Current Topics

New Era of Glycoscience: Intrinsic and Extrinsic Functions Performed by Glycans

Glycan Engineering and Production of 'Humanized' Glycoprotein in Yeast Cells

Yasunori CHIBA* and Hiromi AKEBOSHI

Research Center for Medical Glycoscience, National Institute of Advanced Industrial Science and Technology (AIST); 1–1–1 Higashi, Tsukuba, Ibaraki 305–8566, Japan.

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Protein therapeutics, such as antibodies and cytokines, is the largest class of new drug candidates being developed by pharmaceutical companies. Although most of these glycoproteins are produced in mammalian cells, there is concern that their large-scale production could be affected by an inadequate supply of bovine serum. There is also the risk of viral infection spreading through the use of contaminated protein therapeutics. Consequently, protein expression systems in yeast have been established because protein manufacturing costs are cheaper than in mammalian cells, and yeast systems are virus-free. However, yeasts cannot generate human-type glycans, and thus cannot produce therapeutic glycoproteins for human use. There has therefore been considerable interest in glycan remodeling, from yeast-type to human-type. 'Humanized' glycoproteins can now be generated in yeast by disrupting yeast-specific glycosyltransferases and introducing genes responsible for sugar-nucleotide synthesis, its transported from the cytosol to Golgi lumen, as well as their transfer and hydrolysis. A compound that inhibits yeast *O*-mannosyltransferase suppresses yeast-specific *O*-mannosyl modification, and can produce mucin-type glycoproteins. These systems are just being developed to the stage where the production in glycoengineered yeast of biopharmaceutical glycoproteins such as cytokines, antibodies for therapeutics, and enzymes for replacement therapy for lysosomal diseases are being evaluated for clinical applications. Yeast glycoprotein expression systems are expected to become the dominant approach for the production of human glycoproteins in the near future.

Key words yeast; mammalian-type glycan; production; biopharmaceutical glycoprotein; antibody; lysosomal disease

1. INTRODUCTION

Protein-based therapeutics, such as therapeutic antibodies, enzymes for replacement therapy for lysosomal diseases, and cytokines, has received attention in recent years. For example, sales of antibodies for pharmaceuticals are expected to exceed 30 billion U.S. dollars by 2010.[1] These therapeutic proteins and peptides are almost exclusively produced in mammalian expression systems, in particular Chinese hamster ovary (CHO) cell lines, because the majority of therapeutic proteins are glycosylated post-translationally. Glycosylation is of interest since it is involved in protein folding, the stability of the protein in the bloodstream, and protein–protein interactions, as well as contributing to the activity of some proteins.[2,3] CHO cells produce glycosylated proteins that differ slightly from human proteins; for example, *N*-glycolylneuraminic acid is not observed in humans, but is found in other mammals. Subtle differences in glycan structure can cause an immune response, as evidenced by the generation of antibodies towards that protein in human sera.[4] For this reason, as well as high production costs, an inadequate bovine serum supply, and the risk of infectious diseases, production systems for protein therapeutics are increasingly switching to serum-free cultures, and alternative protein expression systems are being developed worldwide.

Microorganisms such as bacteria and yeasts have been extensively used to produce industrial proteins and enzymes, and are often the expression system of choice when manufacturing costs are of primary concern. However, post-translational modification in microorganisms is quite different from that in mammalian cells. Although it has been assumed that glycan modification of proteins cannot occur in bacterial cells due to their less-developed organelles compared to eukaryotic cells, recent results suggest the presence of similar, but not identical, systems for protein glycosylation in bacteria.[5—7] In contrast, yeasts share the protein glycosylation system in the secretory pathway; however, the sugar chains produced in yeast are composed of mannose polymers, which are somewhat antigenic in humans and are cleared by mannose-specific receptors or lectins. Therefore glycan remodeling to human glycans is required for the production of therapeutic glycoproteins in yeast. In this article, we review recent advances in the 'humanization' of the yeast glycosylation pathway and examine several case studies in which mammalian-type glycoproteins were produced in yeast.

2. 'HUMANIZATION' OF THE GLYCOSYLATION SYSTEM IN YEAST

A typical glycosylation in the budding yeast *Saccharomyces cerevisiae* is shown in Fig. 1. Early steps in *N*-glycan processing, involving the assembly of the core oligosaccharide, its site-specific transfer onto the protein, and its trimming by several glycosidases, are all highly conserved from yeast to humans. However, a series of glycosyltransferase reactions in the Golgi apparatus are distinctly different in humans compared to yeast. In yeast, several mannosyltransferases act on the *N*-glycan intermediate and more than fifty mannose residues are attached.[8] In the case of *O*-glycan, fewer than five mannose residues are attached linearly to

* To whom correspondence should be addressed. e-mail: y-chiba@aist.go.jp

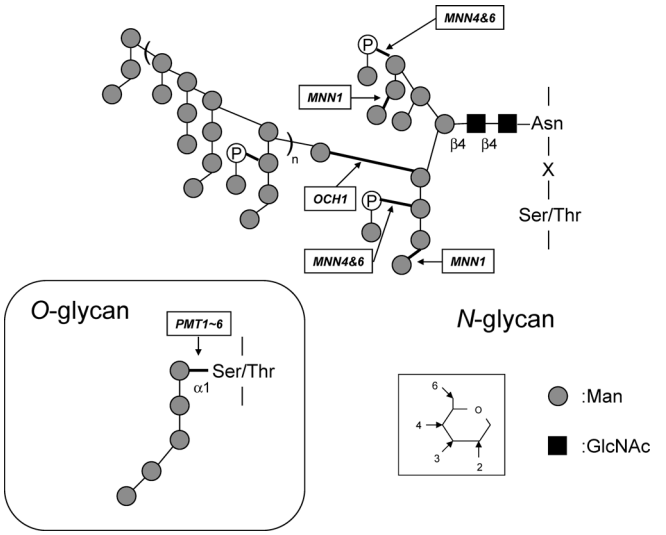

Fig. 1. Schematic Representation of *N*- and *O*-Glycan Structures of *Saccharomyces cerevisiae*

All linkages are alpha-anomeric bond except for beta-linkages indicated in the figure. In the bottom right frame, each linkage is indicated as a number. The genes encoding main enzymes related to yeast-specific modification are represented in bold.

