more embryos into the uterus of a female animal.

35 The term "tissue" refers to a heterogeneous collection of cells responsible for maintaining one or more physiological functions. Of interest for certain embodiments of this invention are organs suitable for transplantation, such as a whole kidney; however, the term also includes organ fragments and other embodiments, such as a piece of connective tissue, or a collection of cells in a medical support device.

General Techniques

In general, the practice of this invention can be carried out using standard techniques of genetic engineering, protein manipulation, and cell culture. Textbooks that describe standard laboratory techniques include the current editions of *"Molecular Cloning: A Laboratory Manual"* (Sambrook et al.);
 5 *"Animal Cell Culture"* (R.I. Freshney, ed.); the series *"Methods in Enzymology"* (Academic Press, Inc.);
"Current Protocols in Molecular Biology" (F.M. Ausubel et al., eds.); and *"Recombinant DNA Methodology II"* (R. Wu ed.). Techniques used in raising, purifying and modifying antibodies, and the design and execution of immunoassays, are described in *Handbook of Experimental Immunology* (D.M. Weir & C.C. Blackwell, eds.); *The Immunoassay Handbook* (Stockton Press NY); and *Methods of Immunological*
 10 *Analysis* (Masseyeff et al. eds., Weinheim: VCH Verlags GmbH).

Texts that describe reproductive techniques and embryo transfer in animals include *Manual of the International Embryo Transfer Society: A procedural guide and general information for the use of embryo transfer technology emphasizing sanitary procedures*, 3rd ed. (Stringfellow et al., Savoy, IL: International Embryo Transfer Society, Savoy IL); and *Embryo transfer in farm animals: A review of techniques and applications* (K.J. Betteridge, ed., Agriculture Canada Monographs No. 16, Ottawa,
 15 1977).

References for human blood group substances include *The Blood Group Antigen Factsbook* (M.E. Reid & C. Lomas-Francis, ed., Academic Press, 1997); and *Blood Cell Biochemistry : Molecular Basis of Human Blood Group Antigens* (J.P. Carton & P. Rouger, eds., Plenum Pub. Corp., 1995). The
 20 synthesis of complex oligosaccharides and carbohydrate-specific enzymes are described in *Oligosaccharides: Their Synthesis and Biological Roles* (H.M.I. Osborn & T.J. Khan, Oxford Univ. Press, 2000) and *Glycoscience: Synthesis of Oligosaccharides and Glycoconjugates* (H. Driguez & J. Thiem, Eds., Springer Verlag, 1999).

Books on the general aspects of nuclear transfer, animal cloning, and xenotransplantation
 25 include *The Second Creation : Dolly and the Age of Biological Control* (I. Wilmut et al., Farrar Straus & Giroux, 2000), and *Xeno: The Promise of Transplanting Animal Organs into Humans* (Cooper & Lanza, Oxford University Press, 2000).

Strategy for engineering tissue with xenocompatible carbohydrate antigens

30 This invention provides cells, animals, and tissues in which expression of $\alpha 1,3$ GT is eliminated by inactivating the endogenous gene, and expression of $\alpha 1,2$ FT is induced by introducing a transgene into the genome. In order to obtain complete suppression of Galc(1,3)Gal expression, the $\alpha 1,3$ GT gene needs to be inactivated on both alleles. At least one copy of an expressible $\alpha 1,2$ FT encoding region under control of a suitable promoter is also present to confer the desired phenotype.

35 A convenient method to make an animal with all these features is to perform all the genetic manipulations on a cell or cell population that is then used for cloning. To complete all the genetic manipulations, it is usually necessary to take the cell through a number of rounds of proliferation and selection. After the desired genotype has been obtained and selected, a cell selected from the

population can be grown into an embryo (if it is an embryonic cell), or used as a donor for nuclear transfer into a suitable recipient cell, which in turn is used to grow the embryo.

The process can be considerably facilitated by increasing the replicative capacity of the cell population. In particular, increasing the expression of telomerase reverse transcriptase sufficiently
5 increases replicative capacity of the nuclear donor, while minimizing risk of transformation to a malignant phenotype, and conferring other advantages described earlier.

Using this approach, genetically modified cells can be created in which the $\alpha 1,3GT$ gene is modified either simultaneously or sequentially on both alleles in the same cell line. One method for generating cells modified on both alleles is to use a single targeting vector in combination with a selection
10 process that requires double integration. The double recombination event is statistically rare, but the extended proliferative capacity of the cell population puts batch screening for such an event within the scope of routine experimentation.

Another method for generating cells modified on both alleles is to use two different targeting constructs. The constructs can each be created with different selection markers that facilitate screening
15 for double integration. For example, the cell can be targeted with a first targeting vector containing a first drug resistance gene, and selected using the corresponding drug. After a round of proliferation, the progeny can then be targeted with a second vector containing a second drug resistance gene, and selected using the second drug. In a variation of this technique, both targeting constructs are used at once, and selection of doubly modified cells is performed in a medium containing both drugs.

The desired phenotype can be obtained by inactivating both $\alpha 1,3GT$ alleles, and separately
20 introducing an $\alpha 1,2FT$ expression cassette. Alternatively, targeting vectors can be used that removes the $\alpha 1,3GT$ start codon, and inserts the $\alpha 1,2FT$ expression cassette simultaneously. The $\alpha 1,2FT$ encoding region can be provided with its own transcription control elements — or it can be placed under control of the endogenous $\alpha 1,3GT$ promoter, which may help direct expression with an appropriate tissue
25 distribution. Once all these manipulations have been made and verified, the cell can be used for cloning the embryo.

It is also possible to arrive at the desired phenotype by making some of the genetic modifications, cloning the embryo, and then making further modifications on a cell from the cloned fetus, newborn animal, adult, or subsequent progeny. For example, the $\alpha 1,3GT$ gene can be inactivated or
30 replaced with $\alpha 1,2FT$ on one allele, and an animal cloned. Cells from the clone could then be harvested for inactivating or replacing the second $\alpha 1,3GT$ allele, and recloned. A further option is to arrive at the desired phenotype by interbreeding — for example, by mating an animal with $\alpha 1,3GT$ substituted with $\alpha 1,2FT$ on one allele with another animal having a similar genotype (or with just an inactivated $\alpha 1,3GT$ allele).

35

Increasing telomerase activity in the nuclear donor

Donor cells for genetic manipulation according to this invention are typically nucleated cells of the desired species with a germ line genotype, selected to be easily maintained in culture. Exemplary are primary fibroblast cells, which are relatively easy to prepare from most species. For example, cells

are collected from sheep or pig fetuses at about 35 days of gestation, and subjected to mild trypsin/EDTA solution, then cultured in a suitable culture medium. Except where explicitly directed otherwise, the techniques of this invention can be applied to any cell type without restriction, including embryonic cells, primary cells from a fetus, offspring, or adult, and established cell lines from any vertebrate.

5 The replicative capacity of the nuclear donor cell is increased by increasing telomerase activity. This assists the cells in maintaining telomere length, thereby expanding the replicative capacity (the number of cell doublings possible before reaching the Hayflick limit and entering crisis). Typically, telomerase activity is modified before inactivation of the target gene, but such modifications are also permitted at a later stage in the procedure.

10 Increasing telomerase activity can be accomplished by a number of strategies, including but not limited to the following:

- a) genetically altering the cell with a nucleotide having an encoding region for telomerase reverse transcriptase (TERT);
- b) artificially placing TERT protein or telomerase holoenzyme into the cell;
- 15 c) altering TERT expression from the endogenous gene; or
- d) altering expression of a telomerase related protein, thereby effectively increasing telomerase activity.

A convenient method for increasing telomerase activity is to genetically alter the cells so that they express TERT, which is usually the limiting component of telomerase enzyme expression. A TERT
20 gene can be cotransfected with a gene for the telomerase RNA component, or a TERT can be selected that is compatible with the RNA component already expressed by the cell.

It has been discovered that when cells from large mammals such as sheep and pigs are genetically altered with human TERT, they express increased telomerase activity, which indicates that the hTERT gene product can combine with endogenous RNA component to create a functional enzyme.
25 It is a hypothesis of this invention that combinations of mammalian TERT into the cells of other mammals will often be effective.

The human TERT gene sequence is provided in U.S. Patent 6,166,178, which also describes the use of TERT to increase replicative capacity of various cell types. The mouse TERT sequence is provided in International Patent Application WO 99/27113. Other publications with telomerase-related
30 sequences include International Patent Application WO 98/21343 (Amgen); WO 98/37181 (Whitehead); WO 98/07838A1 (Mitsubishi); WO 99/01560 (Cambia), and U.S. Patent 5,583,016 (Geron Corp.). U.S. Patent 5,968,506 describes purified telomerase and methods for obtaining it. When TERT is referred to in this description, it is understood to mean a polypeptide comprising a TERT sequence from any mammalian, vertebrate, or other species, with or without alterations, so long as the polypeptide has
35 telomerase activity when associated with telomerase RNA component, as measured by TRAP assay (described below) in the cell line being treated.

Typically, the vector will comprise a TERT encoding region under control of a heterologous transcription control element that promotes transcription in the intended undifferentiated or differentiated cell line. Sequences that can drive expression of the TERT coding region include viral LTRs, enhancers,
40 and promoters (such as MPSV, SV40, MoLV, CMV, MSCV, HSV TK), eukaryotic promoters (such as

β-actin, ubiquitin, elongation factors exemplified by EF1α, and PGK) or combinations thereof (for example, the CMV enhancer combined with the β-actin promoter). Expression of a marker gene can optionally be driven by the same promoter that's driving the TERT gene, either as a separate expression cassette, as part of a polycistronic transcript (in which the coding regions of TERT and the marker gene are separated by an IRES sequence, allowing both individual proteins to be made from a single transcript driven by a single promoter), or as part of the same cassette (a fusion between the coding regions of both TERT and the marker gene, producing a protein that provides the functions of both TERT and the marker gene). Transfection and expression of telomerase in human cells is described in Bodnar et al., Science 279:349, 1998 and Jiang et al., Nat. Genet. 21:111, 1999.

An alternative strategy is to use a vector that substitutes or supplements the promoter in the endogenous TERT gene with a regulatory control element (such as those listed above) that increase expression in the cultured cells. Further illustration of the general strategy of replacing promoters in endogenous genes can be found in U.S. Patent 6,063,630.

When the nucleus of the telomerized cell is transferred to another cell and used to produce a cloned animal or embryo, the tissue will contain alterations to the genome of the donor cell. The presence of a recombinant TERT gene in a donor cell may have other consequences. Accordingly, it may be desirable to provide a mechanism for removing or otherwise inactivating the recombinant TERT gene once the telomeres have been elongated but before nuclear transfer.

This can be accomplished by flanking the TERT gene and/or the transcription control element on both sides with recognition sequences for a site-specific recombinase. Suitable are *lox* sites recognized by Cre recombinase (U.S. Patent 4,959,317), and *flr* sites recognized by Flp recombinase (U.S. Patent 5,929,301). Other site-specific recombinases include XerC (Becker et al., Curr. Microbiol. 32:232, 1996), XerD (Subramanya et al., EMBO J. 16:5178, 1997), xisF (Genes Dev. 8:75, 1994), and Int recombinase (Kolot et al., Mol. Biol. Reprod. 36:207, 1999; Tirumalai et al., Proc. Natl. Acad. Sci. USA 94:6104, 1997). After all the genetic modifications are made to obtain the desired cell-surface carbohydrate antigens, the cell is treated with the corresponding recombinase (or an expression vector for the recombinase) to excise the TERT cassette before nuclear transfer.

Also contemplated are vectors in which a particular gene (such as a selectable marker) is flanked by one type of recombinase recognition site, and the TERT gene or control element is flanked with another type of recognition site. An example is the following:

5'arm – *loxP* – *flr* – *neoP*A – *flr* – pGK promoter – hTERTpA – *LoxP* – 3'arm

This allows the drug resistance marker (*neo*) to be removed from the line after selection using the first recombinase (Flp), while retaining TERT. Further genetic manipulation can then be performed — for example, targeting the other allele of the same gene, possibly using the same vector and selecting for *neo* again. After all manipulation is complete, the TERT encoding region can be removed using the second recombinase (Cre).

Another way of obtaining cells with genomic modifications that do not include TERT is to increase telomerase activity without integrating a TERT gene into the genome. For example, TERT can be transiently expressed using a suitable expression system such as adenovirus, or by introducing TERT protein (or the telomerase holoenzyme) directly into the cell. The TERT will be diluted out as the cell divides, but extension of telomeres in the parent cell should increase replicative capacity of the cell line by several doublings.

Another alternative is to upregulate TERT expression from the endogenous gene by upregulating expression of trans-activating transcriptional regulators. The TERT promoter contains a number of regulator recognition sequences, such as c-Myc, SP1, SRY, HNF-3 β , HNF-5, TFIIID-MBP, E2F and c-Myb. See International Patent Publication WO 00/46355.

A further alternative is not to increase TERT expression, but enhance the effective activity of telomerase already present in the cell. This can be done in cells that have an endogenous level of TERT expression, such as in bone marrow progenitor cells and gonadal tissue. For example, TRF1 and TRF2 are proteins that bind to telomere repeats and regulate access of telomerase (Smogorzewska et al., Mol. Cell Biol. 20:1659, 2000). Decreasing expression of such factors may enhance the ability of telomerase to increase telomere length, thereby increasing replicative capacity of the cell.

Evidence of increased telomerase expression can be obtained by a variety of techniques, including but not limited to determining gene transcript levels (for example, by Northern or RT-PCR analysis), protein expression (for example, by immunocytochemistry), or telomerase activity (for example, by primer extension assay). Extended lifespan or replicative capacity of the treated cells, while often desirable, need not be positively demonstrated for the invention to be put into practice, except where explicitly required.

Telomerase activity can be determined, for example, by TRAP assay (Kim et al., Science 266:2011, 1997; Weinrich et al., Nature Genetics 17:498, 1997), or other suitable technique (e.g., U.S. Patent 5,741,677). Evaluation of hTERT expression by RT-PCR or immunoassay can be done by standard methods, using the sequences disclosed in U.S. Patent 6,166,178. The following assay kits are available commercially for research purposes: TRAPeze® XK Telomerase Detection Kit (Cat. s7707; Intergen Co., Purchase NY); TeloTAGGG Telomerase PCR ELISApplus (Cat. 2,013,89; Roche Diagnostics, Indianapolis IN); and LightCycler TeloTAGGG hTERT quantification kit (Cat. 3,012,344).

