

IMPROVED NUCLEIC ACIDS FOR REDUCING CARBOHYDRATE EPITOPES

The present invention relates to nucleic acids which encode glycosyltransferase and are useful in
5 producing cells and organs from one species which may be used for transplantation into a recipient of another species. Specifically the invention concerns production of nucleic acids which, when present in cells of a transplanted organ result in reduced levels of antibody
10 recognition of the transplanted organ.

The transplantation of organs is now possible due to major advances in surgical and other techniques. However, availability of suitable human organs for transplantation is a significant problem. Demand outstrips
15 supply. This has caused researchers to investigate the possibility of using non-human organs for transplantation.

Xenotransplantation is the transplantation of organs from one species to a recipient of a different species. Rejection of the transplant in such cases is a
20 particular problem, especially where the donor species is more distantly related, such as donor organs from pigs and sheep to human recipients. Vascular organs present a special difficulty because of hyperacute rejection (HAR).

HAR occurs when the complement cascade in the
25 recipient is initiated by binding of antibodies to donor endothelial cells.

Previous attempts to prevent HAR have focused on two strategies : modifying the immune system of the host by inhibition of systemic complement formation (1,2) and
30 antibody depletion (3,4). Both strategies have been shown to temporarily prolong xenograft survival. However, these methodologies are therapeutically unattractive in that they are clinically impractical and would require chronic immunosuppressive treatments. Therefore, recent efforts to
35 inhibit HAR have focused on genetically modifying the donor xenograft. One such strategy has been to achieve high-level expression of species-restricted human complement

- 2 -

inhibitory proteins in vascularized pig organs via transgenic engineering (5-7). This strategy has proven to be useful in that it has resulted in the prolonged survival of porcine tissues following antibody and serum challenge (5,6). Although increased survival of the transgenic tissues was observed, long-term graft survival was not achieved (6). As observed in these experiments and also with systemic complement depletion, organ failure appears to be related to an acute antibody-dependent vasculitis (1,5).

In addition to strategies aimed at blocking complement activation on the vascular endothelial cell surface of the xenograft, recent attention has focused on identification of the predominant xenogeneic epitope recognised by high-titre human natural antibodies. It is now accepted that the terminal galactosyl residue, Gal- $\alpha(1,3)$ -Gal, is the dominant xenogeneic epitope (8-15). This epitope is absent in Old World primates and humans because the $\alpha(1,3)$ -galactosyltransferase (gal-transferase or GT) is non-functional in these species. DNA sequence comparison of the human gene to $\alpha(1,3)$ -galactosyltransferase genes from the mouse (16,17), ox (18), and pig (12) has revealed that the human gene contained two frameshift mutations, resulting in a non-functional pseudogene (20,21). Consequently, humans and Old World primates have pre-existing high-titre antibodies directed at this Gal- $\alpha(1,3)$ -Gal moiety as the dominant xenogeneic epitope.

It appears that different glycosyltransferases can compete for the same substrate. Hence $\alpha(1,2)$ -fucosyltransferase or H transferase (HT) (22) could be an appropriate enzyme to decrease the expression of Gal- $\alpha(1,3)$ -Gal, as both the $\alpha(1,2)$ -fucosyltransferase and the $\alpha(1,3)$ -galactosyltransferase use N-acetyl lactosamine as an acceptor substrate, transferring fucose or galactose to generate fucosylated N-acetyl lactosamine (H substance) or Gal- $\alpha(1,3)$ -Gal, respectively. Furthermore, the $\alpha(1,3)$ -

- 3 -

galactosyltransferase of most animals cannot use the fucosylated N-acetyl lactosamine as an acceptor to transfer the terminal galactose, but will only transfer to N-acetyl lactosamine residues (23). We have previously reported that
5 the simultaneous expression of two glycosyltransferases, $\alpha(1,2)$ -fucosyltransferase (H transferase) and $\alpha(1,3)$ -galactosyltransferase, does not lead to equal synthesis of each monosaccharide, but the activity of the $\alpha(1,2)$ -fucosyltransferase is given preference over that of the
10 $\alpha(1,3)$ -galactosyltransferase, so that the expression of Gal- $\alpha(1,3)$ -Gal is almost entirely suppressed (24).

The $\alpha(1,3)$ -galactosyltransferase (Gal transferase) can galactosylate two types of precursor chains: Type 1: Gal $\beta(1,3)$ GlcNAc and Type 2:
15 Gal $\beta(1,4)$ GlcNAc.

Furthermore, both of these precursors can be transformed into H substance or fucosylated β -D-Gal by two $\alpha(1,2)$ -fucosyltransferases (25,26). These two
fucosyltransferases are H-transferase or FUT1 (22) and
20 secretor (Se) transferase or FUT2 (27). While both enzymes can use both types of precursors, FUT1 HT preferentially utilises Type 2 precursor chains, and FUT2 preferentially utilises Type 1 (28).