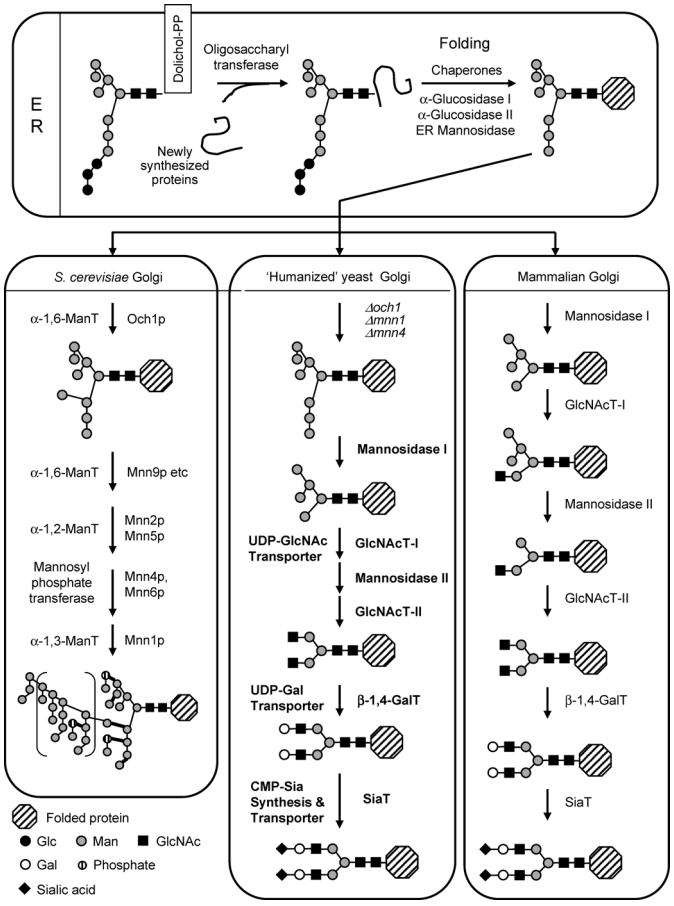

Fig. 2. Comparison of the *N*-Glycosylation Pathway in Mammalian Cells and *S. cerevisiae*, and Strategy for Genetic Manipulation of 'Humanized' Yeast

Deletion of specific yeast genes involved in hyper-mannosylation (*OCH1*, *MNN1* and *MNN4*) and introduction of the genes responsible for the modification of sugar chains to mammalian-type, shown in bold, are required for creation of 'humanized' yeast.

Ser or Thr, where the initial reaction is catalyzed by *O*-mannosyltransferases encoded by *PMT* genes.[9)]

A key technology for humanized *N*-glycan production in yeast was established in 1992, when a mutant strain (*och1*) was isolated.[10)] *OCH1* gene encodes α-1,6-mannosyltransferase, which transfers an initial mannose residue to an *N*-glycan intermediate ($Man_8GlcNAc_2$) in the Golgi.[11)] Disruption of *OCH1* causes a loss of hyper-mannosylated structure in the secreted glycoproteins. Four years later, the whole-genome sequence of *S. cerevisiae* became available[12)] and most genes and encoding proteins responsible for glycosylation were elucidated. During the same period, our group showed that disruption of three genes (*OCH1*, *MNN1*, and *MNN4*) led to the production of an intermediate *N*-glycan structure identical to the human structure.[13,14)] This provided the basis for the humanization of yeast *N*-glycans. To date, all successful efforts to humanize yeast *N*-glycosylation pathways have focused on the deletion of specific yeast genes involved in hyper-mannosylation initiated by Och1p, and of species-specific genes responsible for the modification of sugar chains at the non-reducing end, as shown in Fig. 2.

These findings were followed by the introduction of genes catalyzing the synthesis, transport, and transfer of human sugars. At first, we succeeded in producing the oligomannosyl structure ($Man_5GlcNAc_2$) in *S. cerevisiae* using a triple disrupted strain by introducing α-1,2-mannosidase from *Aspergillus saitoi*.[14)] Next, mammalian-type sugar chain production in the methylotrophic yeast, *Pichia pastoris*, was reported.[15)] This elegant technique uses combinatorial libraries consisting of transmembrane domains of known Golgi and endoplasmic reticulum (ER)-localized proteins, and catalytic domains of several glycosyltransferases and glycosidases from many species. The authors also succeeded in making an erythropoietin containing sialylated biantennary sugar chains by introducing several enzymes required for cytidine 5′-monophosphate (CMP)-sialic acid biosynthesis and sialyltransferase into a previously engineered yeast strain.[16)] Creation of not only CMP-sialic acid but also other sugar-nucleotides such as guanosine 5′-diphosphate (GDP)-fucose (GDP-Fuc),[17)] uridine 5′-diphosphate (UDP)-xylose (UDP-Xyl),[18)] UDP-glucuronic acid (UDP-GlcA),[18)] UDP-galactose (UDP-Gal)[19)] and UDP-β-*N*-acetylgalactosamine (UDP-GalNAc)[19)] have been reported, suggesting that all sugar-nucleotides for production of human-type glycans in yeast are now available, and that the ability to control glycan structure at will in yeast is close to being realized.