The cells can also be characterized as to their replicative capacity by passaging cells and monitoring the number of cell doublings. Unmodified fetal fibroblasts will typically grow through a number of doublings until they reach the Hayflick limit, and then enter into senescence. As illustrated in Figure 5, cells may grow indefinitely if TERT continues to be expressed.

Modifying the galactosyltransferase gene

Once the cell line has been obtained and established in culture, genetic manipulations can be performed to: a) eliminate expression of the endogenous α 1,3GT gene; and b) provide for expression of α 1,2FT to fucosylate the same N-acetyl lactosamine acceptor.

There is a variety of ways the endogenous $\alpha 1,3$ GT gene can be inactivated. For example, a control element that regulates transcription (such as a promoter or transcription start sequence) can be altered or deleted. Alternatively, the gene can be adapted so that any gene product that is produced lacks the essential features of a glycosyltransferase. The encoding region can be interrupted with stop
5 codons, the encoding region can be placed out-of-phase, or critical portions of the protein may be missing, such as a structural component or a signal peptide for secretion. In another alternative, the gene can be adapted so that the protein product lacks the specificity of $\alpha 1,3$ GT — either because the catalytic site is removed, or because substrate binding specificity has been sufficiently altered so that the enzyme is incapable of synthesizing the Gal $\alpha 1,3$ Gal linkage.

10 The $\alpha 1,3$ GT gene can be targeted by homologous recombination, using a vector comprising nucleotide sequence identical or nearly identical to a portion of the gene of interest, linked to another structure capable of introducing the alteration. Such vectors typically have two regions flanking a region of the genome intended for deletion. Between the flanking regions, there is often an additional segment that becomes inserted in the gene in place of the region that is excised.

15 The insert region can include a selectable marker, so that targeted cells can rapidly be separated from untargeted cells. U.S. Patent 5,614,396 describes a method for obtaining a cell containing a desired sequence in the cell's genome, by using a targeting vector having two regions homologous to the targeting sequence, flanking a sequence that is to be inserted, and having a selectable marker. The DNA undergoes homologous recombination at the target site, and recombined cells are recovered under
20 selective culture conditions.

Positive selection markers include the *neo* gene, selectable using G418 or kanamycin; the *hyg* gene, selectable using hygromycin; the *pac* gene, selectable using puromycin; the *gpt* gene, selectable using xanthine; and hypoxanthine-phosphoribosyltransferase (HPRT), selectable using hypoxanthine. Negative selection markers include thymidine kinase (*tk*), selectable using acyclovir or ganciclovir; HPRT,
25 selectable using 6-thioguanine; and cytosine deaminase, selectable using 5-fluoro-cytosine. Markers can also have an intrinsic label, like green fluorescent protein or β -galactosidase, which permit clones of targeted cells to be identified and selected.

Further methodology for homologous recombination is described in the published literature. U.S. Patents 5,464,764 and 5,631,153 provide a double-selection strategy, in which two sequences
30 homologous to the gene target flank a positive selection marker, and a negative selection marker is attached to the 3' terminal of the second flanking region. Homologous integration retains the positive selection marker, but eliminates the negative selection marker, whereas random integration usually retains both markers. Thus, by screening for both markers sequentially or together, cells that have been correctly targeted will be positively selected, and those that have been incorrectly targeted are selected
35 out. U.S. Patent 5,789,215 reports the use of homologous recombinant targeting vectors for modifying the cell genome of mouse embryonic stem cells. See also U.S. Patents 5,589,369 and 5,776,774.

Example 1 illustrates targeting vectors that are capable of inactivating the sheep $\alpha 1,3$ GT gene (SEQ. ID NOs:3 & 4) via homologous recombination. Vectors p0054, p0079, and p0063 (Example 1,
40 Figure 2) are targeted to eliminate Exon 4, which contains the $\alpha 1,3$ GT translation start codon. Other vectors have been obtained that target Exon 8 or Exon 9, which is thought to encode at least part of the

α 1,3GT catalytic site. The α 1,3GT gene in other species can be targeted in a similar fashion, using probes having flanking sequence for the α 1,3GT of that species. The bovine and porcine α 1,3GT cDNA sequences are provided in SEQ. ID NOs:5-8.

5 The vectors comprise flanking regions identical to the targeted α 1,3GT sequence, one side being about 1 kb, the other being at least 1 or 2 kb, in either order. In between the flanking regions is a selectable marker such as *neo*, designed to replace one of the Exons in the α 1,3GT coding sequence. The selectable marker genes are not provided with their own promoter, and require continued translation through the upstream α 1,3GT sequence in order to be expressed. This helps the marker select for properly integrated vector, because vector inserted at a random site will probably not link the marker
10 gene to a suitable promoter, and resistance to the selector drug will not be conferred. In cells that normally express a high level of α 1,3GT and the Gal α (1,3)Gal epitope, the α 1,3GT promoter will be highly active and the drug resistance marker will be strongly expressed. Thus, a higher concentration of selector drug can be used to select out cells that have the vector inserted elsewhere.

The insert region of the targeting vector can also contain an encoding region for α 1,2FT. This
15 way, inactivation of the endogenous α 1,3GT gene and integration of the α 1,2FT will occur simultaneously with a successful targeting event. The α 1,2FT gene can be placed in the targeting vector linked to its own promoter, or the targeting vector can be constructed in such a way that the α 1,2FT gene will be placed under control of the endogenous α 1,3GT promoter once integrated.

Larsen et al. (Proc. Natl. Acad. Sci. USA 86:8227, 1989) describe the molecular cloning,
20 sequence, and expression of human GDP-L-fucose : β -D-galactoside 2- α -L-fucosyltransferase cDNA that can form the H blood group antigen (i.e., α 1,2FT). The nucleic acid sequence and encoded protein sequence is shown in SEQ. ID NOs:9 and 10. Wagner, et al. (Transfusion 37:284, 1997) provide allotypic variants of the human α 1,2FT. Apoil et al. (Mol. Biol. Evol. 17:337, 2000) describe evolution of the α 1,2FT gene in primates, and provide encoding sequences from *Gorilla gorilla*, *Pan troglodytes*,
25 *Macaca mulatta*, and other higher primates. Any fucosyltransferase can be used if it converts the N-acetyl lactosamine acceptor to a determinant that is immunologically equivalent to H substance.

Vectors for replacing the α 1,3GT encoding region in sheep and pigs with the human α 1,2FT are shown in Figure 6 (Example 4). The target cells are contacted with the targeting vector in such a manner that the vector gets entry into the cell nucleus and effects the intended change. Any suitable method of
30 transfection can be used, such as electroporation and lipofection. The vector can also be truncated for insertion into a viral particle (such as an adenovirus vector) that can then be used to transduce the cells. Examples 2 and 5 illustrate the use of targeting constructs on sheep fibroblast cultures suitable for nuclear transfer.

As an alternative to homologous recombination, a target gene can be inactivated using triplex-
35 forming oligonucleotides that induce intrachromosomal gene conversion (Luo et al., Proc. Natl. Acad. Sci. USA 97:9003, 2000; Barre et al., Proc. Natl. Acad. Sci. USA 97:3084, 2000). Other techniques and reagents can be found in Inonue et al., J. Virol. 73:7376, 1999; Cole-Strauss et al., Science 273:1386, 1996; Hasty et al., Mol. Cell Biol. 11: 4509, 1991; and International Patent Publication WO 98/48005.

In instances where the α 1,3GT gene has been inactivated without integrating an α 1,2FT
40 encoding region into the genome, a separate manipulation is required to confer the full phenotype. A

vector is made for introducing the $\alpha 1,2$ FT region, typically under control of a promoter that is active in an appropriate tissue distribution, such as the native $\alpha 1,2$ FT promoter, or one of the model promoters listed earlier. The expression cassette can then be integrated into the genome at any location by any appropriate technique, such as homologous recombination, or transduction with a retroviral or DNA viral vector.

Selection and characterization of targeted cells

Each genetic manipulation can be selected and verified according to genotypic and phenotypic markers.

Where cells have been transfected with a vector bearing a drug resistance marker, successfully targeted clones can be selected by culturing in a medium containing the corresponding drug. Modification of one or both alleles can be confirmed by PCR of genomic DNA, using primers from flanking endogenous $\alpha 1,3$ GT sequence, showing that the segment length has changed, or using a primer for the inserted sequence in combination with a $\alpha 1,3$ GT primer, showing that the sequence is integrated in the correct region. Southern analysis using probes for flanking endogenous $\alpha 1,3$ GT sequence will show altered restriction analysis, and probes for the inserted sequence will confirm the presence and orientation of the insert. In-situ hybridization of genomic DNA can be used to verify the correct location of the modification.

Targeted clones can also be selected and verified based on gene transcripts and the resulting cell phenotype. mRNA can be characterized by Northern analysis or RT-PCR. Cells where the $\alpha 1,3$ GT gene has been inactivated on both alleles will not express the Gal $\alpha(1,3)$ Gal epitope. The determinant can be identified using a specific antibody or lectin. Purified antibody can be obtained from pooled human serum by adsorbing on an affinity column of Synsorb™ 115 (ChemBioMed, Alberta, Canada) or D(+) melibiose (Sigma). An alternative is the "IB4" lectin from *Bandeiraea (Griffonia) simplicifolia* (Sigma Cat. L 3019) which is specific for α -D-galactosyl residues (Hayes et al., J Biol. Chem. 25:1904, 1976), and binds both the Gal $\alpha(1,3)$ Gal epitope, and B blood group substance.

Antibody to Gal $\alpha(1,3)$ Gal can be used to select for homozygous knockouts by complement lysis. Targeted cells are combined with a source of the antibody (such as human serum), and a source of complement, (such as fresh plasma from the same species as the cells, or commercially available guinea pig complement). The mixture is incubated at 37°C for a sufficient period to lyse cells expressing Gal $\alpha(1,3)$ Gal (or halt their growth), using untargeted cells as a control. Surviving cells should have $\alpha 1,3$ GT inactivated on both alleles. Specific antibody or lectin can also be used to isolate homozygous knockout cells by affinity techniques, such as panning, affinity adsorption, or fluorescent-activated cell sorting.

Incorporation and expression of the $\alpha 1,2$ FT encoding region can be determined using antibody or lectin specific for H substance. *Ulex Europaeus* agglutinin I (UEA-1) is a lectin with affinity for the terminal L-fucose on H substance (Matsumoto et al., Biochim. Biophys. Acta 194:180, 1969). The lectin can be used for immunoseparation of cells in which $\alpha 1,2$ FT is active. Most mature pig and sheep cells do not normally bind UEA-1 (Spencer et al., J. Histochem. Cytochem. 40:1937, 1992; K.J. Fahey, Aust. J.

Exp. Biol. Med. Sci. 58:557, 1980). UEA-1 is available from Sigma Chemical Co. or Vector Labs in purified form, labeled with fluorescein or biotin, or insolubilized on beaded agarose. Conrad-Lapostolle et al. (Cell. Biol. Toxicol. 12:189, 1996) describe optimization of UEA-1 magnetic beads for endothelial cell isolation. UEA-1 labeled with a fluorescent tag can be used to separate targeted cells by fluorescence-activated cell sorting.

Using specific antibody and lectin in appropriate combination, it is possible to select cells with the full α 1,3GT negative, α 1,2FT positive phenotype without having drug resistance labels in the targeting vectors. For example, the cells can be targeted with a vector that substitutes α 1,2FT for α 1,3GT on one allele, and selected by positive adsorption to UEA-1. The selected cells are expanded, and then targeted with a similar vector to knock out the second allele. The targeted cells are then subject to complement lysis using antibody to the Gal α (1,3)Gal determinant in human serum, or depleted of cells binding IB4 lectin. Surviving cells are again expanded, cloned, and analyzed for correct genotype, expression of α 1,2FT mRNA, lack of expression of α 1,3GT mRNA, and correct expression of cell surface oligosaccharide determinants. Of course, drug selection, affinity selection, and selection by other criteria can be combined in any effective combination to obtain the phenotype desired.

ABO transferases and other transgenes

Group H substance formed by α 1,2FT in turn can be used as an acceptor for the human ABO histo blood group transferases, to create the allotypic markers characteristic of a particular ABO blood type. Blood group A-transferase adds GalNAc to the Gal residue on Fuca(1,2)Gal-GlcNAc, to form GalNAc α (1,3)[Fuca(1,2)]Gal-GlcNAc (A substance). Blood group B-transferase adds Gal instead to form Gal α (1,3)[Fuca(1,2)]Gal-GlcNAc (B substance).

Certain embodiments of the invention provide animal tissue that not only expresses H substance, but also has at least some of it converted to A or B substance. To provide tissue of the human A blood group, the cells express blood group A-transferase in one or more copies. To provide tissue of the human B blood group, the cells express blood group B-transferase. To provide tissue of the human AB blood group, the cells express both A-transferase and B-transferase.

The nucleotide and protein sequence of human A-transferase and B-transferase are provided in SEQ. ID NOs:11-14. See also U.S. Patents 5,068,191 and 5,326,857. ABO transferase enzymes of other primate species can be found in Sumiyama et al., Gene 259:75, 2000. The transferase encoding sequence is placed in a vector suitable for introducing a transgene into the cell, for example by homologous recombination or retrovirus transduction. The sequence is linked to transcription control elements that promote expression in the appropriate cell types, such as the homologous transferase promoter, the α 1,3GT promoter, or the α 1,2FT promoter. Cells are then selected for successful targeting, and characterized according to whether they express A or B substance (for example, using antibody from human blood group B or A serum, respectively).

If desired, the cells can be adapted with other genetic modifications to enhance its suitability for the ultimate purpose. Xenocompatibility can be enhanced by increasing expression of complement inhibitor such as CD59, DAF or MCP (International Patent Application WO 97/12035). It is also believed

that tissues or organs containing cells that are genetically modified to render them incapable of expressing CD40 antigen have lower risk of chronic xenograft rejection (International Patent Application WO 00/39294). Other xenogeneic antigens, such as that identified in WO 00/57912 or histocompatibility markers, can also be deleted or humanized to increase immunocompatibility.