In work leading up to the present invention the
25 inventors set out to create a nucleic acid which would be useful in reducing unwanted carbohydrate epitopes on the surface of cells. The nucleic acid could be used in production of an organ which would cause reduced levels of rejection when transplanted into another species. The
30 inventors surprisingly found that a glycosyltransferase derived from porcine origin was useful in decreasing unwanted carbohydrate epitopes in cells. The enzyme encoded by the nucleic acid is able to compete effectively with glycosyltransferases which produce unwanted
35 carbohydrate epitopes. In this particular work the inventors cloned a secretor transferase (Se) gene from pig origin, and demonstrated that this is expressed in cells

and results in reduced levels of unwanted epitopes on those cells. The secretor transferase is referred to herein as "pig secretor".

5 Summary of the Invention

In a first aspect the invention provides a nucleic acid encoding a first glycosyltransferase which is able to compete with a second glycosyltransferase for a substrate when said nucleic acid is expressed in a cell
10 which produces said second glycosyltransferase, resulting in reduced levels of a product from said second glycosyltransferase.

The nucleic acid may be DNA or RNA, single or double stranded, or covalently closed circular. It will be
15 understood that the nucleic acid encodes a functional gene (or part thereof) which enables a glycosyltransferase with the appropriate activity to be produced. Preferably the nucleic acid is in an isolated form; this means that the nucleic acid is at least partly purified from other nucleic
20 acids or proteins.

Preferably the nucleic acid comprises the correct sequences for expression, more preferably for expression in a eukaryotic cell. The nucleic acid may be present on any suitable vehicle, for example, a eukaryotic expression
25 vector such as pcDNA (Invitrogen). The nucleic acid may also be present on other vehicles, whether suitable for eukaryotes or not, such as plasmids, phages and the like.

Preferably the first glycosyltransferase is a an enzyme with a higher affinity for the substrate than said
30 second glycosyltransferase. More preferably said first glycosyltransferase preferentially utilises Type 1 substrates. Still more preferably said first glycosyltransferase is Se (also known as FUT2). Preferably the Se originates or is derived from, or is based on, Se
35 from the same species as the cell in which it is intended to be expressed. Thus, the first glycosyltransferase and the cell in which the enzyme is expressed may each

- 5 -

originate from animals of the same species. Such species may be pig, New World monkey, dog or other suitable species. The nucleic acid encoding Se is not necessarily directly derived from the native gene. The nucleic acid
5 sequence for Se may be made by PCR, constructed *de novo* or cloned.

More preferably Se is of porcine origin or based on the porcine enzyme. This means that the enzyme is based on, homologous with, or similar to native porcine Se.

10 More preferably the nucleic acid sequence encoding Se is based on, or similar to a 1.3 kb DNA fragment derived from a pig genomic liver. More preferably the nucleic acid sequence encodes the amino acid sequence shown in Fig. 1. Still more preferably the nucleic acid
15 sequence is that shown in Fig. 1.

It is apparent that the Se gene is not expressed in porcine tissues which are of primary interest for transplantation. Thus Se is not expressed in heart, liver, kidney and pancreas, for example. Thus the invention
20 includes the provision of expression of a gene in a tissue where it is not normally expressed, whereby expression results in reduced levels of unwanted carbohydrate epitopes in that tissue and renders an organ composed of that tissue more suitable for transplantation.

25 The second glycosyltransferase may be any enzyme which produces an unwanted carbohydrate epitope on the cell of interest. This will usually be Gal transferase.

Preferably the cell which expresses the nucleic acid of the invention is a eukaryotic cell. More
30 preferably it is a mammalian cell, still more preferably a New World monkey cell, even more preferably an ungulate cell (pig, sheep, goat, cow, horse, deer, camel, etc.) or a cell from other species such as dogs. Still more preferably the cell is a pig cell.

35 In a related aspect the invention provides a nucleic acid encoding a first glycosyltransferase which is able to compete with a second glycosyltransferase when said

- 6 -

nucleic acid is expressed in a cell which produces said second glycosyltransferase, wherein said first glycosyltransferase is able to utilise more than one substrate, resulting in reduced levels of product from said
5 second glycosyltransferase.

The greater substrate specificity of the first glycosyltransferase means that this enzyme is more efficient at converting substrate to the desired carbohydrate and more effective in reducing the ability of
10 the second glycosyltransferase to produce unwanted carbohydrate epitopes.

Preferably the first glycosyltransferase is Se, still more preferably the Se is as described above.