3. ANTIBODY PRODUCTION IN YEAST

As described above, antibodies for pharmaceutical application have great potential but their production is expensive, in part due to the fact that antibodies are commonly manufactured using batch/fed-batch cultures of mammalian cells. To solve this problem, transgenic plants and animals for monoclonal antibody production have been developed and studied, and antibodies have been successfully expressed in plants, the milk of transgenic goats and the eggs of transgenic chickens.[20—22)] The production of antibodies and partial antibody fragments by several microorganisms has also been studied, including *Escherichia coli*,[23)] fungi[24)] and yeasts.[25)] In the case of yeasts, although the expression of

antigen-binding fragments (Fab) and single chain fragment variants (ScFv) is apparently easy, the production of full length antibodies had not been reported, in part because the structure of a full length antibody (H_2L_2) is very complex, consisting of two heavy chains (H) and two light chains (L) with six intermolecular disulfide bonds.

One of the therapeutic benefits of monoclonal antibodies arises from two binding events. First, the variable region of the antibody binds a specific protein on the target cell, then effector cells such as monocytes and natural killer cells bind to the constant (Fc) region and kill the target cell to which the antibody has bound. This mechanism is called antibody-specific cell cytotoxicity (ADCC). Human monoclonal antibodies contain one *N*-glycan moiety in the Fc region. Terminal sugars such as sialic acids, core fucose, bisecting *N*-acetylglucosamine, and mannose residues in the *N*-glycan affect the binding of immunoglobulin G (IgG) to the FcγRIIIa receptor and thereby influence ADCC activity.[26] In 2006, production of IgG monoclonal antibody in glycoengineered *P. pastoris* was reported.[27] In this study, human IgGs with specific mammalian-type *N*-glycans were expressed in *P. pastoris* harboring mammalian glycosyltransferases. ADCC activity has been shown to optimize the generation of specific glycoforms in *P. pastoris* strains. However, the binding activity of the antibody from *P. pastoris* to the antigen is slightly weaker than that of the same antibody from mammalian cells.

When antibodies were expressed in another methylotrophic yeast, *Ogataea minuta*, we found abnormal *O*-mannosylation on the secreted antibody.[28] As mentioned above, *O*-mannosylation is a common modification in yeast. The initial reaction is catalyzed by *O*-mannosyltransferases encoded by *PMT* genes, but complete disruption of the *PMT* genes is almost impossible because *O*-mannosylation is vital to yeast cells.[29,30] We therefore examined the possibility of inhibiting Pmt activity by adding a Pmt inhibitor, (5-(3,4-bisphenylmethoxyphenylmethylene)-4-oxo-2-thioxo-3-thiazolidineacetic acid; R3A-1c)[31] during cultivation to suppress the addition of *O*-linked sugar chains to antibodies. This Pmt inhibitor partially suppressed *O*-mannosylation of the antibodies. Interestingly, the suppression of *O*-mannosylation was associated with an increased amount of assembled antibody (H_2L_2) and enhanced antigen-binding activity of the secreted antibody. It is possible that the combination of single or double disruption of *PMT* genes and the addition of an optimized concentration of Pmt could completely suppress *O*-mannosylation of antibodies.

4. CREATION OF MAMMALIAN *O*-GLYCOSYLATION SYSTEMS IN YEAST

Several *O*-modifications are found in mammals, such as mucin-type (*O*-linked β-*N*-acetylgalactosamine: *O*-GalNAc), *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc), *O*-linked fucose (*O*-Fuc), *O*-linked glucose (*O*-Glc), *O*-linked galactose (*O*-Gal), *O*-linked xylose (*O*-Xyl) and *O*-mannose (*O*-Man). *O*-GlcNAc modification is observed on serine and threonine side chains of myriad nuclear and cytoplasmic proteins involved in almost all cellular functions. Addition and trimming of *O*-GlcNAc residue on serine or threonine like phosphorylation is nearly abundant, and functions at least partially, *via* its interplay with phosphorylation.[32] *O*-Glc and *O*-Fuc are rare post-translational modifications that have highly functional relevance in the early stages of development and are vital for the physiological functions of certain proteins.[33] Several serum proteins contain these unique modifications. Some *O*-fucose moieties are elongated by the action of members of the Fringe family of β-1,3-*N*-acetylglucosaminytransferases. Although *O*-Fuc occurs on thrombospondin-1 repeats and epidermal growth factor (EGF)-like domains, *O*-Glc and *O*-Fuc together occur only on a single EGF domain in close proximity to each other.[32] *O*-Gal is found in collagen,[34] and is a major modification in plants.[35] *O*-Xyl is an initial modification in the biosynthesis of chondroitin, dermatan and heparan sulfates present in the ER and Golgi apparatus.[36] *O*-Man is a highly conserved modification among eukaryotes, from yeast to humans, where the initial reaction is catalyzed by *O*-mannosyltransferases encoded by *PMT* genes.[8] Although *O*-Man modification has been considered to be specific to eukaryotes, it has been found that several Actinobacteria strains produce glycoproteins that contain mannose residues.[5,37] In mammalian cells, *O*-Man modification was observed in α-dystroglycan, and a major component tetrasaccharide with the structure NeuAcα2-3Galβ1-4GlcNAcβ1-2Man has been characterized[38] and could be the ligand specific for interaction with laminin G domains. It is known that distinct forms of congenital muscle dystrophies (α-dystroglycanopathies) are due to mutation defects of glycosyltransferases involved in *O*-mannosylation of α-dystroglycan (see review[39]).