5

Carbohydrate tags as a selection system for homologous recombination

Surface carbohydrate-based cell selection (as described above and illustrated in Example 5) was conceived and developed as a general system for identifying targeted homologous recombination events in any context. The advantages of using carbohydrate determinants as selection markers are multiple:

- 10 • A change in carbohydrate determinant can be generated *either* by introduction of a new carbohydrate modulating enzyme, *or* by inactivation of carbohydrate modulating enzyme expressed from an endogenous gene. Where a new transgene is introduced into the cell, it can be a close homolog to a naturally occurring enzyme, thereby minimizing antigenic complications from the protein itself.
- 15 • The strategy of sorting by inactivation of an endogenous carbohydrate modifying enzyme is particularly attractive, because the change is effected only by integration of the vector into the correct locus. This is important, because the frequency of integration into random (non-target) sites can be 10-fold higher than the frequency of correct homologous recombination.
- 20 • Many glycosyltransferases (such as those that create the blood group antigens) are expressed in a wide variety of cell types from the endogenous promoter (Ravn et al., APMIS 108:1, 2000), meaning that inactivation screening can be designed that is not cell-type restricted.
- A wide variety of lectins is available for robust separation of the cells either by adsorption or fluorescent tagging techniques. Elution of the selected cells can be accomplished under gentle conditions using carbohydrate competitor ligand.
- 25 • Since cell-surface carbohydrate determinants are modulated by enzymes such as a glycosyltransferase or a glycosidase, the *density* of surface determinants is catalytically (not stoichiometrically) related to the amount of protein translation. Increased antigen density may enhance the likelihood of successful antibody or lectin-based separation.

30 Negative selection according to this system can be accomplished by designing the targeting vector to interrupt the encoding sequence for the target carbohydrate modulating enzyme with a sequence that prevents transcription or translation of the functional gene product. The targeting vector may optionally include other elements to be introduced into the target site, such as a transgene or recognition sequence for a site-specific recombinase.

35 Positive selection according to this system can be accomplished by including in the targeting vector a glycosyltransferase or glycosidase that is not endogenous to the species or phenotype of the cell being targeted. After the targeting reaction, cells are selected for the determinant created by the encoded modulating enzyme, and then checked for proper integration of the vector. Putting the modulating enzyme into the cell need not be the ultimate objective of targeting, it can just piggyback on the vector as a way of following the reaction. The ultimate objective of targeting could be to inactivate an endogenous

gene (which may but need not encode another carbohydrate modulating enzyme). It could also be to introduce another genetic element into a particular locus — such as a different transgene (powered by a different promoter or separated from the enzyme encoding region by an IRES sequence), or a recognition sequence for a site-specific recombinase. Where the enzyme encoding region is present in the targeting
 5 vector only for use as selection tag, it can optionally be removed after the targeted cell line is established — for example, by site specific recombination.

Carbohydrate binding means that detect modulations in carbohydrate determinants include specific antibody (anti-A, present in blood group B serum; anti-B, present in blood group A serum; and anti-Gal α (1,3)Gal, present in virtually all human serum). Also included are specific lectins. The following
 10 are available commercially: H-specific lectins from *Anguilla anguilla*, *Tetragonolobus pupureas*, and *Ulex europaeus* UEA-1, which of course also binds Gal α (1,3)Gal. A-specific lectins from *Helix pomatia*, *Dolichos biflori*, *Helix aspersa*, *Phaseolus limensis*, and *Bandeiraea simplicifolia* (IA4). B-specific lectin: *Ptilota plumosa*, and *Bandeiraea simplicifolia* (IB4).

Methods of separation can involve adhering the antibody or lectin to a solid surface, contacting
 15 the surface with the cells, and collecting cells that adhere or do not adhere to the solid surface. Other methods of separation involve conjugating the antibody or lectin with a fluorescent, phosphorescent, or other labeling means, contacting the cells with the labeled tag, and then separating tagged from non-tagged cells, for example, in a fluorescence-activated cell sorter. Another separation method involves using antibody to bind the distinguishing determinant on the cells, thereby opsonizing them for
 20 complement-mediated lysis.

An illustrative example of the use of this system is the modification of pluripotent stem cells, such as human embryonic stem cells or germ cells (U.S. Patents 6,200,806 and 6,331,406; International Patent Publications WO 99/20741 and WO 01/51616) or their derivatives (WO 01/81549; WO 01/88104). The cells are first genotyped for ABO blood group by PCR amplification (Lee et al., Forensic Sci. Int.
 25 82:227, 1996). Cells that are AO or BO genotype have the advantage that knocking out the single enzymatically active allele will change the surface phenotype of the cell.

The ABO locus is then targeted with a vector containing a genetic element to be introduced into the genome of the cell, flanked on either side by portions of the A- or B-transferase genomic sequence. Cells that are successfully targeted are separated by their ability to bind the *Helix pomatia* lectin for
 30 A-substance, or the IB4 lectin for B-substance. Accuracy of the targeting can be confirmed, for example, by PCR amplification or Southern analysis of genomic DNA. An advantage of this strategy is not only the effective selection of the targeted cells, but the fact that the ABO blood group enzyme is inactivated as a consequence — giving the cells the blood group O phenotype, which makes them universal donor cells with respect to ABO blood group.

In a variation of this example, positive rather than negative selection is used to follow gene targeting. Cells are selected that have the OO genotype, and targeted at the ABO locus by a vector that introduces A- or B-transferase flanked on each side by site-specific recombinase recognition sequences. Cells are positively selected for binding to *Helix pomatia* or IB4 lectin. Then the cells are transfected to transiently express the corresponding recombinase enzyme. As a result, the active transferase is
 40 excised, and the cells revert to the blood group O phenotype, leaving a single recombinase recognition

sequence in the locus. This then can be used to introduce a variety of transgenes into the line by site-specific recombination. It is a theory of this invention that since ABO blood determinants are expressed on most nucleated cells, this site will facilitate stable expression of the transgene — since it may be immune to inactivation that might occur elsewhere in the genome as the cells proliferate and proceed down the differentiation pathway.

A second illustration of the carbohydrate mediated selection system is the use of fluorescently labeled lectins and single-cell sorting to rapidly obtain cells in which the α 1,3GT gene has been replaced with α 1,2FT.

Early passage animal cells are transfected by electroporation with 10 μ g NotI linearized p0090 (a promoterless vector that targets the α 1,3GT locus and inserts α 1,2FT; Example 4). Only cells that integrate the α 1,2FT sequences downstream and in-frame to an active promoter will express α 1,2FT protein, and hence present H substance on the cell surface. Following transfection, a period of 24 hours of growth in complete medium is sufficient to allow expression of α 1,2FT and synthesis of H substance on the cell surface (Figure 7).

Cells expressing α 1,2FT and synthesizing H substance are isolated by their ability to bind UEA-1 lectin as follows. The transfected culture is washed with HEPES buffer (0.15 M NaCl, 0.01 M HEPES, pH 7.5) and then incubated for 30 min with rhodamine conjugated UEA-1 lectin (Vector Labs), diluted 1:50 in HEPES buffer. Excess lectin is removed by three washes with HEPES buffer. Cells binding the lectin are separated by FACS analysis (Becton Dickinson) such that individual fluorescent cells deposited into single wells of a 96-well plate — thereby avoiding cultures that contain mixed populations of targeted and non-targeted cells. As an alternative to the single-cell sorting technique, the targeted cell population can be seeded at a density of ~50 cells in a 10 cm dish. After growth and expansion, the resulting colonies (>100 cells per colony) are ring-cloned and deposited to 96-well plates for DNA analysis.

The cells are allowed to proliferate in complete growth medium until cultures are subconfluent. At this time, the cells are replica plated; one plate for cryopreservation and later recovery, and the other plate for DNA analysis by PCR. Wild type and targeted α 1,3GT alleles are detected using sense (399010, 5'-CAGCTGTGTG GGTATGGGAG GG-3'; SEQ. ID NO:27) and antisense (499006, 5'-CTGAACTGAA TGTTTATCCA GGCCATC-3'; SEQ. ID NO:28) PCR primers, yielding products of 3.0-kb and 2.4-kb, respectively. A second PCR screen with primers 399010 (SEQ. ID NO:27) and 399111 (5'-TGACGATGGC TCCGGAGCCA CAT-3'; SEQ. ID NO:40) produces a fragment of 1.7-kb only in clones that are correctly targeted. Successful targeting can be confirmed by Southern blot analysis.

The genetically altered cells can then be used for nuclear transfer, establishing additional cell lines, or for any other desirable purpose.

Nuclear transfer

Cells that have been successfully targeted and selected according to this invention can be used as nuclear donors by transferring into an enucleated recipient cell.

Suitable recipient cells include oocytes or any other pluripotent cell that is capable of developing into a fertile embryo after transfer and activation. International Patent Application WO 97/07669 (Roslin Institute) describes quiescent cell populations for nuclear transfer. International Patent Application WO 97/07668 (Roslin Institute) describes inactivated oocytes as cytoplasm recipients for nuclear transfer.

Nuclear transfer methods are particularly effective if the nucleus of the donor cell is quiescent, which can be achieved by culturing the donor cell in a serum-free medium (WO 97/07669). In an exemplary method, the nucleus of a donor cell is transferred into an oocyte that is arrested in the metaphase of the second meiotic division, and subsequently activating the reconstituted cell. Briefly, unfertilized metaphase II oocytes are collected as follows: Female animals are synchronized using progestagen sponges for -14 days, and induced to superovulate with single injections of follicle-stimulating hormone on two successive days. Ovulation is induced or synchronized with a suitable dose of gonadotrophin-releasing hormone or an analog thereof (e.g., ~8 mg GnRH Receptal™, Hoechst, UK) on the following day. The oocytes are recovered by flushing from the oviduct one day later, washed, and enucleated by treating with cytochalasin B and aspirating the nucleus using a glass pipette. Enucleated oocytes are then placed into contact with a single cell that acts as the nucleus donor.

Fusion of the donor nucleus into the enucleated recipient cell is effected by placing the couplet in a fusion chamber and aligning it between the electrodes. Electrical pulses are then applied to induce fusion, typically a low-voltage AC pulse for several seconds, followed by a plurality of very short high voltage DC pulses. Following an incubation period, activation is induced by application of an additional electrical pulse. The reconstructed zygote is then cultured for a time before engrafting into a surrogate female. Further details and alternative procedures are described in the patent publications cited above.

Estrus in the surrogate female is typically synchronized artificially using a suitable combination of inducing agents. Cameron et al. (Aust. Vet. J. 66:314, 1989) discuss synchronization methods and other practical aspects for commercial embryo transfer in pigs. Blum-Reckow et al. (J. Anim. Sci. 69:3335, 1991) report experiments relating to transfer of pig embryos after long-term *In vitro* culture. Replacing medium every 12 h during culture improved survival, and pregnancy rate improved if the sexual cycle of recipients was 24 h behind that of the donor.

The embryos are introduced into the uterus of the recipient female using any suitable technique, including devices adapted for the purpose, or appropriate surgical methods. For example, U.S. Patent 4,326,505 describes surgical procedures for embryo transplants in animals, in which the uterine horn is positioned in the peritoneal cavity

5 proximate to the vaginal wall, a cannula is inserted through the vaginal wall and into the uterine horn, and the embryo is introduced through the cannula. Non-surgical methods include using a suitable device to manipulate the injection port through the folds of the cervix to the bifurcation of the uterus. For example, devices and techniques for porcine non-surgical embryo transfer are reported by Li et al. (J. Anim. Sci.

10 74:2263, 1996). Wallenhorst et al. (J. Anim. Sci. 77:2327, 1999) describe the effect of transferring pig embryos to different uterine sites.

Preparation and use of tissue expressing ABO blood group determinants

Once an animal has been obtained that has the desired genetic alterations, tissue can be harvested and characterized.

5 The genomic features of the $\alpha 1,3\text{GT}$ locus, and expression of $\alpha 1,3\text{GT}$ or $\alpha 1,2\text{FT}$ transcripts can be verified using criteria already described. Density of $\text{Gal}\alpha(1,3)\text{Gal}$ and H substance on the cell surface can be determined using specific antibody or lectin in immunocytochemistry or fluorescence labeled flow quantitation methods. Susceptibility of the cells to complement lysis can be determined as follows. Tissue cells from the animal are suspended and labeled with ^{51}Cr . The labeled targets are combined with diluted human serum as a source of both antibody and complement, and then incubated for several
10 hours at 37°C . Release of the ^{51}Cr label correlates with density of $\text{Gal}\alpha(1,3)\text{Gal}$ on the surface of the target cells. For further details of assays for $\alpha 1,3\text{GT}$ inactivation and $\text{Gal}\alpha(1,3)\text{Gal}$ determination, the reader can consult U.S. Patent 5,849,991.

If the animal is confirmed to be $\alpha 1,3\text{GT}$ negative and $\alpha 1,2\text{FT}$ positive, it can be used for investigational purposes, or as a source of any tissue type that is desired for xenotransplantation.
15 Possible harvest tissue includes but is not limited to whole organs, such as kidney, liver, heart, lung, eyes, and pancreas; solid tissue, such as skin, cartilage, pancreatic islets, and vasculature of various types; and cell suspensions, such as progenitor cells for regeneration of neural tissue, hematopoietic tissue, hepatocytes, or other cell types.

If the animal has the $\alpha 1,3\text{GT}$ gene replaced with $\alpha 1,2\text{FT}$ on both chromosomes, the phenotype
20 should breed true. However, if there is only one $\alpha 1,2\text{FT}$ gene, then of course it will segregate in the progeny of the cloned animal according to mendelian genetics. The $\alpha 1,2\text{FT}$ positive phenotype can be maintained by testing the phenotype of each offspring, in combination with an appropriate cross-breeding strategy. Alternatively, the $\alpha 1,3\text{GT}$ negative $\alpha 1,2\text{FT}$ positive parent can be cloned as needed to provide the required amount of tissues and organs for research and commercial use.

25 Cells and tissue harvested from $\alpha 1,3\text{GT}$ inactivated, $\alpha 1,2\text{FT}$ expressing tissue can be tested for compatibility according to standard protocols. Antigen expression can be determined by immunocytochemistry, using the IB4 lectin or antibody obtained from human serum. Compatibility with the potential recipient is assessed in part using recipient's serum to test the tissue for cytochemical staining, or in a cytotoxicity assay. Xenotransplantation can be modeled in non-human animals that do
30 not normally express the $\text{Gal}\alpha(1,3)\text{Gal}$ antigen, including GAL knockout mice (see Gock et al., Xenotransplantation 7:237, 2000) or catharine non-human primates.

*The following examples provided as further non-limiting illustrations
of particular embodiments of the invention.*

35

EXAMPLESExample 1: Construction of vectors for inactivating galactosyltransferase

This example describes vectors that inactivate the $\alpha(1,3)$ galactosyltransferase ($\alpha(1,3)$ GT) gene by
 5 homologous recombination.