Still more preferably the first
15 glycosyltransferase has a higher affinity for one or more of its substrates than the second glycosyltransferase.

The invention also extends to isolated proteins produced by the nucleic acid of the invention. It further extends to biologically or functionally active fragments of
20 such proteins.

In another aspect the invention provides a method of producing a nucleic acid encoding a first glycosyltransferase which is able to compete with a second glycosyltransferase for a substrate when said nucleic acid
25 is expressed in a cell which produces said second glycosyltransferase, resulting in reduced levels of product from said second glycosyltransferase, said method comprising operably linking a nucleic acid sequence encoding a first glycosyltransferase to an appropriate
30 vector or other nucleic acid in order to obtain expression of said first glycosyltransferase.

Those skilled in the art will be aware of the techniques for producing the nucleic acid. Standard techniques such as those described in Sambrook et al may be
35 employed.

Preferably the nucleic acid sequences are the preferred sequences described above.

- 7 -

In another aspect the invention provides a method of reducing the level of a carbohydrate exhibited on the surface of a cell, said method comprising the step of causing a nucleic acid to be expressed in said cell wherein
5 said nucleic acid encodes a first glycosyltransferase which is able to compete for substrate with a second glycosyltransferase and wherein said cell produces said second glycosyltransferase which is capable of producing said carbohydrate.

10 The cell may be any suitable cell, preferably those described above.

The invention also extends to cells produced by the above method and organs comprising the cells.

The nucleic acid of the invention may be present
15 in the cell with another nucleic acid construct which also down-regulates production of unwanted carbohydrates in the surface of the cells, such as that disclosed in PCT/US95/07554, or that of an International application based on Australian provisional application P01402 filed 2
20 August 1996 in the name of The Austin Research Institute.

In another aspect the invention provides a method of producing a cell from one species, such as a donor, which cell is immunologically acceptable to another species which is a recipient, comprising the step of reducing
25 levels of carbohydrate on said cell which cause it to be recognised as non-self by the recipient species, said method comprising causing a nucleic acid to be expressed in said cell, wherein said nucleic acid encodes a first glycosyltransferase which is able to compete for a
30 substrate with a second glycosyltransferase and wherein said cell produces said second glycosyltransferase which is capable of producing said carbohydrate.

The cell may be from any of the species mentioned above. Preferably the cell is from a New World primate or a
35 pig. More preferably the cell is from a pig.

The invention also extends to non-human transgenic animals comprising or harbouring the nucleic

- 8 -

acid of the invention.

In another aspect the invention provides an expression unit such as a retroviral packaging cell or retroviral packaging cassette, a retroviral construct or a retroviral producer cell which expresses the nucleic acid of the invention, resulting in a cell which is immunologically acceptable to an animal by having reduced levels of a carbohydrate on its surface, which carbohydrate is recognised as non-self by said animal.

Preferably the animal is a human, ape or Old World monkey.

The retroviral packaging cells or retroviral producer cells may be cells of any animal origin in which it is desired to reduce the level of carbohydrates on the cell surface to make it more immunologically acceptable to a host. Such cells may be derived from mammals such as canine species, rodent or ruminant species and the like.

The invention also extends to a method of producing a retroviral packaging cell or a retroviral producer cell having reduced levels of a carbohydrate on its surface, wherein the carbohydrate is recognised as non-self by an animal, comprising transforming/transfecting the retroviral packaging cell or the retroviral producer cell with the nucleic acid of the invention under conditions such that the chimeric enzyme is produced. The "chimeric enzyme" means the enzyme encoded by the nucleic acid of the invention.

The term "nucleic acid" refers to any nucleic acid comprising natural or synthetic purines and pyrimidines.

The terms "originates", "based on", or "derived from" mean that enzyme is homologous to, or similar to, the enzyme from that species.

The term "glycosyltransferase" refers to a polypeptide with an ability to move carbohydrates from one molecule to another.

The term "operably linking" means that the

- 9 -

nucleic acid sequences are ligated such that a functional protein is able to be transcribed and translated.

The term "reducing the level of a carbohydrate" refers to lowering, minimising, or in some cases, ablating the amount of carbohydrate displayed on the surface of the cell. Preferably said carbohydrate is in the absence of the first glycosyltransferase of the invention, capable of stimulating recognition of the cell as "non-self" by the immune system of an animal. The reduction of such a carbohydrate therefore renders the cell, or an organ composed of said cells, more acceptable to the immune system of an animal in a transplant situation or gene therapy situation.

The term "causing a nucleic acid to be expressed" means that the nucleic acid is introduced into the cell (i.e. by transformation/transfection or other suitable means) and contains appropriate signals to allow expression in the cell.

