Glycosylation engineering in yeast: the advent of fully humanized yeast
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Yeasts have been extensively used as model organisms to elucidate cellular processes and their mechanism in lower eukaryotes. Consequently, a large number of powerful genetic tools have been developed to engineer yeast and improve its utility. These tools and the development of efficient large-scale fermentation processes have made recombinant protein expression in yeast an attractive choice. However, for the production of glycoproteins for human use, native high-mannose yeast glycosylation is not suitable and therefore represents a major limitation for yeast based protein expression systems. Over the last two decades several groups have attempted to overcome this problem, yet with limited success. Recently however, major advances in the glycoengineering of the yeast Pichia pastoris, have culminated in the production of fully humanized sialylated glycoproteins.

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Introduction
Protein-based therapeutics are emerging as the fastest growing class of compounds within the pharmaceutical industry [1]. Since the introduction of the first recombinant therapeutic protein, human insulin, numerous proteins have been approved for therapeutic use in humans, including erythropoietin, various interferons, blood-clotting factors, as well as monoclonal antibodies. Traditionally, Escherichia coli and mammalian cell expression systems have been developed to express aglycosylated and glycosylated proteins, respectively. With the majority of therapeutic proteins being glycosylated, mammalian cell culture, in particular Chinese hamster ovary (CHO) cell lines, emerged as the expression host of choice for the production of therapeutic glycoproteins. N-glycosylation is of particular interest since it is involved in protein folding, in vivo half-life and often contributes to protein function [2,3]. To date mammalian cell culture has been the only process to yield human-like glycoproteins, but differences to proteins isolated from humans have been found, and have been the source of some concern. For example, most animals produce glycoproteins containing the sialic acid N-glycolylneuraminic acid, which is absent in humans [4]. As a consequence, glycoproteins produced by mammalian cell culture contain this aberrant sialic acid which has been found to lead to an immune response, evidenced by the generation of Hanganutziu-Deicher antibodies [5,6]. In addition, high production costs, the requirement for complex media and the concern of viral contamination have made mammalian cell culture a choice of necessity rather than one of preference. To overcome the shortcomings of both systems, many researchers have turned to alternative protein expression systems, including insects, yeast and plants (reviewed in ref. [7]).

Yeast and filamentous fungi have found wide industrial use for the production of recombinant proteins. The ability to grow on chemically defined media in the absence of animal-derived growth factors, the ease of scale-up, and the high yields of secreted protein have all contributed to the attractiveness of yeast based protein expression [8]. For example, the yeast Pichia pastoris has been shown to produce recombinant rat gelatin at 14.8 g/l [9]. Yeasts are capable of performing many human post-translational modification reactions, including N-linked glycosylation. However, the N-linked glycans from yeast differ significantly from those of mammalian cells and humans, which has compromised their therapeutic utility. The challenge for the glycoengineer has been the elimination of the endogenous hyper-mannosylated yeast glycans, followed by the introduction of elements required to generate human-like sialylated complex glycans [10–12]. This review will discuss seminal advances in the humanization of yeast glycosylation pathways.

N-glycosylation in yeast and humans
Mammalian cells and yeast share the initial biosynthetic pathway for the synthesis of N-glycans. The process is initiated by the transfer of N-acetylglucosamine (GlcNAc) from UDP-GlcNAc onto dolichol phosphate, on the cytoplasmic face of the endoplasmic reticulum (ER). Subsequent glycosyltransferase reactions, that involve the addition of GlcNAc and mannose (Man), mature the structure to Man₅GlcNAc₂-P-dolichol, which
is then translocated to the luminal face of the ER membrane by a flipase enzyme. Once inside the ER the structure is further extended to Glc3Man9GlcNAc2-P-dolichol, at which point the glycans is transferred to the N-X-S/T motif of the target peptide by the oligosaccharyltransferase complex. Following transfer to the nascent polypeptide, three glucose residues and one mannose residue are removed to produce Man5GlcNAc2, at which stage the glycopeptidase is transferred to the Golgi. Although this glycan structure arriving in the Golgi is identical in yeast and mammals, the pathways diverge significantly as the protein proceeds through the remainder of the respective secretory pathways. In mammalian cells the Man8GlcNAc2 glycan is trimmed to Man5GlcNAc2 by the action of several α-1,2-mannosidases, at which stage GlcNAc is added by N-acetylgalcosaminyltransferase I (GnT1). Subsequent trimming and extension reactions lead to the production of a sialylated structure (as exemplified in Figure 1a). Although a host of other glycosyltransferase reactions exist, the pathway exemplified in Figure 1a can be viewed as the core human glycosylation pathway. In contrast to mammals, yeast do not further trim the Man5GlcNAc2 glycan in the Golgi, but rather extend the existing high mannose structure with additional mannose sugars, to produce hyper-mannosylated glycans (Figure 1a, reviewed in [13]).