Unlike *N*-glycosylation pathways, *O*-glycosylated modifications (other than *O*-Man) had not been attempted in yeast because of the lethality of the deletion of *O*-mannosylation, as mentioned above. Recently, our group demonstrated two production systems for mammalian-type *O*-glycoproteins in *S. cerevisiae*: a mucin-type glycosylation[18] and an *O*-fucosylation (*O*-Fuc).[40] In the latter system, modification with *O*-Fuc was achieved by introducing several genes involved in the conversion of GDP-Fuc from GDP-mannose, together with the protein *O*-fucosyltransferase-1 (*O*-FucT-1). O-FucT-1 recognizes Ser or Thr residues adjacent to the third conserved cysteine within the consensus sequence $C^2X_{4-5}(S/T)C^3$.[41,42] Furthermore, *O*-Fuc has been pro-posed to play a role in protein quality control and thereby affects secretion.[43—45] Expression of EGF domain mutants by this system revealed that *O*-fucosylation occurs without competition with the endogenous *O*-mannosylation pathway, and that the three disulfide bonds in the EGF domain contribute differently to *in vivo* *O*-fucosylation. Further introduction of the human Manic fringe (β-1,3-*N*-acetylglycosaminyltransferase) gene into yeast equipped with the *in vivo* *O*-fucosylation system facilitated the addition of *N*-acetylglucosamine to the EGF domain from factor IX, but not from factor VII. This indicates that the three-dimensional structure of the *O*-fucosylated EGF domain, in addition to the amino acid sequence as reported previously,[43] may affect recognition by Manic fringe. These peptides may be useful for basic studies of the Notch signaling pathway, including substrate specificity analysis and the production of antibodies recognizing *O*-glycosylated EGF domains.

Mucin-type glycans are a common *O*-linked sugar chain in mammals.[46] Mucin-type modification is initiated by *O*-

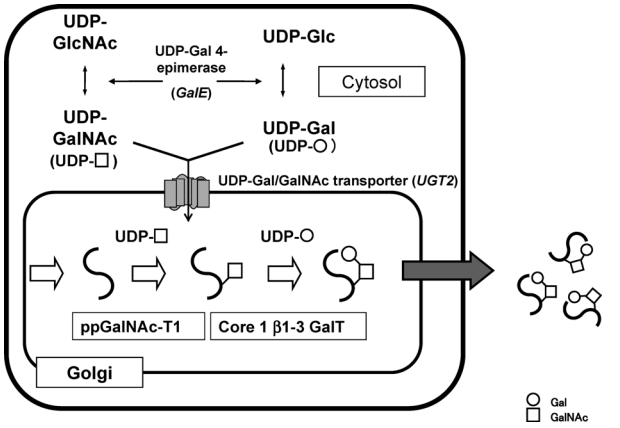

Fig. 3. Schematic Representation of Production of Glycoprotein with Mucin-Type *O*-Glycan in Yeast

Each gene encodes: GalE, *Bacillus subtilis* UDP-Gal 4-epimerase; UGT2, human UDP-Gal transporter 2; ppGalNAc-T1, human UDP-GalNAc:polypeptide α-*N*-acetylgalactosaminyltransferase-1; Core 1 β1-3GalT, *Drosophila melanogaster* β-1,3-galactosyltransferase.

linked GalNAc to Ser or Thr residues on a peptide backbone. Although its function remains unclear, it is believed to be involved in the processing of hormones,[47] in endocytosis,[48] and in the sorting of apical proteins in the *Drosophila* embryo.[49] Mucin-type glycans are sometimes clustered, forming the "mucin domain" found on mucins,[50] which function as a selective molecular barrier at the epithelial surface[51] and are involved in morphogenetic signal transduction.[52] Changes both in expression of mucin and in their glycosylation state are closely associated with the development of cancer and cancer-related processes such as cell growth, differentiation, adhesion, invasion and immune surveillance.[46] Since there is interest both in mucin function and its application as a pharmaceutical, a yeast strain producing a mucin-type glycan has been created.[19] A strategy of mucin-type glycan production is shown in Fig. 3. We first generated a system for *in vivo* production of MUC1a peptides containing *O*-linked GalNAc and the core1 structure (Galβ1-3GalNAcα1-*O*-Ser/Thr). Surprisingly, little (less than 10%) competition with *O*-mannosylation was observed, and use of the Pmt inhibitor (R3A-1c) as described in Section 3 caused complete suppression of *O*-mannosylation. We then engineered a yeast capable of producing functional podoplanin. Podoplanin (also called aggrus)[53,54] is a very interesting mucin-type glycoprotein that acts as a platelet-aggregating factor for cancer cells and may be involved in tumor metastasis. Additionally, podoplanin is a potential diagnostic marker for many tumors including testicular tumors, several squamous cell carcinomas and brain tumors, and may be associated with malignancy.[55,56] We showed that yeast-produced podoplanin with a specific *O*-glycan structure (sialyl core1 (NeuAcα2-3Galβ1-3GalNAcα1-*O*-Ser/Thr) and sialyl Tn-antigen (NeuAcα2-6GalNAcα1-*O*-Ser/Thr) structures) on a specific amino acid residue (Thr52) possesses platelet aggregation activity. Our study indicated that a combination of glycosyltransferases introduced into yeast cells allowed the analysis of the structure-function relationship of *O*-glycans on proteins. We believe that production of mucin-type glycoproteins in yeast will further the course of basic research and pharmaceutical applications in the near future.