The term "immunologically acceptable" refers to producing a cell, or an organ made up of numbers of the cell, which does not cause the same degree of immunological reaction in the other species as a native cell from the one species. Thus the cell may cause a lessened immunological reaction, only requiring low levels of immunosuppression therapy to maintain such a transplanted organ or no immunosuppression therapy may be necessary.

It is contemplated that the nucleic acid of the invention may be useful in producing the chimeric nucleic acids disclosed in an application based on Australian provisional application PO1402 filed 2 August 1996 in the name of The Austin Research Institute.

The retroviral packaging cell and/or producer cells may be used in applications such as gene therapy. General methods involving use of such cells are described in PCT/US95/07554 and the references discussed therein.

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Detailed Description of the Invention

The invention will now be described by way of reference only to the following non-limiting figures and example.

5 Fig 1 shows the nucleic acid sequence and corresponding amino acid sequence of porcine secretor. The rows in each panel represent pig, human and rabbit FUT2 and pig, human and rabbit FUT1 from the top to bottom.

10 Fig 2 shows the amino acid sequences of pig, human and rabbit glycosyltransferases.

Fig 3 shows a typical FACS profile of pig endothelial cells which express $\alpha(1,2)$ -fucosyltransferase.

15 Fig 4 is a dot blot showing the presence of $\alpha(1,2)$ -fucosyltransferase in six offspring of mice injected with a transgenic construct.

20 The work presented below is surprising in that the inventors had previously attempted to clone human secretor but were unsuccessful. A non-functional human pseudogene for secretor was cloned. This raised the question of whether other species such as pigs have a functional gene for secretor. The fact that the inventors were able to successfully clone the pig secretor gene and use it to down regulate unwanted epitopes was surprising. Because of the differences in blood group antigens between pigs and humans, it was not known whether pigs have secretor antigens. The cloning of a functional gene indicates that pigs do have the epitope produced by the secretor glue.

25 Furthermore, although FUT1 had been cloned, it did not permit the pig secretor gene to be isolated. FUT1 and FUT2 are sufficiently different in that probes based on the sequence of FUT1 do not hybridise with that of FUT2.

Example 1 Cloning of Pig Secretor

35 Cloning. The gene encoding the sequence for the human secretor gene (Sec2) (27) was cloned from human genomic DNA using a PCR strategy according to the published sequence, primers, and conditions. A pig genomic liver library in

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- 11 -

EMBL-3 (Clontech Laboratories, Palo Alto, Ca) was screened using this human clone. Nine clones were obtained after screening 5×10^5 plaques. Two of these were randomly chosen for further examination. Limited restriction mapping showed identical banding patterns for both clones, with a 3.3 kb PstI fragment specifically hybridising with the human (Sec 2 $\alpha(1,2)$ -fucosyltransferase) probe. This fragment (PSe 16.1) was sequenced using the ABI automated sequencer.

For functional studies the coding segment of the genomic clone was subcloned into an expression vector. Utilising the polymerase chain reaction (PCR), and the Pig Se sequence as obtained above, 1048 bp gene product was derived using primers : 5' primer homologous to the 5'UTR: 5'CAGAAGCTTATGCTCAGCATGCAGGC in which the underlined sequence contains a unique Hind III site; 3' primer homologous to the 3'UTR: 5'-5'-GTCCTGCAGTGAGTGCTTAAGGAGTGG where the underlined sequence contains a PstI site. This PCR product was purified as above, digested with Hind III and PstI, ligated with similarly digested pcDNA1 (Invitrogen Corporation, San Diego, CA), and then used to transform MC1061/P3. One clone, designated pPSeT, was selected for transfections. Also used were pPGT, which encodes the cDNA for the porcine $\alpha(1,3)$ -galactosyltransferase (19), and pPHT, which encodes the cDNA for the porcine "H" $\alpha(1,2)$ -fucosyltransferase (33).

Transfection. COS cells were maintained in Dulbecco's modified Eagles Medium (DMEM) (Cytosystems Pty. Ltd., Castle Hill, NSW, Australia). COS cells were transfected using the DEAE-dextran method, using DMEM medium supplemented with Foetal Clone II (Hy clone Utah), and 48 h later cells were examined for cell surface expression.

Serology. Direct fluorescence staining of cell surface carbohydrate epitopes was performed with FITC or TRITC conjugated lectins: IB4 lectin isolated from Griffonia

- 12 -

simplicifolia (Sigma, St. Louis, MO) detects Gal- α (1,3)-Gal and UEA1 lectin isolated from *Ulex europaeus* (Sigma, and EY Laboratories, Inc., San Mateo, CA) detects H substance. H substance was also detected by indirect immunofluorescence using a monoclonal antibody (mAb) specific for the H-epitope (ASH-1952) developed at the ARI, and FITC conjugated goat anti-mouse IgG (Zymed Laboratories, San Francisco, CA) used to detect murine antibody binding.

