low affinity to core ß1→4 and core ß1→6 branches of the tri- and tetra-antennary N-glycans, expression of ST6Gal1 sialyltransferase is not efficient enough, resulting in recombinant proteins which differ from the plasma protein not only regarding their N-glycan antennarity but also regarding the sialylation linkage.

Therefore, the technical problem underlying the present invention is the provision of recombinant glycoproteins having homogenous post-translational modifications with primarily di-antennary N-glycans, as well as cell lines that are capable of recombinantly producing such proteins.

The solution to the above technical problem is achieved by the embodiments characterized in the claims.

In particular, in one aspect, the present disclosure relates to a cell line that is genetically modified to reduce the expression of at least one of GnTIVa/b and GnTV.

In another aspect, the present disclosure relates to a recombinant glycoprotein having N-glycans with a significantly increased proportion of di-antennary N-glycans when compared to the same recombinant glycoprotein expressed in a cell line with non-reduced expression of GnTIVa/b and/or GnTV.

In another aspect, the present disclosure relates to a method for the expression of a recombinant glycoprotein of interest in a cell, said glycoprotein having N-glycans with a significantly increased proportion of di-antennary N-glycans when compared to the same recombinant glycoprotein expressed in a cell line with non-reduced expression of GnTIVa/b and/or GnTV, comprising the steps of providing a cell line as disclosed herein; expressing the glycoprotein of interest in said cell line; and isolating the glycoprotein from the cell or the cell culture supernatant.

GnTIVa and GnTIVb are glycosyltransferases that participate in the transfer of N-acetylglucosamine (GlcNAc) to the core mannose residues of N-linked glycans.

They are isoenzymes that are often equally expressed. They catalyze the formation of the GlcNAc ß1-4 branch on the GlcNAc ß1-2 Man α 1-3 arm of the core structure of N-linked glycans. GnTV catalyzes the addition of GlcNAc in ß 1-6 linkage to the α-linked mannose of di-antennary N-linked oligosaccharides. Both enzymes (GnTIVa/b and GnTV) are essential for the production of tetra-antennary N-linked sugar chains (Figure 1).

In this context, in cell lines wherein GnTIVa and GnTIVb are more or less equally expressed, preferably the expression of both is reduced. However, in cell lines that predominantly express one of GnTIVa and GnTIVb, such as CAP cells expressing predominantly GnTIVb, reduction of the expression of the predominantly expressed isoenzyme may be sufficient.

In preferred embodiments, the cell line is a suspension cell line, *i.e.*, a cell line that can be cultured in suspension culture, e.g. serum-free suspension culture. Respective cell lines are known in the art.

In preferred embodiments, the cell line is further genetically modified to overexpress β-galactoside α-2,6-sialyltransferase 1 (ST6Gal1) and/or α-2,3-sialyltransferase 4 (ST3Gal4).

In this context, the term "overexpressed" either refers to protein expression in cells that prior to genetic modification did not express the respective protein at all, or to an increase in endogenous expression of at least 1.2-fold, preferably at least 1.4-fold, 1.6-fold, 1.8-fold, 2-fold or more.

Methods for the expression/overexpression of a given protein in a cell are not particularly limited and are known in the art. Suitable genes encoding ST3Gal4 and suitable genes encoding ST6Gal1 are not particularly limited and include any genes from any origin that encode a protein having the respective activity, wherein mammalian, in particular human genes are particularly preferred.

In a further embodiment, the present disclosure relates to methods for the expression of recombinant glycoproteins of interest in a cell, said glycoproteins having N-glycans with a significantly increased proportion of di-antennary N-glycans when compared to the same recombinant glycoprotein expressed in a cell line with non-reduced expression of GnTIVa/b and/or GnTV, said methods comprising the step of genetically modifying said cell to reduce the expression of GnTIVa/b and /or GnTV in said cell, and optionally to overexpress ST6Gal1 and/or ST3Gal4.

In particular embodiments, said methods comprise the steps of:

(a) providing a cell line according to the present invention;
(b) expressing the glycoprotein of interest in said cell line; and
(c) isolating the glycoprotein from the cell or the cell culture supernatant.

Means for recombinantly expressing glycoproteins in a given cell line, as well as for isolating said glycoproteins from the cell or the cell culture supernatant, are not particularly limited and are known in the art.

In these aspects, all of the definitions and preferred and/or specific embodiments described below for the recombinant glycoproteins of the present invention, and described above for the cell lines of the present invention apply in an analogous manner where applicable.

In a further embodiment, the present disclosure relates to a recombinant glycoprotein having N-glycans with a significantly increased proportion of di-antennary N-glycans when compared to the same recombinant glycoprotein expressed in a cell line with non-reduced expression of GnTIVa/b and/or GnTV.

As used herein, the term “recombinant glycoprotein” indicates that the respective glycoproteins are biotechnologically produced in genetically modified or cells.

According to the present invention, the proportion of di-antennary N-glycans in the glycoproteins is preferably increased at least 1.2-fold, more preferably at least 1.4-fold, 1.6-fold, 1.8-fold, 2-fold or more, when compared to the same recombinant glycoprotein expressed in a cell line with non-reduced expression of GnTIVa/b and/or GnTV.

In related preferred embodiments, the proportion of tetra-antennary N-glycans in the glycoproteins is significantly reduced when compared to the same recombinant glycoprotein expressed in a cell line with non-reduced expression of GnTIVa/b and/or GnTV, Preferably, said proportion is reduced at least 2-fold, more preferably at least 5-fold, at least 10-fold, at least 100-fold (corresponding to a reduction by at least 50%, more preferably at least 80%, at least 90%, at least 99%) or more, wherein any remaining N-glycans besides di-antennary N-glycans, if present at all, are tri-antennary N-glycans.

Preferably, the glycoprotein is selected from the group consisting of α1-antitrypsin (AAT), hepatocyte growth factor (HGF), erythropoietin (EPO), Factor VII (FVII), Factor VIII (FVIII), Factor IX (FIX), von-Willebrand-Factor (vWF), and C1 esterase inhibitor (C1-inhibitor; C1 Inh), wherein C1 Inh is particularly preferred.

In a further embodiment, the present disclosure relates to a method for the generation of a cell line for the recombinant expression of glycoproteins according to the present invention, comprising steps of genetically modifying a cell line to reduce the expression of at least one of GnTIVa/b and GnTV, and optionally to overexpress ST6Gal1 and/or ST3Gal4.

In further embodiments, the present disclosure relates to methods for decreasing the micro-heterogeneity of N-glycans of recombinant glycoproteins produced in a cell, said method comprising the step of genetically modifying said cell to reduce the expression of GnTIVa/b and /or GnTV in said cell, and optionally to overexpress ST6Gal1 and/or ST3Gal4.

In particular embodiments, said methods comprise the steps of:

(a) providing a cell line according to the present invention;
(b) expressing the glycoprotein of interest in said cell line; and
(c) isolating the glycoprotein from the cell or the cell culture supernatant.

In these aspects, all of the definitions and preferred and/or specific embodiments described for the recombinant glycoproteins of the present invention and the cell lines of the present invention apply in an analogous manner where applicable.

As used herein, the term "about" is intended to be a modifier of ± 10%, preferably ± 5% of the specified value. As an example, the term "about 0.2" is intended to encompass the range of 0.18 to 0.22, preferably 0.19 to 0.21.

The terms "comprising/comprises", "consisting of/consists of", and "consisting essentially of/consists essentially of" are used herein in an interchangeable manner,