5. PRODUCTION OF RECOMBINANT ENZYMES IN YEAST FOR TREATMENT OF LYSOSOMAL DISEASES

Treatment of Lysosomal Storage Diseases In the lysosome, glycoconjugates are catabolized by exohydrolases. Each step of the catabolic pathway is mediated by an enzyme with different substrate specificity, with or without the interaction of cofactors. However, when there is insufficient or defective hydrolase or cofactor due to a genetic disorder, the catabolic pathway is blocked and the hydrolase substrate accumulates in the cell. Blockage of the degradation pathway results in cellular dysfunction leading to various symptoms. Over 40 diseases due to deficiency in lysosomal proteins have been identified and are collectively called lysosomal storage diseases (LSDs). The onset, progression, and severity of symptoms are dependent on the type of disease and the mutation site on the responsible protein. Some examples of pathologies are neurological symptoms or retardation, skeletal deformities, edema, hepatomegaly, anemia and apasticity.

Numerous approaches have been attempted to treat LSDs such as chemical chaperones,[57,58] substrate deprivation,[59,60] enzyme replacement,[61—64] gene therapy,[65—68] and bone-marrow transplantation,[69—71] but all suffer from problems in their clinical application.

Chemical chaperone therapy (CCT) and substrate deprivation therapy (SDT) involve the administration of an enzyme inhibitor. In CCT, the enzyme inhibitor forms a complex with the target enzyme, leading to stable trafficking to the lysosome. The basis of SDT is the reduction of accumulated glucosylceramide by partial inhibition of glucosylceramide synthesis with imino-sugars, thereby reducing the burden of subsequent enzyme reactions.[72] These therapies may be effective for the brain and central nervous system, since iminosugar inhibitors can pass through the blood–brain barrier (BBB). However, the application of CCT and SDT is limited to patients expressing a destabilized enzyme with reduced activity.[58,73] Deficient hydrolysis due to low enzyme activity in α-galactosidosis, β-glucocerebrosidosis, β-galactosidosis, and β-hexosaminidosis (which cause Fabry, Gaucher, GM1-, and GM2-gangliosidosis, respectively), can be controlled by SDT. Low side effects and high therapeutic effects have been reported following the administration of appropriate doses,[74,75] although administration of *N*-butyldeoxynojirimicin to mice resulted in side effects such as reduced body weight and toxic effects on some tissues, which limits dosage-escalation in this therapy.[76] Derivatives of *N*-butyldeoxynojirimicin have been approved for treatment of Gaucher disease and are now in clinical trials for some other LSDs.[72]

Experiments using gene therapy and bone-marrow transplantation (BMT) have provided good results in delivering normal enzymes into the brain and neural cells. BMT treatment is more effective to perform before development of disease for which quick decision is required.[77,78] However, the identification of suitable donors is time consuming,[77] which is contradictory to above suggestion. Likewise, problems arise during the clinical application of gene therapy, such as difficulties in controlling the expression and localization of the enzyme,[77] and BMT is a potentially dangerous procedure.[79] Differences in the degree of improvement among LSD patients following treatment[70,78,80—83] suggest the need for careful consultation prior to treatment.

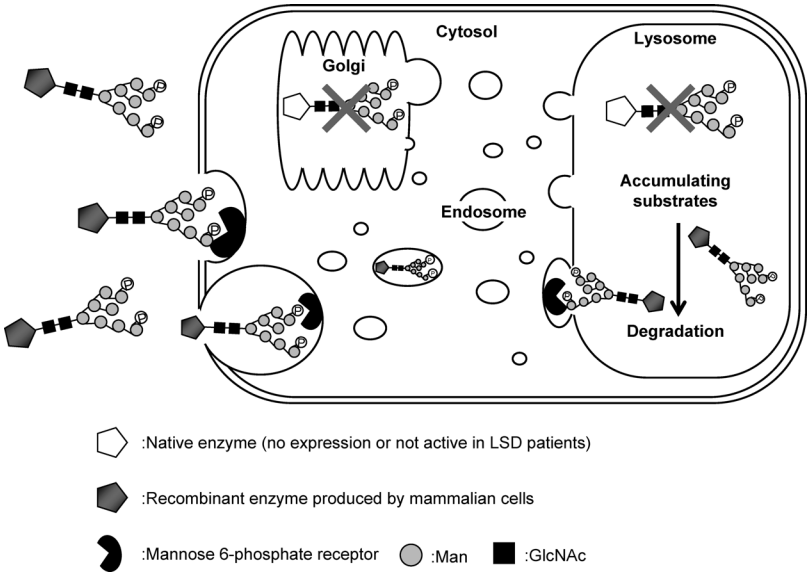

Fig. 4. Schematic Diagram for Enzyme Replacement Therapy of Lysosomal Storage Disease

In the patient's cell, a lysosomal enzyme is not produced at all or produced only in inactive form, thus its substrate is not hydrolyzed and accumulates in the cell. To take place of the native enzyme, recombinant enzyme is administrated and incorporated to the cell through the receptor on the cell surface, which basically recognizes terminal mannose 6-phosphate residues on *N*-glycan. Recombinant enzyme is then transported to the lysosome through the endosome and degrades accumulating substrates.