10 **Enzyme assays.** Cells were washed twice with phosphate buffered saline and lysed in either 1% Triton X100/100mM Tris pH7.0 or 1% Triton X100/100mM sodium cacodylate pH 6.5/25mM MnCl₂ at 4°C for 30 min, lysates centrifuged and the supernatant collected and stored at -70°C. Protein
15 concentration was determined by the Bradford test, using bovine serum albumin as a standard; 5-20µg of cell extract was used per transferase assay. The assay for α -1,2fucosyltransferase involved mixing cell extracts and acceptor (75mM phenyl- β -Dgalactoside (Sigma)) in 50µl 50mM
20 MOPS (3-[N-Morpholino]propanesulphonic acid) pH 6.5; 20mM MnCl₂; 5mM ATP; 3µM GDP [¹⁴C]-Fuc (specific activity 287mCi/mmol, Amersham International plc, Amersham, UK) and incubation for 2h at 37°C. The reaction was terminated by the addition of ethanol, and the incorporated ¹⁴C-Fuc
25 determined by liquid scintillation counting after separation in Sep-Pak C18 cartridges (Waters-Millipore, Millford, MA). In all cases the parallel reactions were performed in the absence of added acceptor molecules, to allow for the calculation of specific incorporation.

30

Results

Cloning of pig FUT2 (Se)

Two clones were obtained after screening 5x10⁵ plaques of a pig genomic liver library in EMBL-3 (Clontech
35 Laboratories, Palo Alto, Ca) with the cDNA fragment encoding the full length human FUT2 (27). Limited restriction mapping showed identical banding patterns for

- 13 -

both clones, with a 3.3 kb *Pst* I fragment specifically hybridising with the human FUT2 probe. This fragment was subcloned to generate the clone pSe16.1, which was sequenced. The complete nucleotide sequence of the pig FUT2 DNA contains 1269 bp of nucleotide sequence (Fig. 1): a 8 bp 5' untranslated (UT) region, an open reading frame of 1060 bp encoding a 340 amino acid protein with the initiation codon being nucleotide 9, succeeded by 156 bp of 3'UT. The predicted protein sequence of the pig FUT2 suggests a type II integral membrane protein, typical of other glycosyltransferases. There are three distinct structural features of the predicted protein: (i) a short (4 amino acid) amino-terminal cytoplasmic tail; (ii) a putative transmembrane region composed of 21 hydrophobic amino acids (residues 5-26), flanked on either side by charged amino acid residues; (iii) a 314 amino acid carboxyl-terminal domain which contains three potential N-linked glycosylation sites.

Comparison of the amino acid sequences of pig FUT2 with the human (22,27) and rabbit (29) $\alpha(1,2)$ -fucosyltransferases shows the highest identity with the *Se* transferase rather than the *H* transferase (Fig. 2): the pig FUT2 shows 83.2% identity with human FUT2, 74.1 % identity with rabbit FUT2, 58.5% identity with pig FUT1, 57.1% identity with human FUT1, and 58.8% identity with rabbit FUT1. We note that the highest sequence identity is in the carboxyl portion of the molecule, which contains the catalytic domain (30).

The pig FUT2 nucleotide sequence shows about 36% homology with human FUT1.

Expression of H substance after transfection with pig FUT2

The 1.3 kb *Pst* I fragment containing the coding sequence was subcloned into the COS cell expression vector pCDNA-1 (Invitrogen Corporation San Diego, CA). COS cells transfected with the cloned genomic DNA encoding the pig FUT2 expressed H substance, as indicated by staining with

fluoresceinated UEA I lectin, which detects H substance (31) (~65% positive as shown in Table 1). After transfection with the pig FUT1 cDNA clone similar staining was observed while no staining was seen with the reagent on

5 COS cells transfected with the cDNA for the pig $\alpha(1,3)$ -galactosyltransferase (19). In contrast, staining with fluoresceinated IB4 lectin, which detects Gal $\alpha(1,3)$ Gal (32), was detected on COS cells transfected with pig $\alpha(1,3)$ -galactosyltransferase cDNA but not with the pig FUT1

10 or FUT2 DNA.