The sequence of the sheep cDNA for $\alpha(1,3)$ GT is shown in SEQ. ID NOs:3 & 4. To develop genomic constructs, DNA was isolated from Black Welsh Mountain fetal fibroblasts, and a λ DASHII phage library was constructed. Sau3A partially digested genomic DNA was dephosphorylated and ligated to compatible BamHI vector arms (Stratagene). The ligation products were packaged to produce
 10 recombinant phage, which were propagated on *spi* selective XL1-Blue-MRA(P2) bacterial cells. The resulting library had a complexity of 1.4×10^6 recombinants, and was subsequently amplified once. Six phage clones were isolated that spanned Exon-4, Exon-6-7 and Exon-9.

Recombinant phage designated B, C and G, have been deposited as a pooled sample with the National Collections of Industrial and Marine Bacteria Limited (NCIMB), Aberdeen, U.K, under Accession
 15 No. NCIMB 41056. The phage can be separated using the oligonucleotide probes 5'-GGGAGGAAGC GAAGGTGCA-3' (SEQ. ID NO:15), 5'-CTTGATGGGT TTATCCAGAA CA-3' (SEQ. ID NO:16) and 5'-TGATAATCCC AGCAGTATTC-3' (SEQ. ID NO:17), respectively. Each recombinant phage has also been deposited separately with the NCIMB under the following Accession numbers: Clone B, No. 41059; Clone C, No. 41060; and Clone G, No. 41061.

20 **Figure 1** maps the sequenced intron regions of $\alpha(1,3)$ GT to their positions in the gene. Exon 4 contains the translation start codon. Also shown are designs for exemplary targeting vectors that disrupt gene expression by excising Exons 4 and 9 by homologous recombination.

Several recombinant vectors were constructed for targeting Exon 4 of the sheep $\alpha(1,3)$ GT gene. The vector comprises two regions that are complementary to genomic sequence; a 1.2-kb 5' arm, which
 25 includes sequence from Intron 3 leading up to and including the start codon in Exon 4, and a ~9-kb 3' arm that initiates 1.5-kb into Intron 4, continuing to Intron 5. Separating these regions is *neo^R*-polyA sequence. After homologous recombination, the vector confers neomycin phosphotransferase resistance to the cells and deletes 1.5-kb of genomic sequence, including the first coding exon of $\alpha(1,3)$ GT gene. The entire cassette was cloned into pBlueScript™ for propagation in DH5 α bacterial cells.

30 **Figure 2, Top Panel**, shows the vector designated p0054. It was constructed by amplifying a truncated left arm (300bp, includes EcoRI site) (using primers 199001, 5'-ACGTGGCTCC AAGAATTCTC CAGGCAAGAG TACTGG-3', SEQ. ID NO:18; and 199006, 5'-CATCTTGTTT AATGGCCGAT CCCATTATTT TCTCCTGGGA AAAGAAAAG-3', with tail complementary to the start of *neo* coding sequence, SEQ. ID NO:19), and a *neo*-polyA sequence obtained from Stratagene (using primers 199005,
 35 5'-CTTTTCTTTT CCCAGGAGAA AATAATGGGA TCGGCCATTG AACAGATG-3', SEQ. ID NO:20, with tail complementary to left arm; and 199004, 5'-CAGGTCGACG GATCCGAACA AAC-3', SEQ. ID NO:21). These fragments were used to prime from each other to give a 1.2-kb fusion product. This was ligated to Intron 3 sequence, to extend the left arm, and to ~9-kb of 3' sequence to create the right arm, which initiates 1.5-kb into Intron 4, continuing to Intron 5.

Figure 2, Middle Panel, shows the promoterless *neo*-polyA insertion vector designated plasmid p0079. This vector contains the same left arm-*neo*-polyA fusion as in vector p0054, but with a modified right arm of 3.9-kb. The 3' region comprises a 1.5-kb fragment, generated by PCR (using primers 200011, 5'-CAGATCTAAC GAGGATTCAA TGTCAAAGGA AAAGTGATTC TGTCAAT-3', SEQ. ID NO:22; and 499006, 5'-CTGAACTGAA TGTTTATCCA GGCCATC-3', SEQ. ID NO:23), which extends from the second codon in Exon 4 into Intron 4, replacing the sequence deleted in p0054. The 3' arm was extended by ligation to a 2.4-kb EcoRV downstream fragment.

Figure 2, Lower Panel, shows the promoterless *pac*-polyA replacement vector designated plasmid p0063, also directed towards Exon 4. Construction of this vector was similar as for p0054, except that it contains the *pac* gene, which codes for puromycin N-acetyltransferase, rather than the *neo* gene. The *pac* sequence is available in plasmid pPUR from ClonTech. The oligonucleotide primers used to generate the 5'-*pac*-polyA fusion were, for the 5' region, 199001 (SEQ. ID NO:18) and 699002 (5'-GCGCACCGTG GGCTTGTA CTGTCATTAT TTTCTCCTGG GAAAAGAAAA G-3', SEQ. ID NO:24), with tail complementary to the start of *pac* coding sequence; and, for *pac*-polyA, 699004 (5'-GAGAAAATAA TGACCGAGTA CAAGCCCACG GTGC-3' SEQ. ID NO:25), with tail complementary to left arm, and 699005 (5'-CTGGGGATCC AGACATGATA AGATAC-3' SEQ. ID NO:26).

Example 2: Targeting the galactosyltransferase gene

Electroporation conditions were optimized using a β -galactosidase marker plasmid, pCMV-Sport- β gal (Gibco). Using a 0.4 cm cuvette with 3×10^5 cells (0.8mL, 6 μ g plasmid DNA) and a setting of 250 μ F : 400 Volts (Gene Pulser, BioRad), 10-30% of the surviving fibroblasts stained positive for β -gal expression.

For targeting the $\alpha 1,3$ GT gene, 10, 25 or 100 μ g of NotI linearized p0054 vector was mixed with 1×10^7 early passage Black Welsh Mountain fetal fibroblasts and pulsed. Cells were grown on tissue culture plastic for 24 h before G418 (300 μ g/mL) was applied. After 10-14 days, colonies were isolated.

Figure 3 shows the results of site specific recombination detected by PCR amplification. Wild type and targeted $\alpha 1,3$ GT alleles were detected using sense (399010, 5'-CAGCTGTGTG GGTATGGGAG GG-3'; SEQ. ID NO:27) and antisense (499006, 5'-CTGAACTGAA TGTTTATCCA GGCCATC-3'; SEQ. ID NO:28) PCR primers, yielding products of 2.8-kb and 2.2-kb, respectively. A second PCR screen with primers 399010 (SEQ. ID NO:29) and 399005 (5'-AGCCGATTGT CTGTTGTGCC CAGTCAT-3'; SEQ. ID NO:30) produced a fragment of 1.5-kb only in clones that were correctly targeted. The frequency of site-specific recombination was 1 in 52 (6 in 312) clones in the 1 μ g experiment or 1 in 88 (10 in 877) from all electroporations.

In parallel experiments, sheep fibroblasts were targeted with a vector designed to inactivate the prion protein (PrP) gene. This gene is heavily implicated in disease pathology of spongiform diseases such as scrapie, bovine spongiform encephalopathy, and Creutzfeldt-Jakob disease (CJD). The sheep PrP gene sequence is provided in Goldmann et al., Proc. Natl. Acad. Sci. USA 87:2476, 1990.

The frequency of site-specific recombination observed in these experiments is shown in Table 1:

TABLE 1: Gene Targeting Efficiency in Primary Sheep Fibroblast Cultures

Parental culture	Target locus	Drug resistant colonies	Targeting events detected	Colonies suitable for nuclear transfer
Black Welsh	$\alpha 1,3GT$	877	10 (1.1%)	0 (0%)
Black Welsh	PrP	533	55 (10.3%)	1 (0.2%)
Finn Dorset	$\alpha 1,3GT$	568	35 (6.2%)	2 (0.4%)

Nuclear transfer is typically conducted as follows. Oocytes are harvested from adult female breeding sheep treated with an analogue of gonadotrophin releasing hormone (Buserelin™, given 24 h after sponge removal). The oocytes are stripped of cumulus cells by triturating with a pipette and incubating with hyaluronidase. They are then enucleated by removing the first polar body and metaphase plate. A single targeted nuclear donor cell is introduced under the zona of each oocyte. The cell combination is subject to simultaneous electrofusion and activation (0.25 kV cm⁻¹ AC for 5 sec. to align oocyte and donor cell, followed by 3 pulses of 1.25 kV cm⁻¹ DC for 80 µsec to fuse and activate the reconstructed embryo). The activated cell is maintained in culture overnight at 39°C. Next day, the cells are embedded in agar chips to protect from macrophages, and then transferred to the ligated oviduct of a temporary recipient.

Estrous is controlled in the temporary recipient by treatment with intravaginal progestagen sponge for 11 to 16 days, with or without subcutaneous or intramuscular injection of 500 i.u. of PMSG. The timing brings the temporary recipients to estrus ~3 days before the oocyte donors. Cells are collected under general anesthesia using barbiturate followed by gaseous anesthetics. The reproductive tract is exposed by midventral laparotomy; placing ligatures of silk at each uterotubal junction, and embryos are transferred through the fimbriated end of the oviduct. The laparotomy is then closed, and a long-acting antibiotic is administered. The embryos are flushed from the temporary recipient after 6 days, and developing embryos are removed from the agar chip.

Blastocysts and morula are then transferred into the recipients that will carry the embryo to term. Estrus is controlled by treatment with an intravaginal progestagen sponge for 11 to 16 days, bringing the final recipients to estrus simultaneously with the oocyte donor. The permanent recipients are anesthetized by intravenous barbiturate and gaseous anesthetics, the reproductive tract is exposed by mid-ventral laparotomy, and the oviduct or uterus is temporarily cannulated for transfer of the embryos. Alternatively, three small puncture incisions are made anterior to the udder, and a laparoscope, manipulating forceps and needle are inserted for manipulation of the uterus. The oviduct or uterus is temporarily cannulated for transfer of the embryos, and the incision is sutured closed.

Recipients of oocytes with a targeted nucleus, engrafted in the manner outlined, were monitored for the status of their pregnancy by subcutaneous ultrasonic scanning on a weekly basis. For animals maintaining their pregnancy, the progress of the fetus is monitored regularly by ultrasound, and brought to term. Results are shown in Table 2. The longest-lived animal born with a PrP knockout survived 12 days.

TABLE 2: Nuclear Transfer from Gene Targeted Primary Cells

Stage of Animal Cloning	Nuclear donor cell		
	Parental Finn Dorset	α 1,3GT targeted	PrP targeted
Reconstructions	126	142	454
Morula and blastocyst	33	21	43
Fetuses at day 60	5	5	8
Lambs at birth; live (dead)	0 (2)	0	3 (1)
Lambs alive at 1 week	0	0	1

Example 3: Telomerizing nuclear donor cells

5 A vector containing an expression cassette for telomerase reverse transcriptase was found to increase functional telomerase activity and replicative capacity in sheep fibroblasts suitable for nuclear transfer.

Figure 4 is a map of plasmid pGRN145. It contains sequences encoding telomerase reverse transcriptase (abbreviated here as hTERT) with a consensus Kozak sequence downstream of the myeloproliferative sarcoma virus (MPSV) promoter. It also contains puromycin and hygromycin resistant gene sequences and allows drug selection of the transfected clones.

Primary sheep fibroblast cell line designated BW6F2 (passage 6, obtained from a Black Welsh sheep) was transfected with linearized pGRN145. The cells were plated in 96 well plates, and selected using puromycin at 1 μ g/mL. PCR screening with puromycin primers showed that all but one of the selected clones contained the vector sequence.

Fourteen of the clones were developed into cell lines. hTERT expression was measured in the cloned sheep fibroblasts by Western blot and by immunocytology. Functional telomerase activity was measured by TRAP assay, and was found to be positive in 10 of these clones, compared with the original BW6F2 line.

20 In order to determine the replicative capacity of the cloned fibroblast cell lines, the cells were passaged continuously using standard culture conditions.

Figure 5 shows the growth curves for these cells. Each line represents a single clone designation, except BW6F23, which is the parental (untransfected) line. The solid circles represent telomerase-expressing clones, and the solid triangles represent telomerase-negative clones. Open squares represent clones that were telomerase-negative initially, but became positive later. All telomerase-negative clones became senescent towards the end of the growth curve, as did the parental BW6F2 cells.

The clones expressing hTERT have been grown through at least 260 population doublings (PDs) and still grow like young cells. Cells transfected with a control plasmid without hTERT cDNA or the transfected cells not expressing hTERT grew less than 83 PDs. The parental cells only replicate through 127 PDs, when they become senescent.

5 The hTERT expressing sheep fibroblasts were also analyzed to determine whether or not the hTERT expressing cells showed signs of transformation to a malignant phenotype by karyotype analysis, response to serum starvation (0.1% serum for 7 days, followed by resynchronization for 24 h in 10% serum). Telomere length was assessed by extracting DNA from cloned cells using a blotting assay. The DNA was digested with RsaI and HinfI, separated on 0.7% agarose, blotted onto a nylon membrane, and
10 probed with ³²P-labeled (TTAGGG)₃ oligonucleotide.

It was found that by passage ~150, some clones have telomere shortening (GRN 1-1, 2-7 and 2-8), while others show no change (GRN 2-1, 2-5 and 2-10), or show elongated telomeres (GRN 2-2). Clones with higher hTERT expression levels (detected by Western blot and immunostaining with 1A4 antibody) maintained their telomere length, while clones with lower hTERT expression levels were
15 typically the ones showing shortened telomeres.

A summary of results from these experiments is shown in Table 3.

TABLE 3: Characteristics of Telomerized Sheep Fibroblast Clones

Designation	PCR for puromycin gene	TRAP assay (telomerase activity)	Population doublings observed	Response to serum starvation	Contact inhibition	Karyotype
GRN 1-1	+	—	354	Normal (p54-56)	Normal (p72)	Normal (p14, p49)
GRN 1-2	+	— → +	289	Normal (p50)	Normal (p47)	Normal (p8, p35)
GRN 2-1	+	+	264	Normal (p50)	Normal (p43)	Normal (p13, p80)
GRN 2-2	+	— → +	294	Normal (p48)	Normal (p52)	Normal (p30) Abnormal (p90)
GRN 2-3	+	—	37 ^a	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
GRN 2-4	+	—	75 ^a	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
GRN 2-5	+	+	279	Normal (p54)	Normal (p46)	Normal (p12, p86)
GRN 2-7	+	+	314	Normal (p62)	Normal (p64)	Normal (p15, p97)
GRN 2-8	+	+	318	Normal (p60)	Normal (p52-53)	Normal (p15)
GRN 2-10	+	+	166 ^b	<i>n.d.</i>	<i>n.d.</i>	Abnormal (p13)
GRN 2-12	+	+	293	Normal (p50)	Normal (p51-53)	<i>n.d.</i>
GRN 2-13	+	+	258	Normal (p47)	Normal (p48-49)	Normal (p16)
GRN 2-18	—	—	83 ^a	Abnormal (p18)	<i>n.d.</i>	<i>n.d.</i>
GRN 2-20	+	— → ?	113 ^b	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
^a – Cells became senescent ^b – Growth curve stopped						

Example 4: Vectors for substituting the galactose transferase gene with fucosyltransferase

Vectors for substituting the encoding sequence for α 1,2FT into the α 1,3GT behind the endogenous promoter have been made by modifying the knockout vectors for *neo* (p0054) and *puro* (p0063).