Enzyme replacement therapy (ERT) is the most advanced therapeutic for treating LSDs and has been applied to Gaucher disease,[61] Fabry disease,[62,63] Pompe disease,[84,85] and mucopolysaccharidosis I,[64] II[86] and VI.[87] Since fully active recombinant enzymes produced mainly in mammalian cells are administrated in ERT, this treatment is useful for most patients who either lack expression of the enzyme or express too little of it. Additionally, no complicated surgery is required. The administered recombinant enzymes have mannose-6-phosphate (M6P) residues at the non-reducing end of the *N*-glycan which are recognized by the M6P receptors on the cell surface, resulting in their incorporation into the cell (Fig. 4). ERT is effective mainly on somatic cells and not the brain or central nervous system, since enzyme molecules cannot cross the BBB. However, there have been some reports that the long-term administration of an enzyme at high dose improves the therapeutic response in the brain.[88,89] It has also been noted that antibodies are sometimes raised against the administrated protein, for example, when the therapy was applied to a knockout mouse model,[90] or to patients lacking expression of the target enzyme.[91] A study of ERT for Fabry disease indicated that more than half of male patients showed high titers of IgG antibodies that cross-react *in vitro* similarly with the recombinant enzymes after 6 months of treatment.[91] The raised antibody circulates in the blood and sometimes reduces the therapeutic effect by capturing newly administrated enzyme.

Regardless, ERT is a well-established and proven method and is theoretically applicable to all LSDs. We therefore believe that affordable enzymes would help make LSD a more curable disease by making this treatment available to all patients. What is required is a system in which a large amount of enzyme can be produced at low cost.

Production of Recombinant α-Galactosidase A in Yeast for ERT of Fabry Disease To date, recombinant enzymes for replacement therapy have been isolated from mammalian cells because the glycan structure of these mammalian enzymes is similar to that of human enzymes. However, the production of adequate amounts of protein for therapeutic purposes in mammalian cells is expensive, and careful monitoring for viral infection is essential.[92] Therefore, a more convenient protein-expression host is desirable. We have produced recombinant human α-galactosidase A (α-GalA) in the yeast *S. cerevisiae*, manipulated to produce protein with human-like glycan structures (Fig. 5). The *S. cerevisiae* HPY21 strain produces glycoprotein in which a polymannosylated structure specific to yeast (and not found in humans) was eliminated by deletion of the *OCH1* (initial α-1,6-mannosyltransferase) and *MNN1* (terminal α-1,3-mannosyltransferase) genes, thereby potentially solving the antigenicity of yeast-specific *N*-glycan structures.[93] In ERT, enzymes enter the cell *via* the M6P receptor on the cell surface, so recombinant α-GalA with highly phosphorylated *N*-glycan would be expected to be an effective therapeutic. *S. cerevisiae MNN4* is a positive regulator for phosphomannosyltransferase (Mnn6p), which facilitates phosphorylation of the sugar chain. Overexpression of ScMnn4p increases phosphorylated *N*-glycan content in *S. cerevisiae*.[94] The HPY21 strain contains a mutation in the promoter region of *MNN4*, so that Mnn4p is constitutively produced.[93] The non-reducing end of the M6P residue in recombinant α-GalA *N*-glycan is covered by a mannose residue, but it must be exposed for M6P receptor recognition. To remove the outer mannose residue from M6P, recombinant α-GalA was treated with an α-mannosidase. This α-mannosidase was produced by a *Cellenomonas* species isolated from soil. Supernatant from an enriched culture grown on Baker's yeast mannan was used for treatment.[92,93]

α-GalA deficiency causes Fabry disease, with ceramide trihexoside (CTH) accumulation. The pathology of Fabry disease includes painful neuropathy and renal, cardiovascular and cerebrovascular dysfunction.[95] Purified recombinant α-GalA was introduced into Fabry patient fibroblasts *via* the M6P receptor, and its dose-dependency and pharmacokinet-

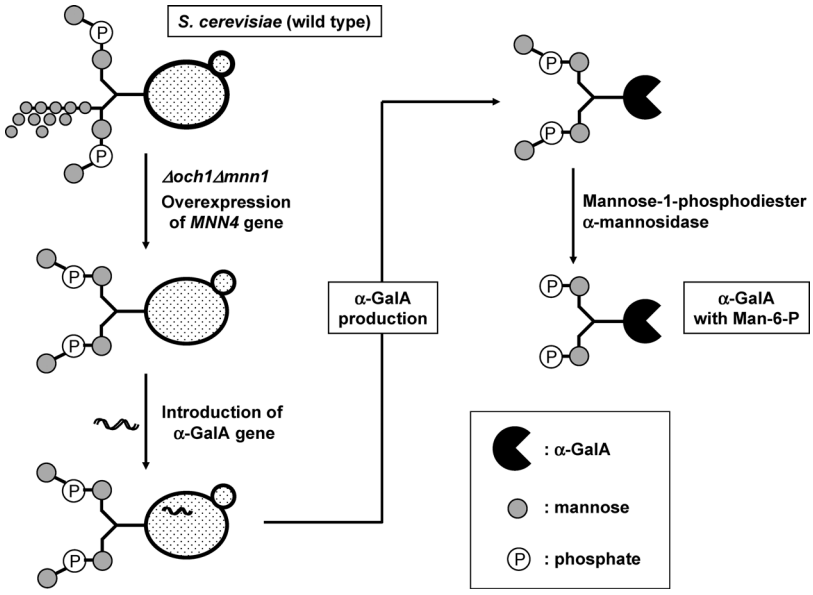

Fig. 5. Production of the Recombinant Human α-Galactosidase (α-GalA) in *S. cerevisiae*

Deletion of specific yeast genes involved in hyper-mannosylation (*OCH1* and *MNN1*) and overexpression of *MNN4* genes responsible for mannosylphosphate transfer in *S. cerevisiae* are required for production of highly phosphorylated *N*-glycan. The recombinant α-GalA is treated with a cultural supernatant of *Cellenomonas* species that contains mannose-1-phosphodiester α-mannosidase activity to remove the outer mannose residue from mannose 6-phosphate.

ics of incorporation were also examined.[93,96] Furthermore, weekly enzyme administration to a Fabry mouse model for 4 weeks revealed that recombinant α-GalA was distributed into the liver, kidney, heart and spleen. Immunohistochemical analysis of viscera from mice treated with α-GalA showed CTH degradation in all the organs examined, except for insufficient CTH cleavage in the glomeruli.[96] These results suggest the possibility of using yeast as a host to produce recombinant enzymes for replacement therapy.