Table 1. Cell surface staining of transfected COS cells

<u>Transfection with cDNA encoding¹</u>			<u>% Staining</u>	
<u>FUT1</u>	<u>FUT2</u>	<u>GT</u>	<u>UEA1</u>	<u>IB4</u>
+	-	-	75	0
-	+	-	68	0
-	-	+	0	65
+	-	+	72	8
-	+	+	73	9
+	+	+	76	<1

1. cDNA encoding pig FUT1, FUT2 and GT used

Enzymatic studies

Cell lysates prepared from COS cells transfected with pFUT2 and pFUT1 were assayed for $\alpha(1,2)$ -
5 fucosyltransferase activity. Using mock-transfected COS cells to show baseline activity ($1.1 \text{ nmol hr}^{-1}\text{mg}^{-1}$), significant $\alpha(1,2)$ -fucosyltransferase activity was observed in lysates from both pFUT2 ($151.1 \text{ nmol hr}^{-1}\text{mg}^{-1}$) and pFUT1 ($140.0 \text{ nmol hr}^{-1}\text{mg}^{-1}$) transfected COS cells, but not in ppGT
10 transfected COS cells ($6.7 \text{ nmol hr}^{-1}\text{mg}^{-1}$). The enzyme activity measured in these lysates reflects the expression of H substance on the cell surface as shown in Example 2.

Cotransfection of COS cells

15 COS cells transfected with the pig $\alpha(1,3)$ -galactosyltransferase cDNA clone expressed Gal- $\alpha(1,3)$ -Gal as indicated by reactivity with the IB4 lectin (65% of cells reactive) (Table 1). COS cells was also able to express H substance, as after transfection with either the
20 pig FUT2 or FUT1 clones they stained with the UEA1 lectin (68 and 75% of cells respectively reactive, Table 1). However, when the COS cells were simultaneously transfected with the pig $\alpha(1,3)$ -galactosyltransferase cDNA clone and either pig FUT2 or pig FUT1, and examined for cell surface
25 staining of either carbohydrate, the cells predominantly expressed H substance (72% of cells positive, Table 1), compared with 8% of cells expressing Gal $\alpha(1,3)$ -Gal (Table 1). When both pig FUT2 and pig FUT1 were cotransfected together with the pig $\alpha(1,3)$ -galactosyltransferase cDNA,
30 only one H substance was detected (76%) and <1% Gal $\alpha(1,3)$ -Gal (Table 1). This reduction observed using FUT1 and FUT2 was specific and not due to amount of DNA used for transfection, because using twice the amount of DNA for either FUT1 or FUT2 alone had no effect on the expression
35 of Gal $\alpha(1,3)$ -Gal. Thus expression of both FUT2 and FUT1 resulted in a major decrease in expression of Gal $\alpha(1,3)$ -Gal.

Example 2 Enzyme Kinetics

Cell lysates prepared from COS cells transfected in the manner described in Example 1 with pFUT2 (pig Se),
5 pFUT1 (pig H transferase), or with vector alone were assayed for $\alpha(1,2)$ -fucosyltransferase activity, and the kinetic values were calculated. The K_m values (reflecting the affinity for substrate) obtained for pFUT1, and pFUT2 are shown in Table 2. These values were compatible with
10 those of human and rabbit homologues that have been reported.

The respective K_m values obtained for pFUT1, and pFUT2 with various substrates were:

(a) $\text{Gal}\beta(1,3)\text{GlcNAc}$ (Type I): 6.0mM for pFUT1 and 1.3
15 mM for pFUT2.

The K_m values reported for rabbit FUT1 and rabbit FUT2 were 3.1mM and 1.5mM respectively (34) and 2mM and 1mM for human FUT1 and human FUT2 respectively (35).

(b) $\text{Gal}\beta(1,4)\text{GlcNAc}$ (Type II): 3.7mM for pFUT1 and
20 4.4mM for pFUT2.

The K_m values reported for rabbit FUT1, and rabbit FUT2 were 4.2mM and 6.7mM respectively (34) and 1.9mM and 5.7mM for human FUT1 and human FUT2 respectively (37).

(c) $\text{Gal}\beta(1,3)\text{GalNAc}$ (Type III): 14mM for pFUT1 and
25 for pFUT2 0.2mM.

The K_m values reported for rabbit FUT1, and rabbit FUT2 were 5.8mM and 1mM respectively (34).

(d) $\text{Gal}\beta(1,4)\text{Gal}$: 4.2mM and 1.5mM for pFUT1 and pFUT2
30 respectively.

(e) $\text{Gal}\beta(1,4)\text{Glc}$, 1.9mM and 7.4mM for pFUT1 and pFUT2 respectively.

Thus, pFUT1 can be distinguished from pFUT2 on
35 the basis of substrate preference; pFUT1 is relatively specific for type II and type IV substrates, while pFUT2 (and other Secretor homologues), although having greater

affinity for type I and III acceptors, will use other substrates.

Table 2. Enzyme Kinetics of pFUT1 and pFUT2

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Apparent Km of pig $\alpha(1,2)$ -fucosyltransferases, pFUT1 (H type) and pFUT2 (Secretor type), obtained with various substrates.