5 **Figure 6**, Top Panel, shows a map of the sheep α 1,2FT substitution vector. A 1kb RV-RI fragment was cloned into KpnI-RI cleaved modified pBS (pBluescript™, Stratagene), hence destroying the original RV site as follows. The 5' arm (gal) – FT fusion, which lacks a polyA site, was produced by PCR. The 5' arm was amplified with primers 199001 (5'-ACGTGGCTCCA AGAATTCTCCA GGCAAGAGTAC TGG-3', SEQ. ID NO:18) and 700001 (5'-CTG ACG ATG GCT CCG GAG CCA CAT
10 TAT TTT CTC CTG GGA AAA GAA AAG-3', SEQ. ID NO:31), the latter having complementarity to human α 1,2FT.

The human α 1,2FT sequence (GenBank accession NM000148, SEQ. ID NO:9) was amplified from mRNA prepared from the 293 cell line (a permanent line of primary human embryonal kidney transformed by human adenovirus type 5 DNA; ATCC Accession No. CRL-1573). The following primers
15 were used: 700002 (5'-ATA ATG TGG CTC CGG AGC CAT CGT CA-3'; SEQ. ID NO:32) and 700003 (5'-AAA GGA TCC TCA AGG CTT AGC CAA TGT CCA GAG T-3'; SEQ. ID NO:33). The products of 0.3 kb and 1.1 kb, respectively, were mixed in a PCR reaction in which they primed from each other to give a 1.4 kb fragment that was cloned into RI – BHI cut vector from above. This fusion has been sequenced and is correct.

20 A fragment containing the SV40 poly A site, produced from synthetic oligos (700004, 5'-GAT CCG GGG ATC GGC AAT AAA AAG ACA GAA TAA AAC GCA CGG GTG TTG GGT CGT TTG TTC CTC GAG GTC GAC GAT-3', SEQ. ID NO:34; 700005, 5'-ATC GTC GAC CTC GAG GAA CAA ACG ACC CAA CAC CCG TGC GTT TTA TTC TGT CTT TTT ATT GCC GAT CCC CG -3', SEQ. ID NO:35), was ligated 3' of the FT coding sequence between BHI and RV sites. Finally, to complete the 3' arm of
25 the vector, two separate fragments (a ~7kb RV-NotI then a 2.4kb RV) were added. The vector was designated p0090.

Figure 6 also shows construction of a pig promoterless α 1,2FT substitution vector. The middle panel of the figure shows pPAGTarget1, a vector comprising porcine α 1,3GT sequence for inactivating α 1,3GT in pig cells. The vector is digested with StuI/NsiI to release a small amount of 5 and 3' α 1,3GT sequence, and also the neoA cassette. The NsiI site is blunt ended with DNA polymerase. A PCR
30 fragment fusing 5' pig α 1,3GT with α 1,2FT-polyA is then made. Oligonucleotides galF (CCTATGCAAA TTAAGGTAG AACGCAC, SEQ. ID NO:36) and galR (5'-CTGACGATGG CTCCGGAGCC ACATTATTTT CTCCTGGGA AAAGAAAAG-3', SEQ. ID NO:37), with part of the latter being complementary to the α 1,2FT sequence, produce a 200bp fragment. Oligonucleotides FTF
35 (5'-ATAATGTGG CTCCGGAGC CATCGTCA-3', SEQ. ID NO:38) and FTR (5'-CTCGAGGAA CAAACGACCC AACACCCGTG-3', SEQ. ID NO:39), directed to SV40 poly-A, produce a 1.2 kb α 1,2FT poly-A fragment.

These fragments are fused by PCR, polished with T4 DNA polymerase, 5' phosphorylated, and ligated into the *StuI* / *NsiI* polished vector, to produce the targeting vector shown at the bottom of Figure 5. The vector is linearized with *NotI* or *Sall* when used for targeting.

5 Example 5: α 1,2FT gene expression in telomerized fibroblasts

Primary Black Welsh fibroblasts (designation BW6F2) were transfected with the hTERT gene as described in Example 3. The characteristics of telomerized clone GRN1.1 are described in Example 3.

GRN1.1 cells at passage 5 or 25 were resuscitated into T175 flasks and grown to subconfluency. Cells (2.8×10^6 , passage 5; 8.3×10^6 , passage 25) were electroporated with 10 μ g of *NotI* linearized p0054 targeting vector, using a setting of 125 μ F: 350 V in Flowgen™ 0.4 cm / 800 μ l cuvettes. Diluted cells were plated to 20 $\times$ 96 well plates. The next day, G418 (600 μ g / mL) was added to the medium to begin the selection process. Cell death appeared after 8-10 days in G418, much longer than when using parental BW6F2 cells. Colonies were observed after ~2 weeks and replica plated (41 colonies from passage 5 cells; 2 colonies from passage 25 cells) on day 20 of selection.

PCR analysis was conducted on DNA isolated from selected colonies. One correct targeting event (clone B9) was detected from the passage 5 electroporation. This clone and eight non-targeted clones were resuscitated in 24 well plates. All clones grew to confluency. The B9 (correctly targeted) cell line, and the C9 cell line (one of the eight containing randomly integrated α 1,3GT) grew fastest. Clones B9 and C9 have been karyotyped, and both are 54XY.

Thus, telomerized sheep fibroblasts were successfully targeted with the promoterless neo α 1,3GT targeting vector, p0054. The targeted clone (B9) has been expanded, and retains a stable karyotype. This clone exists as a pure population of targeted cells and continues to grow at passage 17 (~80 doublings). Successfully targeted clones can be used for replacing the α 1,3GT gene on the other allele with α 1,2FT, using the targeting vectors obtained in Example 4.

To ensure that the α 1,2FT encoding sequence in the fusion vector produces functional enzyme, the α 1,2FT sequence was PCR'ed and subcloned between the *NheI* and *BamHI* sites of pEGFP-C1 (ClonTech), thereby replacing the GFP sequence in the vector to form a pCMV- α 1,2FT-pA cassette. This plasmid was designated p105. The vector was transfected into the B9 and C9 telomerized α 1,3GT-targeted lines by lipofection. After 48 h, the cells were washed in PBS and fixed in cold acetone for 10 min at 4°C. Samples were washed in Hepes buffer (0.15 M NaCl, 0.01 M Hepes, pH 7.5) and incubated with UEA-1 rhodamine (Vector Labs) diluted 1:50 in Hepes buffer. After washing in Hepes buffer, the samples were mounted in DAPI containing Vector Shield™.

Figure 7 shows the results. Normally, sheep fibroblasts do not stain with the UEA-1 lectin, since they do not bear H substance. Staining of human 293 cells is shown here as a positive control. As a result of transfection, both the B9 and C9 sheep cell lines now specifically bind UEA-1, showing that expression of the human α 1,2FT gene in sheep fibroblasts does indeed cause synthesis of H substance carbohydrate on the cell surface.

The compositions and procedures provided in the description can be effectively modified by those skilled in the art without departing from the spirit of the invention embodied in the claims that follow.

SEQUENCE DATA

5

TABLE 4: Sequences listed in this Disclosure		
SEQ. ID NO:	Designation	Reference
1	Human Telomerase Reverse Transcriptase cDNA sequence	GenBank Accession NM 003219 U.S. Patent 6,166,178
2	Human Telomerase Reverse Transcriptase amino acid sequence	GenBank Accession NM 003219 U.S. Patent 6,166,178
3	Sheep α 1,3GT cDNA sequence	This Disclosure.
4	Sheep α 1,3GT amino acid sequence	This Disclosure.
5	Bovine α 1,3GT cDNA sequence	GenBank Accession J04989 Joziassse et al. "Bovine α 1- $\rightarrow$ 3-galactosyltransferase" J. Biol. Chem. 264, 14290 (1989)
6	Bovine α 1,3GT amino acid sequence	GenBank Accession P14769 Joziassse et al. (1989), supra.
7	Pig α 1,3GT cDNA sequence	GenBank Accession L36152 Sus scrofa alpha-1,3-galactosyltransferase mRNA. Strahan et al. "cDNA sequence and chromosome localization of pig α 1,3 galactosyltransferase" Immunogenetics 41, 101 (1995) See also GenBank Accession L36535 Sandrin et al. "Characterization of cDNA clones for porcine α (1,3)galactosyltransferase" Xenotransplantation (1994)
8	Pig α 1,3GT amino acid sequence	GenBank Accession L36152 Strahan et al., supra.
9	Human α 1,2FT (FUT1) cDNA sequence	Larsen, et al. "Molecular cloning, sequence, and expression of a human GDP-L-fucose:beta-D-galactoside 2-alpha-L-fucosyltransferase cDNA that can form the H blood group antigen" Proc. Natl. Acad. Sci. USA 87, 6674 (1990) GenBank Accession NM 000148
10	Human α 1,2FT (FUT1) amino acid sequence	Larsen, et al., supra. GenBank Accession NM 000148
11	Human Fuc- α 1,2Gal- α 1,3GalNAc (Blood Group A) transferase cDNA sequence	Yamamoto, et al. "Cloning and characterization of DNA complementary to human UDP-GalNAc: Fuc- α 1,2Gal- α 1,3GalNAc transferase" J. Biol. Chem. 265:1146-1151 (1989) GenBank Accession J05175
12	Human Blood Group A-transferase amino acid sequence	Yamamoto, et al., supra. GenBank Accession J05175
13	Human	Yamamoto. "Homo sapiens B-specific alpha 1- $\rightarrow$ 3 galactosyltransferase (ABO) mRNA, ABO-*B101

TABLE 4: Sequences listed in this Disclosure

SEQ. ID NO:	Designation	Reference
	Fuc- α 1,2Gal- α 1,3Gal (Blood Group B) transferase cDNA sequence	allele, complete cds." (direct submission) GenBank Accession AF134414
14	Human Blood Group B-transferase amino acid sequence	Yamamoto, supra. GenBank Accession AF134414
15 to 40	Probes and PCR primers	This Invention.

SEQ. ID NO:1

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SEQ. ID NO:6

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. An ovine or porcine cell that is homozygous for inactivation of the $\alpha 1,3$ GT gene, and expresses an $\alpha 1,2$ FT transgene.
5
2. The cell of claim 1, wherein at least part of the encoding sequence in the $\alpha 1,3$ GT gene is replaced with an $\alpha 1,2$ FT encoding sequence.
3. The cell of claim 1, wherein the $\alpha 1,2$ FT encoding sequence is placed under control of the endogenous $\alpha 1,3$ GT promoter.
10
4. The cell of any preceding claim, containing a transgene for blood group A-or B-transferase.
- 15 5. The cell of any one of claims 1 to 4, which is an ovine cell.
6. The cell of any one of claims 1 to 4, which is a porcine cell.
7. Tissue from an ovine or porcine mammal that expresses
20 $\alpha(1,2)$ fucosyltransferase ($\alpha 1,2$ FT) and is homozygous for inactivation of $\alpha(1,3)$ galactosyltransferase ($\alpha 1,3$ GT).
8. The tissue of claim 7, which also expresses blood group A-or B-transferase, or both.
25
9. The tissue of claim 7 or claim 8, which is ovine tissue.
10. The tissue of claim 7 or claim 8, which is porcine tissue.
- 30 11. An ovine or porcine mammal that is homozygous for inactivation of the $\alpha 1,3$ GT gene, and expresses a $\alpha 1,2$ FT transgene.
12. The mammal of claim 11, wherein at least part of the encoding sequence in the $\alpha 1,3$ GT gene is replaced with an $\alpha 1,2$ FT encoding sequence.
35

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13. The mammal of claim 11 or claim 12, which is transgenic for the ABO blood group A-transferase, B-transferase, or both.
14. The mammal of any one of claims 11 to 13, which is a sheep.
- 5 15. The mammal of any one of claims 11 to 13, which is a pig.
- 10 16. A method for producing the tissue of any one of claims 7 to 10, comprising transferring the nucleus of a donor cell wherein at least part of the encoding sequence of a $\alpha 1,3$ GT gene is replaced with a $\alpha 1,2$ FT encoding sequence, to a suitable recipient cell; engrafting the cell into the uterus of a surrogate host; obtaining an embryo or neonate from the host that has grown from the engrafted cell; and harvesting the tissue from the embryo or neonate, or its progeny.
- 15 17. Use of the cell or tissue of any one of claims 1 to 10, for xenotransplantation into a human or animal body.
18. Use of the cell or tissue of any one of claims 1 to 10, in the preparation of a medicament for tissue regeneration.
- 20 19. A method for treating a subject in need of tissue regeneration, comprising administering to the subject the cell or tissue of any one of claims 1 to 10.
- 25 20. A cell, tissue or mammal according to any one of claims 1 to 15, substantially as hereintofores described with reference to any one of the Examples.