Production of Recombinant HexA in Yeast for ERT of GM2-Gangliosidosis Next, we focus on yeast recombinant enzymes for the treatment of GM2-gangliosidosis. This disease is caused by a deficiency of β-hexosaminidase A (HexA, E.C. 3.2.1.52), which hydrolyzes β-glycosidically linked *N*-acetylglucosamine or *N*-acetylgalactosamine residues at the non-reducing end of glycoconjugates. Upon coactivation by the GM2 activator protein, HexA degrades GM2 gangliosides in the lysosome.[97] HexA is composed of two subunits, α and β, which have 57% similarity in their amino acid sequences. Three isozymes are normally found in mammalian cells: HexA ($\alpha\beta$ heterodimer), HexS ($\alpha\alpha$ homodimer) and HexB ($\beta\beta$ homodimer), with HexA and HexB as the major forms and HexS as a labile minor form.[98,99] Unlike HexA, the two homodimers are incapable of hydrolyzing GM2 ganglioside, but all three isozymes can hydrolyze the fluorescent substrate, 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide (MUG). There are three types of GM2 gangliosidosis: Tay-Sachs (TS), Sandhoff (SD), and AB variant, with defects in the α-subunit, β-subunit, and GM2 activator protein, respectively. The pathology of GM2-gangliosidosis is exhibited as neural disorders, and its rapid progression leads to a vegetative state within a few years. Macular red cherry red spots are commonly observed. In the case of slower progression of the disease, ataxias, dysarthrias and muscle weakness are also observed. At present, there is no effective treatment for this disease.

Enzyme replacement using recombinant human β-hexosaminidase (mixture of all isozymes) from CHO cells has been examined in SD mouse microglia cells,[100] SD mouse Schwann cells, and fibroblast cells from SD patients.[101] Since further ERT experiments in mouse models and molecular studies on recombinant HexA are required, the β-hexosaminidase was expressed in a yeast expression system in order to obtain the required large amounts of enzyme (Fig. 6).

In addition to production in *S. cerevisiae*, recombinant α-GalA was also produced in the methylotrophic yeast *P. pastoris* with a productivity of 4.5 mg/l,[102] which is higher than that in *S. cerevisiae* (1 mg/l[93]). Since high expression of recombinant enzyme was expected, we chose the methylotrophic yeast *O. minuta* as a host for recombinant HexA expression. As with *S. cerevisiae*, an *OCH1* disrupted strain, *O. minuta* TK5-3,[103] was used as a host. The genes coding for the α and β subunits of HexA were co-expressed in *O. minuta* TK5-3 under the alcohol oxidase (*AOX1*) promoter. As in other mammalian expression systems, co-expression of the two HexA subunits produces not only HexA but also the homodimeric isozymes HexS ($\alpha\alpha$) and HexB ($\beta\beta$).[100,104] The *O. minuta* strain produced 14.3 mg of β-hexosaminidase isozymes from 1 l of culture broth; HexA constituted 23% of the total isozymes, based on the MUG hydrolyzing activities of the isolated isozymes.[105]

The host *O. minuta* strain was manipulated to increase the amount of phosphorylation on recombinant HexA *N*-glycans. As in the case of α-GalA expression in *S. cerevisiae*, expression of *MNN4* was increased. *OmMNN4*, a gene homologous to *ScMNN4*, was introduced under the AOX promoter (Om4 strain) to the HexA expressing strain (mock). Overexpression of OmMnn4p resulted in expression of recombinant HexA with highly phosphorylated *N*-glycans (Akeboshi *et al.*, manuscript in preparation). M6P receptor-blotting analysis of HexA and M6P-exposed HexA (M6PHexA) using recom-