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Substrate		Km	
		pFUT1	pFUT2
		(Km in mM)	
Type I	Gal β (1,3)GlcNAc	6.0	1.3
Type II	Gal β (1,4)GlcNAc	3.7	4.4
15 Type III	Gal β (1,3)GalNAc	14	0.2
Type IV	Gal β (1,4)Gal	4.2	1.5
Lactose	Gal β (1,4)Glc	1.9	7.4

15

Example 3 Generation of pig endothelial cells expressing chimeric $\alpha(1,2)$ -fucosyltransferase

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The pig endothelial cell line PIEC expressing the Secretor type $\alpha(1,2)$ -fucosyltransferase were produced by lipofectamine transfection of pFUT2 plasmid DNA (20 μ g) and pSV2NEO (2 μ g). Cells with stable integration were selected by growing the transfected PIEC in media containing G418 (500 μ g/ml; Gibco-BRL, Gaithersburg, MD).

25

Fourteen independent clones were examined for cell surface expression of H substance by staining with UEA-1 lectin. >95% of cells of each of these clones were found to be positive: Fig. 3 shows a typical FACS profile obtained for these clones.

30

Example 4 Production of the transgenic construct, purification, and microinjection.

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A 1023 bp NruI/NotI DNA fragment, encoding the full length pFUT2 was generated utilising the Polymerase Chain Reaction and the pH7T plasmid (36) using the primers:

- 18 -

5' primer homologous to the 5'UTR:

5'-CATGCGGCCGCTCAGTGCTTAAGGAGTGGGGAC-3'.

The underlined sequence contains a unique NruI site;

3' primer homologous to the 3'UTR:

5 5'-GAGTCGCGAATGCTCAGCATGCAGGCATCTTTC-3'

The underlined sequence contains a NotI site.

The DNA was purified on gels before being electroeluted and subcloned into a NruI/NotI cut genomic H-2K^b containing vector (38), resulting in the plasmid clone (pH-2K^b-pFUT2) encoding the pFUT2 gene directionally cloned into exon 1 of the murine H-2K^b gene. This produced a transcript that commences at the H-2K^b transcriptional start site, continuing through the pFUT2 cDNA insert. The construct was engineered such that translation would begin at the initiation codon (ATG) of the pFUT2 cDNA and terminate at the stop codon (TGA) 1023bp downstream.

DNA was prepared for microinjection by digesting pH-2K^b-pFUT2 with XhoI and purification of the H-2K^b-pFUT2 DNA from the vector by electrophoretic separation in agarose gels, followed by extraction with chloroform, and precipitation in ethanol to decontaminate the DNA. Injections were performed on the pronuclear membrane of (C57BL/6xSJL)F₁ zygotes at concentrations between 2-5ng/μl, and the zygotes were then transferred to pseudopregnant (C57BL/6xSJL)F₁ females.

Screening for the transgene

The presence of the transgene in live offspring was detected by dot blotting. 5μg of genomic DNA was transferred to nylon filters and hybridized with the insert from pFUT2, using a final wash comprising 0.1xSSC/1% SDS at 68°C. Fig 4 shows the results of testing 16 live offspring, of which six were found to have the transgenic construct integrated into the genome. Expression of transgenic protein is examined by haemagglutination and fucosyltransferase activity.

It will be apparent to the person skilled in the art that while the invention has been described in some detail for the purposes of clarity and understanding,
5 various modifications and alterations to the embodiments and methods described herein may be made without departing from the scope of the inventive concept disclosed in this specification.

References cited herein are listed on the
10 following pages, and are incorporated herein by this reference.

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CLAIMS

1. A nucleic acid encoding a first
5 glycosyltransferase which is able to compete with a second
glycosyltransferase for a substrate when said nucleic acid
is expressed in a cell which produces said second
glycosyltransferase, resulting in reduced levels of a
product from said second glycosyltransferase.
- 10 2. A nucleic acid according to claim 1, wherein the
first glycosyltransferase is an enzyme with a higher
affinity for the substrate than said second
glycosyltransferase.
3. A nucleic acid according to claim 1 or claim 2,
15 wherein the first glycosyltransferase preferentially
utilises a Type 1 substrate.
4. A nucleic acid according to claim 3, wherein the
first glycosyltransferase is Se (FUT2).
5. A nucleic acid according to claim 4, wherein the
20 nucleic acid sequence encoding Se is based on, or similar
to a 1.3 kb DNA fragment derived from a pig genomic liver.
6. A nucleic acid according to claim 5, encoding the
amino acid sequence shown in Fig 1.
7. A nucleic acid according to claim 6, having the
25 sequence shown in Fig 1.
8. A nucleic acid according to any one of claims 1
to 7, wherein the second glycosyltransferase is an enzyme
which produces an unwanted carbohydrate epitope on the
cell.
- 30 9. A nucleic acid according to claim 8, wherein the
second glycosyltransferase is Gal transferase.
10. A nucleic acid according to any one of claims 1
to 9, wherein the first glycosyltransferase and/or the cell
originate from a mammal selected from the group consisting
35 of primates, ungulates and dogs.
11. A nucleic acid according to any one of claims 1
to 10, wherein the mammal is a pig.