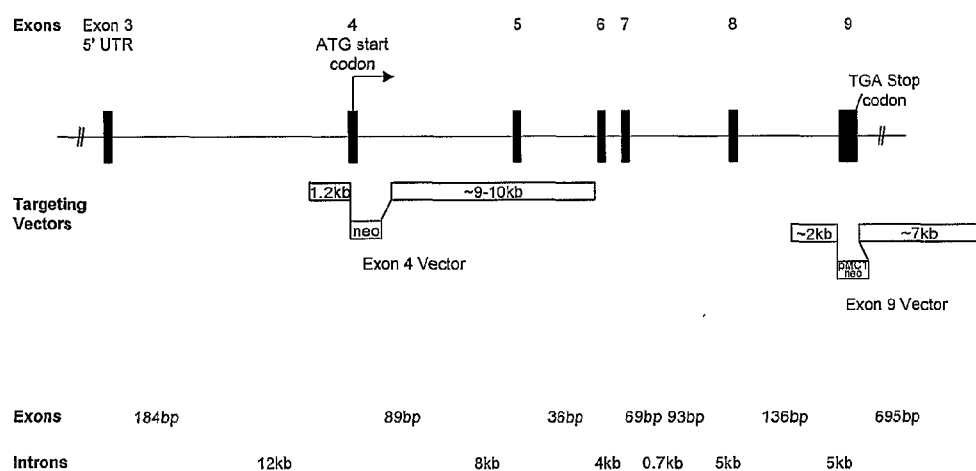
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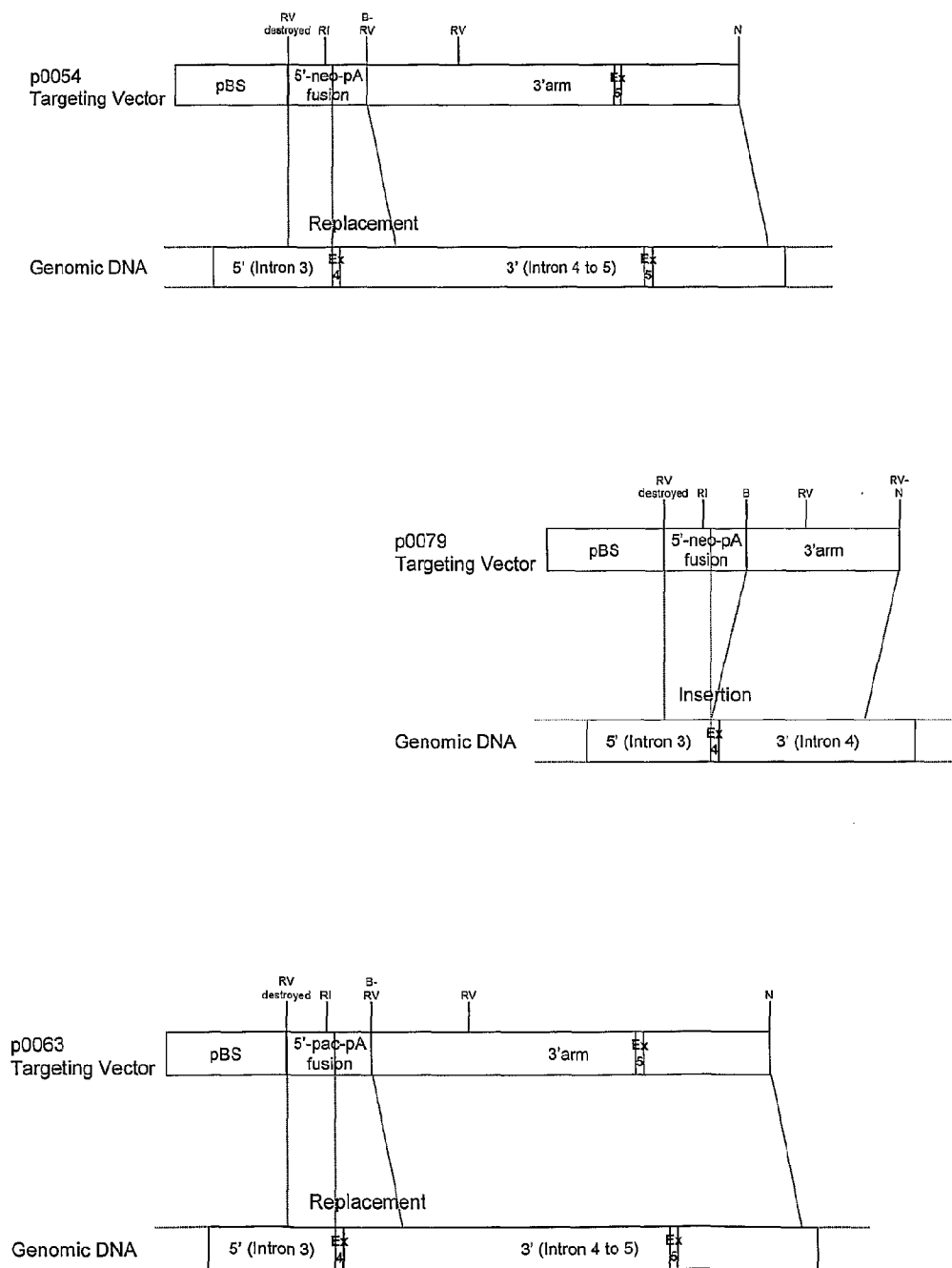
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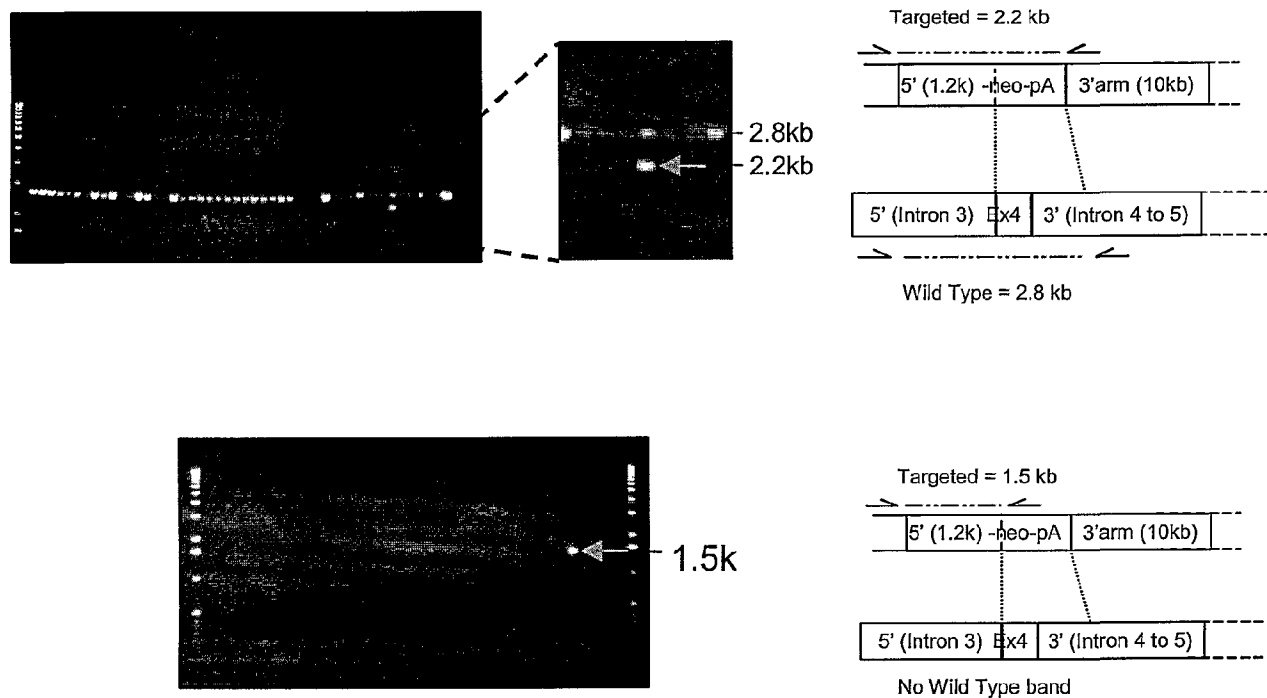


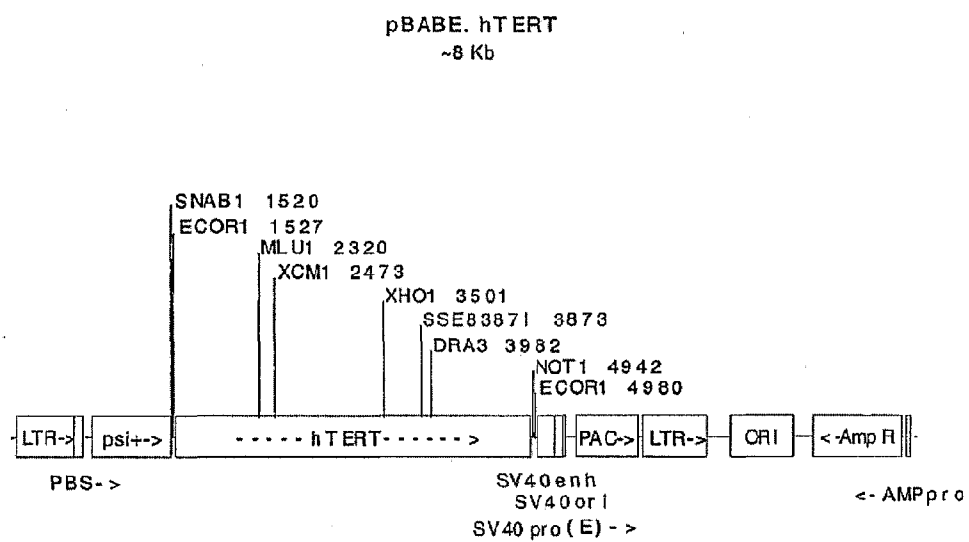
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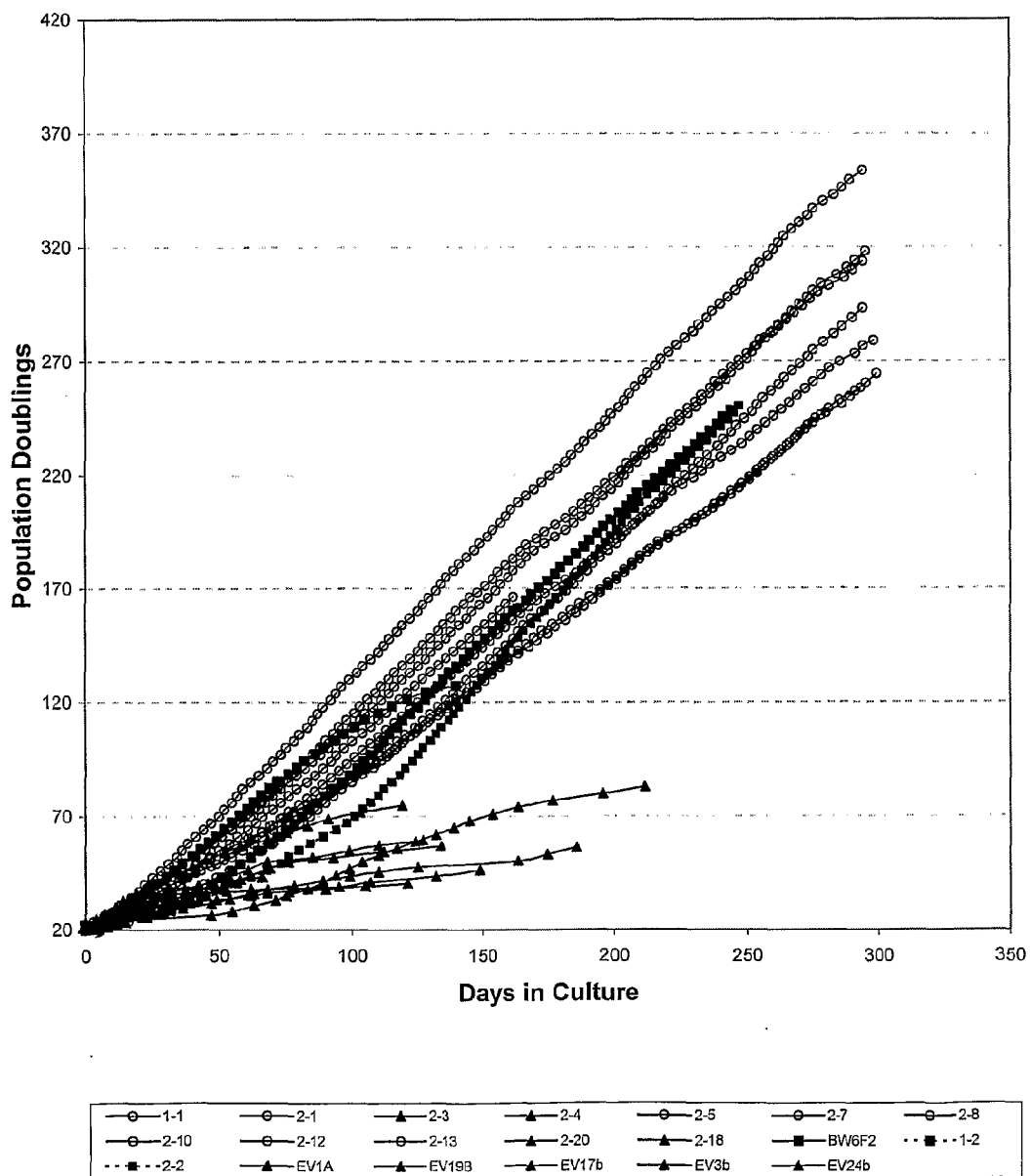
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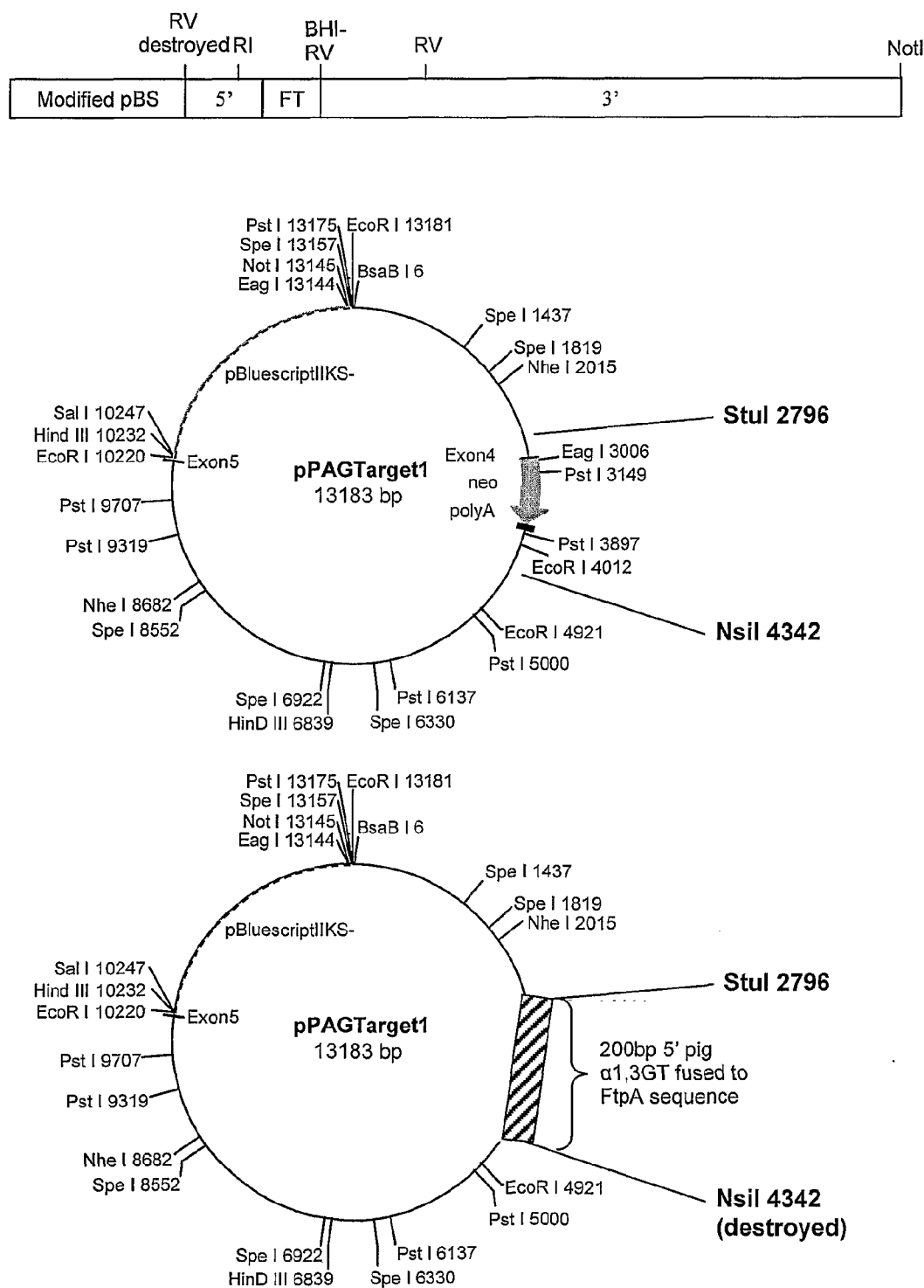
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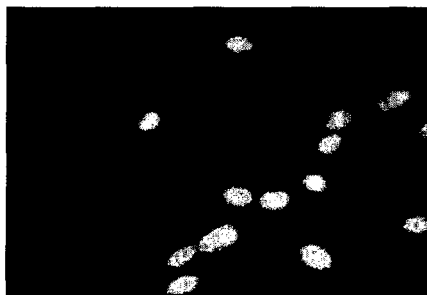
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293 human cells