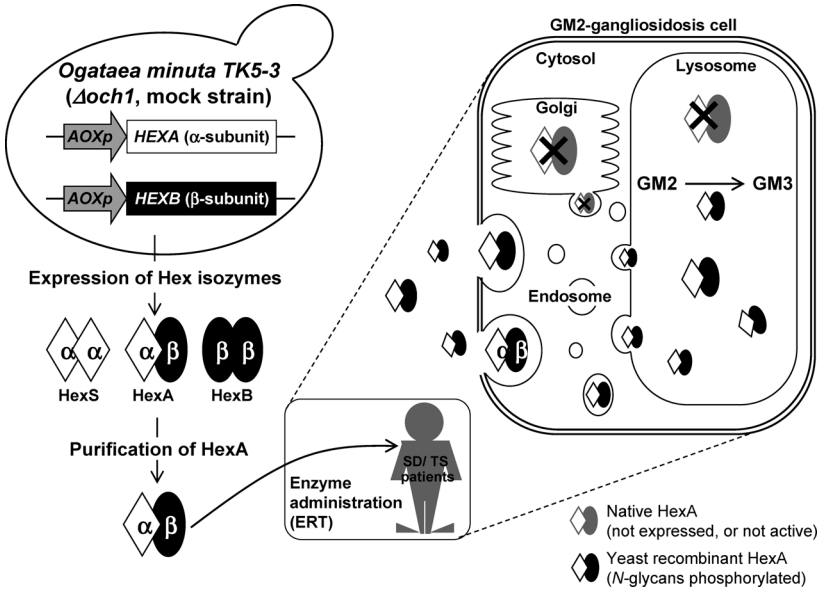

Fig. 6. Outline for ERT of GM2-Gangliosidosis by Yeast Recombinant HexA

Yeast recombinant HexA is produced by overexpression of *HEXA* and *HEXB* genes, which encodes α- and β-subunits of HexA, respectively. Crude enzyme is produced as the mixture of three isozymes, HexB, HexS and HexA. HexA is isolated from the crude mixture and M6PHexA is prepared by exposure of M6P residues of HexA *N*-glycans. Sandhoff and Tay-Sachs patients accumulate GM2-ganglioside (GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1Cer) in the cell. When M6PHexA is administrated to the patients, M6PHexA is incorporated into the cell through M6P receptor and transported to the lysosome. It degrades the accumulated GM2-ganglioside to GM3-ganglioside (NeuAcα2-3Galβ1-4Glcβ1-1Cer).

binant domain 9 of the M6P receptor (M6P binding domain[106]) detected signals specific for M6PHexA.[105]

Cellular uptake of purified M6PHexA was examined. Fibroblasts derived from SD and TS patients were cultured in HexA- or M6PHexA-containing medium. Only M6PHexA was incorporated into the cells, and incorporation was inhibited by the co-addition of M6P, suggesting that this enzyme is incorporated *via* the M6P receptor.[105] HexA untreated with α-mannosidase was not incorporated, which further supports M6P receptor-mediated incorporation.[105] The incorporation of mock- and Om4-M6PHexA in GM2-gangliosidosis human fibroblasts and SD mouse neuronal cell lines was compared. Immunostaining for GM2-gangliosides in the fibroblasts after Om4-M6PHexA incorporation suggested their rapid degradation in administrated cells, which is much better than the effect of the same dose of mock-M6PHexA (Akeboshi *et al.*, manuscript in preparation). Therefore, phosphorylation-dependence is more important than dose-dependence for enzyme incorporation.

Neural pathologies arising from diseases such as GM2-gangliosidosis can be markedly improved by enzyme replacement to the brain and central nervous system, although there are technical challenges due to the BBB. There is no established method for the clinical application of enzyme replacement, and experimental approaches continue to be studied.[107,108] One approach for delivering enzymes to the brain is the conjugation of tags that can cross the BBB to the target protein. These tags include the atoxic fragment C of tetanus toxin,[107] monoclonal antibodies to the human insulin receptor,[109] monoclonal antibodies to the rat transferrin receptor,[109] and an acidic amino acid (AAA) tag.[110] In another experimental approach, the protein is encapsulated in a liposome conjugated with a BBB-crossing tag such as transferrin,[111] and is delivered into the brain. Enveloping or tagging proteins or peptides with polyethylene glycol (PEGylation) has also been attempted, and has been combined with other techniques.[109,111] Sawada *et al.* found that intra-arterially injected microglia migrated specifically to the brain, but were rarely found in the liver. This system is therefore a candidate for brain-specific delivery of medicines and other bioactive materials,[92] including lysosomal enzymes for replacement therapy. Since effective degradation of GM2 has been confirmed in neuronal cells (Akeboshi *et al.*, manuscript in preparation), it is predicted that once recombinant M6PHexA passes through the BBB, efficient treatment can be expected. In conjunction with BBB penetration techniques, our yeast-derived recombinant enzymes can be applied to the treatment of LSDs with severe neuronal pathologies.

For diseases like LSDs, for which ongoing administration of enzyme is necessary, inexpensive and adequate amounts of enzyme are required. It will also be necessary in the future to produce various types of enzymes to treat different types of LSDs. Modification of the target protein, such as changing the glycan structure, facilitating *N*-glycan phosphorylation, raising productivity and/or adding tags or introducing mutations, can easily be performed by disrupting the target gene or introducing foreign target genes into a yeast expression system. Yeast is a very promising system for producing therapeutic enzymes for LSDs given their versatility for producing clinically useful enzymes.

6. CONCLUSIONS AND PERSPECTIVE

This review has provided a brief overview of 'humanized' glycoprotein production in yeast. A new endeavour in this field is the production of human-type glycosaminoglycans (GAGs). Since UDP-glucuronic acid (GlcA) and UDP-xylose can be synthesized in yeast by introducing the genes encoding UDP-Glc dehydrogenase and UDP-GlcA decarboxylase from *Arabidopsis thaliana*,[18] it should be possible to

produce GAGs using a strategy similar to that used in *N*-glycan engineering. However, sulfation of glycans, especially GAGs, in yeast is problematic. A future issue will be how to produce more complex glycans, such as sialyl LeX on a polylactosamine structure, and fully sialylated tetraantennary *N*-glycan. The most important challenge is to create a homogenous glycan on the target protein in yeast, although this is an issue common to all cellular expression systems. Further studies are required to fully understand the mechanisms controlling intracellular trafficking and localization of glycosyltransferases.

The development of analytical instruments for the analysis of glycans, such as lectin arrays and mass spectrometry, has simplified the determination of glycan structures. In addition, many genome projects have also provided information about the function of glycan-related genes. For example, we used *Arabidopsis* genes for conversion of GDP-Man to GDP-Fuc, and a *Drosophila* gene for the synthesis of the core 1 structure. New findings and developments in other research areas will help to establish better production systems in yeast. Since the scale-up production of recombinant proteins in yeast is a well-established technology, further development of glycoengineered yeast systems will lead to the production of lower-priced pharmaceutical products. We also expect that expression of targeted gene or cDNA libraries by our system will lead to the identification of additional functional glycopeptides and glycoproteins that can be developed into therapeutics.

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