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| ☒ | Claim(s) | : paged | 35 | to paged | 38 |
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12. A nucleic acid according to any one of claims 1 to 11, wherein said first glycosyltransferase is able to utilise more than one substrate, resulting in reduced levels of product from said second glycosyltransferase.
- 5 13. A nucleic acid encoding a first glycosyltransferase which is able to compete with a second glycosyltransferase when said nucleic acid is expressed in a cell which produces said second glycosyltransferase, wherein said first glycosyltransferase is able to utilise
- 10 more than one substrate, resulting in reduced levels of product from said second glycosyltransferase.
14. A nucleic acid according to claim 13, wherein the first glycosyltransferase has a higher affinity for one or more of its substrates than the second glycosyltransferase.
- 15 15. A nucleic acid according to claim 14, wherein the first glycosyltransferase is Se.
16. A vehicle comprising a nucleic acid according to any one of claims 1 to 15, selected from the group consisting of an expression vector, a pcDNA, a plasmid and
- 20 phage.
17. A vehicle according to claim 16, which enables said nucleic acid to be expressed in prokaryotes or in eukaryotes.
18. An isolated protein or functionally active
- 25 fragment thereof, produced by expression of the nucleic acid according to any one of claims 1 to 15.
19. A method of expressing a gene encoding a glycosyltransferase in a tissue where said gene is not normally expressed, comprising the step of introducing said
- 30 gene into cells of said tissue, whereby expression results in reduced levels of unwanted carbohydrate epitopes in the tissue and renders an organ composed of that tissue more suitable for transplantation.
20. A method according to claim 19, wherein the gene
- 35 is the Se gene and the tissue is selected from the group consisting of pig heart, liver, kidney and pancreas.
21. A method of producing a nucleic acid encoding a

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- first glycosyltransferase which is able to compete with a second glycosyltransferase for substrate when said nucleic acid is expressed in a cell which produces said second glycosyltransferase, resulting in reduced levels of product
- 5 from said second glycosyltransferase, said method comprising operably linking a nucleic acid sequence encoding a first glycosyltransferase to a vector or nucleic acid in order to obtain expression of said first glycosyltransferase.
- 10 22. A method of reducing the level of a carbohydrate exhibited on the surface of a cell, said method comprising causing a nucleic acid to be expressed in said cell wherein said nucleic acid encodes a first glycosyltransferase which is able to compete for a substrate with a second
- 15 glycosyltransferase and wherein said cell produces said second glycosyltransferase which is capable of producing said carbohydrate.
23. A cell or organ produced according to the method of claim 22.
- 20 24. A nucleic acid according to any one of claims 1 to 15, further comprising a nucleic acid construct which also down-regulates production of unwanted carbohydrates on the surface of said cell.
- 25 25. A method of producing a cell from a donor species, which cell is immunologically acceptable to a recipient species, comprising the step of reducing levels of carbohydrate on said cell which cause it to be recognised as non-self by the recipient species, said method comprising causing a nucleic acid to be expressed in
- 30 said cell wherein said nucleic acid encodes a first glycosyltransferase which is able to compete for a substrate with a second glycosyltransferase and wherein said cell produces said second glycosyltransferase which is capable of producing said carbohydrate.
- 35 26. A non-human transgenic animal, comprising a nucleic acid according to any one of claims 1 to 15.
27. An expression unit which expresses the nucleic





acid according to any one of claims 1 to 15, resulting in a cell which is immunologically acceptable to an animal as a result of having reduced levels of a carbohydrate on its surface, which carbohydrate is recognised as non-self by
5 said animal.

28. An expression unit according to claim 27, selected from the group consisting of a retroviral-packaging cell, a retroviral packaging cassette, a retroviral construct and a retroviral producer cell.

10 29. A method of producing a retroviral packaging cell or a retroviral producer cell according to claim 28, having reduced levels of a carbohydrate on the cell surface wherein the carbohydrate is recognised as non-self by an animal, comprising the step of transforming or transfecting
15 said cell with the nucleic acid according to any one of claims 1 to 15, under conditions such that a chimeric enzyme encoded by the nucleic acid is produced.

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