B9 telomerized sheep cells expressing $\alpha 1,2\text{FT}$



C9 telomerized sheep cells expressing $\alpha 1,2\text{FT}$



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 <212> PRT
 <213> human

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 35 40 45
 Ala Leu Val Ala Gln Cys Leu Val Cys Val Pro Trp Asp Ala Arg Pro
 50 55 60
 Pro Pro Ala Ala Pro Ser Phe Arg Gln Val Ser Cys Leu Lys Glu Leu
 65 70 75 80

Val Ala Arg Val Leu Gln Arg Leu Cys Glu Arg Gly Ala Lys Asn Val
 85 90 95
 Leu Ala Phe Gly Phe Ala Leu Leu Asp Gly Ala Arg Gly Gly Pro Pro
 100 105 110
 Glu Ala Phe Thr Thr Ser Val Arg Ser Tyr Leu Pro Asn Thr Val Thr
 115 120 125
 Asp Ala Leu Arg Gly Ser Gly Ala Trp Gly Leu Leu Leu Arg Arg Val
 130 135 140
 Gly Asp Asp Val Leu Val His Leu Leu Ala Arg Cys Ala Leu Phe Val
 145 150 155 160
 Leu Val Ala Pro Ser Cys Ala Tyr Gln Val Cys Gly Pro Pro Leu Tyr
 165 170 175
 Gln Leu Gly Ala Ala Thr Gln Ala Arg Pro Pro Pro His Ala Ser Gly
 180 185 190
 Pro Arg Arg Arg Leu Gly Cys Glu Arg Ala Trp Asn His Ser Val Arg
 195 200 205
 Glu Ala Gly Val Pro Leu Gly Leu Pro Ala Pro Gly Ala Arg Arg Arg
 210 215 220
 Gly Gly Ser Ala Ser Arg Ser Leu Pro Leu Pro Lys Arg Pro Arg Arg
 225 230 235 240
 Gly Ala Ala Pro Glu Pro Glu Arg Thr Pro Val Gly Gln Gly Ser Trp
 245 250 255
 Ala His Pro Gly Arg Thr Arg Gly Pro Ser Asp Arg Gly Phe Cys Val
 260 265 270
 Val Ser Pro Ala Arg Pro Ala Glu Glu Ala Thr Ser Leu Glu Gly Ala
 275 280 285
 Leu Ser Gly Thr Arg His Ser His Pro Ser Val Gly Arg Gln His His
 290 295 300
 Ala Gly Pro Pro Ser Thr Ser Arg Pro Pro Arg Pro Trp Asp Thr Pro
 305 310 315 320
 Cys Pro Pro Val Tyr Ala Glu Thr Lys His Phe Leu Tyr Ser Ser Gly
 325 330 335
 Asp Lys Glu Gln Leu Arg Pro Ser Phe Leu Leu Ser Ser Leu Arg Pro
 340 345 350
 Ser Leu Thr Gly Ala Arg Arg Leu Val Glu Thr Ile Phe Leu Gly Ser
 355 360 365
 Arg Pro Trp Met Pro Gly Thr Pro Arg Arg Leu Pro Arg Leu Pro Gln
 370 375 380
 Arg Tyr Trp Gln Met Arg Pro Leu Phe Leu Glu Leu Leu Gly Asn His
 385 390 395 400

Ala Gln Cys Pro Tyr Gly Val Leu Leu Lys Thr His Cys Pro Leu Arg
 405 410 415
 Ala Ala Val Thr Pro Ala Ala Gly Val Cys Ala Arg Glu Lys Pro Gln
 420 425 430
 Gly Ser Val Ala Ala Pro Glu Glu Glu Asp Thr Asp Pro Arg Arg Leu
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 Val Gln Leu Leu Arg Gln His Ser Ser Pro Trp Gln Val Tyr Gly Phe
 450 455 460
 Val Arg Ala Cys Leu Arg Arg Leu Val Pro Pro Gly Leu Trp Gly Ser
 465 470 475 480
 Arg His Asn Glu Arg Arg Phe Leu Arg Asn Thr Lys Lys Phe Ile Ser
 485 490 495
 Leu Gly Lys His Ala Lys Leu Ser Leu Gln Glu Leu Thr Trp Lys Met
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 Ser Val Arg Asp Cys Ala Trp Leu Arg Arg Ser Pro Gly Val Gly Cys
 515 520 525
 Val Pro Ala Ala Glu His Arg Leu Arg Glu Glu Ile Leu Ala Lys Phe
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 Leu His Trp Leu Met Ser Val Tyr Val Val Glu Leu Leu Arg Ser Phe
 545 550 555 560
 Phe Tyr Val Thr Glu Thr Thr Phe Gln Lys Asn Arg Leu Phe Phe Tyr
 565 570 575
 Arg Lys Ser Val Trp Ser Lys Leu Gln Ser Ile Gly Ile Arg Gln His
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 Leu Lys Arg Val Gln Leu Arg Glu Leu Ser Glu Ala Glu Val Arg Gln
 595 600 605
 His Arg Glu Ala Arg Pro Ala Leu Leu Thr Ser Arg Leu Arg Phe Ile
 610 615 620
 Pro Lys Pro Asp Gly Leu Arg Pro Ile Val Asn Met Asp Tyr Val Val
 625 630 635 640
 Gly Ala Arg Thr Phe Arg Arg Glu Lys Arg Ala Glu Arg Leu Thr Ser
 645 650 655
 Arg Val Lys Ala Leu Phe Ser Val Leu Asn Tyr Glu Arg Ala Arg Arg
 660 665 670
 Pro Gly Leu Leu Gly Ala Ser Val Leu Gly Leu Asp Asp Ile His Arg
 675 680 685
 Ala Trp Arg Thr Phe Val Leu Arg Val Arg Ala Gln Asp Pro Pro Pro
 690 695 700
 Glu Leu Tyr Phe Val Lys Val Asp Val Thr Gly Ala Tyr Asp Thr Ile
 705 710 715 720

Pro Gln Asp Arg Leu Thr Glu Val Ile Ala Ser Ile Ile Lys Pro Gln
 725 730 735

Asn Thr Tyr Cys Val Arg Arg Tyr Ala Val Val Gln Lys Ala Ala His
 740 745 750

Gly His Val Arg Lys Ala Phe Lys Ser His Val Ser Thr Leu Thr Asp
 755 760 765

Leu Gln Pro Tyr Met Arg Gln Phe Val Ala His Leu Gln Glu Thr Ser
 770 775 780

Pro Leu Arg Asp Ala Val Val Ile Glu Gln Ser Ser Ser Leu Asn Glu
 785 790 795 800

Ala Ser Ser Gly Leu Phe Asp Val Phe Leu Arg Phe Met Cys His His
 805 810 815

Ala Val Arg Ile Arg Gly Lys Ser Tyr Val Gln Cys Gln Gly Ile Pro
 820 825 830

Gln Gly Ser Ile Leu Ser Thr Leu Leu Cys Ser Leu Cys Tyr Gly Asp
 835 840 845

Met Glu Asn Lys Leu Phe Ala Gly Ile Arg Arg Asp Gly Leu Leu Leu
 850 855 860

Arg Leu Val Asp Asp Phe Leu Leu Val Thr Pro His Leu Thr His Ala
 865 870 875 880

Lys Thr Phe Leu Arg Thr Leu Val Arg Gly Val Pro Glu Tyr Gly Cys
 885 890 895

Val Val Asn Leu Arg Lys Thr Val Val Asn Phe Pro Val Glu Asp Glu
 900 905 910

Ala Leu Gly Gly Thr Ala Phe Val Gln Met Pro Ala His Gly Leu Phe
 915 920 925

Pro Trp Cys Gly Leu Leu Leu Asp Thr Arg Thr Leu Glu Val Gln Ser
 930 935 940

Asp Tyr Ser Ser Tyr Ala Arg Thr Ser Ile Arg Ala Ser Leu Thr Phe
 945 950 955 960

Asn Arg Gly Phe Lys Ala Gly Arg Asn Met Arg Arg Lys Leu Phe Gly
 965 970 975

Val Leu Arg Leu Lys Cys His Ser Leu Phe Leu Asp Leu Gln Val Asn
 980 985 990

Ser Leu Gln Thr Val Cys Thr Asn Ile Tyr Lys Ile Leu Leu Leu Gln
 995 1000 1005

Ala Tyr Arg Phe His Ala Cys Val Leu Gln Leu Pro Phe His Gln
 1010 1015 1020

Gln Val Trp Lys Asn Pro Thr Phe Phe Leu Arg Val Ile Ser Asp
 1025 1030 1035

Thr Ala Ser Leu Cys Tyr Ser Ile Leu Lys Ala Lys Asn Ala Gly
 1040 1045 1050
 Met Ser Leu Gly Ala Lys Gly Ala Ala Gly Pro Leu Pro Ser Glu
 1055 1060 1065
 Ala Val Gln Trp Leu Cys His Gln Ala Phe Leu Leu Lys Leu Thr
 1070 1075 1080
 Arg His Arg Val Thr Tyr Val Pro Leu Leu Gly Ser Leu Arg Thr
 1085 1090 1095
 Ala Gln Thr Gln Leu Ser Arg Lys Leu Pro Gly Thr Thr Leu Thr
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 1115 1120 1125
 Thr Ile Leu Asp
 1130

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 <213> sheep

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 tgtcattgtt gtgttttggg aatatatcca cagcccagaa ggctctttgt tctggataaa 300
 cccatcaaga aaccagaag tcagtggcgg cagcagcatt cagaagggtt ggtggtttcc 360
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 catggttggc caccagtc tcttttacgt catggtggac gacgtctcca ggatgccttt 720
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 <211> 369
 <212> PRT
 <213> sheep

<400> 4
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 35 40 45
 Ile Gln Lys Gly Trp Trp Phe Pro Arg Trp Phe Asn Asn Gly Tyr Gln
 50 55 60
 Glu Glu Asp Glu Asp Val Asp Glu Glu Lys Glu Gln Arg Lys Glu Asp
 65 70 75 80
 Lys Ser Lys Leu Lys Leu Ser Asp Trp Phe Asn Pro Phe Lys Arg Pro
 85 90 95
 Glu Val Val Thr Met Thr Asp Trp Lys Ala Pro Val Val Trp Glu Gly
 100 105 110
 Thr Tyr Asn Arg Ala Val Leu Asp Asp Tyr Tyr Ala Lys Gln Lys Ile
 115 120 125
 Thr Val Gly Leu Thr Val Phe Ala Val Gly Arg Tyr Ile Glu His Tyr
 130 135 140
 Leu Glu Glu Phe Leu Thr Ser Ala Asn Lys His Phe Met Val Gly His
 145 150 155 160
 Arg Val Ile Phe Tyr Val Met Val Asp Asp Val Ser Arg Met Pro Leu
 165 170 175
 Ile Glu Leu Gly Pro Leu Arg Ser Phe Lys Val Phe Glu Val Lys Pro
 180 185 190
 Glu Arg Arg Trp Gln Asp Val Ser Met Val Arg Met Lys Thr Ile Gly
 195 200 205
 Glu His Ile Val Ala His Ile Gln Arg Glu Val Asp Phe Leu Phe Cys
 210 215 220

Met Asp Val Asp Gln Val Phe Gln Asp Glu Phe Gly Val Glu Thr Leu
 225 230 235 240

Gly Glu Ser Val Ala Gln Leu Gln Ala Trp Trp Tyr Lys Ala Asp Pro
 245 250 255

Asp Glu Phe Thr Tyr Glu Arg Arg Lys Glu Ser Ala Ala Tyr Ile Pro
 260 265 270

Phe Gly Glu Gly Asp Phe Tyr Tyr His Ala Ala Ile Phe Gly Gly Thr
 275 280 285

Pro Thr Gln Val Leu Asn Ile Thr Gln Glu Cys Phe Lys Gly Ile Leu
 290 295 300

Lys Asp Lys Lys Asn Asp Ile Glu Ala Gln Trp His Asp Glu Ser His
 305 310 315 320

Leu Asn Lys Tyr Phe Leu Leu Asn Lys Pro Thr Lys Ile Leu Ser Pro
 325 330 335

Glu Tyr Cys Trp Asp Tyr His Ile Gly Leu Pro Ala Asp Ile Lys Leu
 340 345 350

Val Lys Met Ser Trp Gln Thr Lys Glu Tyr Asn Val Val Arg Asn Asn
 355 360 365

Val

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 <211> 1617
 <212> DNA
 <213> bovine

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 ggcagcagca ttcagaaggg ctggtggctt ccgagatggt ttaacaatgg ttaccatgaa 660
 gaagatggag acataaacga agaaaaggaa caaagaaacg aagacgaaag caagcttaag 720

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<210> 6
 <211> 368
 <212> PRT
 <213> bovine

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Phe Trp Ile Asn Pro Ser Arg Asn Pro Glu Val Gly Gly Ser Ser Ile
35              40              45

Gln Lys Gly Trp Trp Leu Pro Arg Trp Phe Asn Asn Gly Tyr His Glu
50              55              60

Glu Asp Gly Asp Ile Asn Glu Glu Lys Glu Gln Arg Asn Glu Asp Glu
65              70              75              80

Ser Lys Leu Lys Leu Ser Asp Trp Phe Asn Pro Phe Lys Arg Pro Glu
85              90              95

Val Val Thr Met Thr Lys Trp Lys Ala Pro Val Val Trp Glu Gly Thr
100             105             110

Tyr Asn Arg Ala Val Leu Asp Asn Tyr Tyr Ala Lys Gln Lys Ile Thr
115             120             125

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Val Gly Leu Thr Val Phe Ala Val Gly Arg Tyr Ile Glu His Tyr Leu
 130 135 140
 Glu Glu Phe Leu Thr Ser Ala Asn Lys His Phe Met Val Gly His Pro
 145 150 155 160
 Val Ile Phe Tyr Ile Met Val Asp Asp Val Ser Arg Met Pro Leu Ile
 165 170 175
 Glu Leu Gly Pro Leu Arg Ser Phe Lys Val Phe Lys Ile Lys Pro Glu
 180 185 190
 Lys Arg Trp Gln Asp Ile Ser Met Met Arg Met Lys Thr Ile Gly Glu
 195 200 205
 His Ile Val Ala His Ile Gln His Glu Val Asp Phe Leu Phe Cys Met
 210 215 220
 Asp Val Asp Gln Val Phe Gln Asp Lys Phe Gly Val Glu Thr Leu Gly
 225 230 235 240
 Glu Ser Val Ala Gln Leu Gln Ala Trp Trp Tyr Lys Ala Asp Pro Asn
 245 250 255
 Asp Phe Thr Tyr Glu Arg Arg Lys Glu Ser Ala Ala Tyr Ile Pro Phe
 260 265 270
 Gly Glu Gly Asp Phe Tyr Tyr His Ala Ala Ile Phe Gly Gly Thr Pro
 275 280 285
 Thr Gln Val Leu Asn Ile Thr Gln Glu Cys Phe Lys Gly Ile Leu Lys
 290 295 300
 Asp Lys Lys Asn Asp Ile Glu Ala Gln Trp His Asp Glu Ser His Leu
 305 310 315 320
 Asn Lys Tyr Phe Leu Leu Asn Lys Pro Thr Lys Ile Leu Ser Pro Glu
 325 330 335
 Tyr Cys Trp Asp Tyr His Ile Gly Leu Pro Ala Asp Ile Lys Leu Val
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 Lys Met Ser Trp Gln Thr Lys Glu Tyr Asn Val Val Arg Asn Asn Val
 355 360 365

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 <211> 1269
 <212> DNA
 <213> pig

<400> 7
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 <211> 371
 <212> PRT
 <213> pig

<400> 8
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 Phe Trp Ile Tyr Gln Ser Lys Asn Pro Glu Val Gly Ser Ser Ala Gln
 35 40 45
 Arg Gly Trp Trp Phe Pro Ser Trp Phe Asn Asn Gly Thr His Ser Tyr
 50 55 60
 His Glu Glu Glu Asp Ala Ile Gly Asn Glu Lys Glu Gln Arg Lys Glu
 65 70 75 80
 Asp Asn Arg Gly Glu Leu Pro Leu Val Asp Trp Phe Asn Pro Glu Lys
 85 90 95

Arg Pro Glu Val Val Thr Ile Thr Arg Trp Lys Ala Pro Val Val Trp
 100 105 110
 Glu Gly Thr Tyr Asn Arg Ala Val Leu Asp Asn Tyr Tyr Ala Lys Gln
 115 120 125
 Lys Ile Thr Val Gly Leu Thr Val Phe Ala Val Gly Arg Tyr Ile Glu
 130 135 140
 His Tyr Leu Glu Glu Phe Leu Ile Ser Ala Asn Thr Tyr Phe Met Val
 145 150 155 160
 Gly His Lys Val Ile Phe Tyr Ile Met Val Asp Asp Ile Ser Arg Met
 165 170 175
 Pro Leu Ile Glu Leu Gly Pro Leu Arg Ser Phe Lys Val Phe Glu Ile
 180 185 190
 Lys Ser Glu Lys Arg Trp Gln Asp Ile Ser Met Met Arg Met Lys Thr
 195 200 205
 Ile Gly Glu His Ile Leu Ala His Ile Gln His Glu Val Asp Phe Leu
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 Phe Cys Met Asp Val Asp Gln Val Phe Gln Asn Asn Phe Gly Val Glu
 225 230 235 240
 Thr Leu Gly Gln Ser Val Ala Gln Leu Gln Ala Trp Trp Tyr Lys Ala
 245 250 255
 His Pro Asp Glu Phe Thr Tyr Glu Arg Arg Lys Glu Ser Ala Ala Tyr
 260 265 270
 Ile Pro Phe Gly Gln Gly Asp Phe Tyr Tyr His Ala Ala Ile Phe Gly
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 Gly Thr Pro Thr Gln Val Leu Asn Ile Thr Gln Glu Cys Phe Lys Gly
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 Ile Leu Gln Asp Lys Glu Asn Asp Ile Glu Ala Glu Trp His Asp Glu
 305 310 315 320
 Ser His Leu Asn Lys Tyr Phe Leu Leu Asn Lys Pro Thr Lys Ile Leu
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 Ser Pro Glu Tyr Cys Trp Asp Tyr His Ile Gly Met Ser Val Asp Ile
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 Asn Asn Ile
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<210> 9.
 <211> 3373
 <212> DNA
 <213> human
 <400> 9

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<211> 365
<212> PRT
<213> human

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Cys Val Leu Ser Val Ile Phe Phe Leu His Ile His Gln Asp Ser Phe
20          25          30

Pro His Gly Leu Gly Leu Ser Ile Leu Cys Pro Asp Arg Arg Leu Val
35          40          45

Thr Pro Pro Val Ala Ile Phe Cys Leu Pro Gly Thr Ala Met Gly Pro
50          55          60

Asn Ala Ser Ser Ser Cys Pro Gln His Pro Ala Ser Leu Ser Gly Thr
65          70          75          80

Trp Thr Val Tyr Pro Asn Gly Arg Phe Gly Asn Gln Met Gly Gln Tyr
85          90          95

Ala Thr Leu Leu Ala Leu Ala Gln Leu Asn Gly Arg Arg Ala Phe Ile
100         105         110

Leu Pro Ala Met His Ala Ala Leu Ala Pro Val Phe Arg Ile Thr Leu
115         120         125

Pro Val Leu Ala Pro Glu Val Asp Ser Arg Thr Pro Trp Arg Glu Leu
130         135         140

Gln Leu His Asp Trp Met Ser Glu Glu Tyr Ala Asp Leu Arg Asp Pro
145         150         155         160

Phe Leu Lys Leu Ser Gly Phe Pro Cys Ser Trp Thr Phe Phe His His
165         170         175

Leu Arg Glu Gln Ile Arg Arg Glu Phe Thr Leu His Asp His Leu Arg
180         185         190

Glu Glu Ala Gln Ser Val Leu Gly Gln Leu Arg Leu Gly Arg Thr Gly
195         200         205

Asp Arg Pro Arg Thr Phe Val Gly Val His Val Arg Arg Gly Asp Tyr
210         215         220

Leu Gln Val Met Pro Gln Arg Trp Lys Gly Val Val Gly Asp Ser Ala
225         230         235         240

Tyr Leu Arg Gln Ala Met Asp Trp Phe Arg Ala Arg His Glu Ala Pro
245         250         255

Val Phe Val Val Thr Ser Asn Gly Met Glu Trp Cys Lys Glu Asn Ile
260         265         270

Asp Thr Ser Gln Gly Asp Val Thr Phe Ala Gly Asp Gly Gln Glu Ala
275         280         285

Thr Pro Trp Lys Asp Phe Ala Leu Leu Thr Gln Cys Asn His Thr Ile
290         295         300

Met Thr Ile Gly Thr Phe Gly Phe Trp Ala Ala Tyr Leu Ala Gly Gly
305         310         315         320

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<210>	12
<211>	353
<212>	PRT
<213>	human

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Met Ala Glu Val Leu Arg Thr Leu Ala Gly Lys Pro Lys Cys His Ala
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Leu Arg Pro Met Ile Leu Phe Leu Ile Met Leu Val Leu Val Leu Phe
20          25          30

Gly Tyr Gly Val Leu Ser Pro Arg Ser Leu Met Pro Gly Ser Leu Glu
35          40          45

Arg Gly Phe Cys Met Ala Val Arg Glu Pro Asp His Leu Gln Arg Val
50          55          60

Ser Leu Pro Arg Met Val Tyr Pro Gln Pro Lys Val Leu Thr Pro Trp
65          70          75          80

Lys Asp Val Leu Val Val Thr Pro Trp Leu Ala Pro Ile Val Trp Glu
85          90          95

Gly Thr Phe Asn Ile Asp Ile Leu Asn Glu Gln Phe Arg Leu Gln Asn
100         105         110

Thr Thr Ile Gly Leu Thr Val Phe Ala Ile Lys Lys Tyr Val Ala Phe
115         120         125

Leu Lys Leu Phe Leu Glu Thr Ala Glu Lys His Phe Met Val Gly His
130         135         140

Arg Val His Tyr Tyr Val Phe Thr Asp Gln Leu Ala Ala Val Pro Arg
145         150         155         160

Val Thr Leu Gly Thr Gly Arg Gln Leu Ser Val Leu Glu Val Arg Ala
165         170         175

Tyr Lys Arg Trp Gln Asp Val Ser Met Arg Arg Met Glu Met Ile Ser
180         185         190

Asp Phe Cys Glu Arg Arg Phe Leu Ser Glu Val Asp Tyr Leu Val Cys
195         200         205

Val Asp Val Asp Met Glu Phe Arg Asp His Val Gly Val Glu Ile Leu
210         215         220

Thr Pro Leu Phe Gly Thr Leu His Pro Gly Phe Tyr Gly Ser Ser Arg
225         230         235         240

Glu Ala Phe Thr Tyr Glu Arg Arg Pro Gln Ser Gln Ala Tyr Ile Pro
245         250         255

Lys Asp Glu Gly Asp Phe Tyr Tyr Leu Gly Gly Phe Phe Gly Gly Ser
260         265         270

Val Gln Glu Val Gln Arg Leu Thr Arg Ala Cys His Gln Ala Met Met
275         280         285

Val Asp Gln Ala Asn Gly Ile Glu Ala Val Trp His Asp Glu Ser His
290         295         300

Leu Asn Lys Tyr Leu Leu Arg His Lys Pro Thr Lys Val Leu Ser Pro
305         310         315         320

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Glu Tyr Leu Trp Asp Gln Gln Leu Leu Gly Trp Pro Ala Val Leu Arg
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Pro

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 <211> 1065
 <212> DNA
 <213> human

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 <211> 354
 <212> PRT
 <213> human

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 Met Ala Glu Val Leu Arg Thr Leu Ala Gly Lys Pro Lys Cys His Ala
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 20 25 30
 Gly Tyr Gly Val Leu Ser Pro Arg Ser Leu Met Pro Gly Ser Leu Glu
 35 40 45
 Arg Gly Phe Cys Met Ala Val Arg Glu Pro Asp His Leu Gln Arg Val
 50 55 60
 Ser Leu Pro Arg Met Val Tyr Pro Gln Pro Lys Val Leu Thr Pro Cys
 65 70 75 80
 Arg Lys Asp Val Leu Val Val Thr Pro Trp Leu Ala Pro Ile Val Trp
 85 90 95
 Glu Gly Thr Phe Asn Ile Asp Ile Leu Asn Glu Gln Phe Arg Leu Gln
 100 105 110
 Asn Thr Thr Ile Gly Leu Thr Val Phe Ala Ile Lys Lys Tyr Val Ala
 115 120 125
 Phe Leu Lys Leu Phe Leu Glu Thr Ala Glu Lys His Phe Met Val Gly
 130 135 140
 His Arg Val His Tyr Tyr Val Phe Thr Asp Gln Pro Ala Ala Val Pro
 145 150 155 160
 Arg Val Thr Leu Gly Thr Gly Arg Gln Leu Ser Val Leu Glu Val Gly
 165 170 175
 Ala Tyr Lys Arg Trp Gln Asp Val Ser Met Arg Arg Met Glu Met Ile
 180 185 190
 Ser Asp Phe Cys Glu Arg Arg Phe Leu Ser Glu Val Asp Tyr Leu Val
 195 200 205
 Cys Val Asp Val Asp Met Glu Phe Arg Asp His Val Gly Val Glu Ile
 210 215 220
 Leu Thr Pro Leu Phe Gly Thr Leu His Pro Ser Phe Tyr Gly Ser Ser
 225 230 235 240
 Arg Glu Ala Phe Thr Tyr Glu Arg Arg Pro Gln Ser Gln Ala Tyr Ile
 245 250 255
 Pro Lys Asp Glu Gly Asp Phe Tyr Tyr Met Gly Ala Phe Phe Gly Gly
 260 265 270
 Ser Val Gln Glu Val Gln Arg Leu Thr Arg Ala Cys His Gln Ala Met
 275 280 285
 Met Val Asp Gln Ala Asn Gly Ile Glu Ala Val Trp His Asp Glu Ser
 290 295 300
 His Leu Asn Lys Tyr Leu Leu Arg His Lys Pro Thr Lys Val Leu Ser
 305 310 315 320
 Pro Glu Tyr Leu Trp Asp Gln Gln Leu Leu Gly Trp Pro Ala Val Leu
 325 330 335

Arg Lys Leu Arg Phe Thr Ala Val Pro Lys Asn His Gln Ala Val Arg
340 345 350

Asn Pro