**Genetic manipulation of N-linked glycosylation in yeast**

The first challenge for glycoengineers was the elimination of the α-1,6 mannose extension, which leads to hypermannosylation in yeast. The enzyme OCH1 (an α-1,6 mannosyltransferase) is involved in the transfer of mannos from GDP-Man onto the α-1,3 branch of the tri-mannose core leading to an α-1,6 extension that is the substrate for additional mannosyltransferases throughout the yeast secretory pathway. The actions of these mannosyltransferases can often lead to the generation of glycans with over 30 additional mannose residues (reviewed in [13]). Therefore, preventing the addition of this first mannose to initiate the outer-chain was recognized as a critical step in preventing hypermannosylation in yeast. Identification of a temperature sensitive mutant in *Saccharomyces cerevisiae* led to the observation that outer chain elongation was deficient, and that a mutation in the och1 gene was responsible [14]. Subsequent cloning and expression of the wildtype OCH1 gene demonstrated that it was (i) able to complement the temperature sensitivity and (ii) was responsible for the transfer of the initial α-1,6 mannose of the outer chain [15]. The same group went on to show that elimination of OCH1 and MNN1 in *S. cerevisiae* produced a strain with a single glycan, Man8GlcNAc2, which is a suitable starting point for the production of mammalian glycoproteins [16*].

The next advance in the glycoengineering of yeast was the introduction of an α-1,2 mannosidase from *Aspergillus
daitoi* into *S. cerevisiae* [17**]. By expressing the enzyme in the early secretory pathway, using a C-terminal HDEL retention signal, Chiba et al. were able to demonstrate the first step in the mammalian-specific glycosylation pathway, albeit at low efficiency. Although the glycans were not homogeneous their work demonstrated that Man5GlcNAc2 structures can be produced by yeast. By using an identical strategy in *Pichia pastoris*, which involved the expression of *Trichoderma reesei* α-1,2 mannosidase, fused to an HDEL retention signal, Professor Contreras’ group in Ghent demonstrated a decrease of over 85% in α-1,2 mannose [18]. However, since OCH1 activity was not eliminated, the authors observed the presence of residual non-human α-1,6 linked terminal mannose. Neither of these two reports determined the extent to which the observed α-1,2 mannosidase trimming occurred in vivo versus ex vivo, which is of concern since the HDEL retrieval mechanism is known to be leaky and could result in the formation of Man5GlcNAc2 ex vivo [19]. A significant advance followed when *Pichia pastoris* was used to screen a combinatorial library of α-1,2 mannosidases [20**]. Following the elimination of OCH1, a library of α-1,2 mannosidase catalytic domains was fused to a library of yeast ER and Golgi localization signals, and screened for optimal in vivo mannose trimming. Although the majority of fusion constructs displayed little or no activity, a few strains were found to produce mostly Man5GlcNAc2. Further analysis of these strains demonstrated (i) complete absence of α-1,6 linked terminal mannose (i.e. och1p activity), and (ii) the absence of α-1,2 mannosidase activity in the medium, ensuring in vivo mannose trimming. Subsequently, the strains possessing the most efficient mannosidase/leader fusions were transformed with a construct encoding a UDP-GlcNAc transporter, and used to screen a library of catalytic domains encoding N-acetylgalcosaminyltransferase I fused to a library of yeast localization signals. The result of this comprehensive screen was the production of a strain capable of converting essentially all glycans to GlcNAcMan5GlcNAc2. This work demonstrated the first production of hybrid glycans in yeast, but also showed that glycosylation pathways can be engineered in vivo to generate uniform glycans, which provided the basis for the development of an efficient industrial platform.

The further humanization of yeast glycosylation was achieved when Hamilton et al. used the combinatorial library approach to introduce mannosidase II and N-acetylgalcosaminyl transferase II (GnTII) [21**]. The resulting strain secreted glycoproteins containing uniform GlcNAc2Man9GlcNAc2 glycans; thereby demonstrating the first production of a complex glycoprotein in yeast. Subsequently this technology was further developed to demonstrate the first in vivo transfer of galactose onto complex glycans [22**]. In this report, an alternative strategy was pursued to obtain GlcNAc2Man9GlcNAc2, the precursor for β-1,4-galactosyl transfer in humans.
By deleting the yeast ALG3 gene, mannosidase II activity was obviated, allowing for the generation of GlcNAc₂Man₃GlcNAc₂ by the sequential action of mannosidase I, GnTI and GnTII. Finally galactose transfer was demonstrated by introducing a β-1,4-galactosyltransferase fusion construct using the aforementioned library approach [23]. Unlike previous engineering efforts, where the activated sugar nucleotide precursor was already present in the yeast, UDP-galactose is not present in *P. pastoris*. Bobrowicz et al. reasoned that UDP-
glucose, which is required to produce Glc₃Man₉GlcNAc₂ in the ER, would be present in the Golgi and by localizing a UDP-galactose epimerase in the Golgi would allow for the generation of UDP-galactose from UDP-glucose. The authors generated a triple fusion containing (i) the yeast localization signal of *S. cerevisiae* Mnn2, (ii) UDP-galactose epimerase from *Schizosaccharomyces pombe* and (iii) the catalytic domain from human β-1,4-galactosyltransferase. Introduction of this triple fusion protein confirmed their hypothesis and demonstrated the first synthesis of galactosylated complex glycans in yeast [22**]. By contrast, Vervecken *et al.* showed that by localizing a β-1,4 galactosyltransferase, in the absence of a UDP-galactose epimerase or UDP-galactose transporter, the transfer of a monosaccharide that could be hydrolyzed by β-galactosidase could be observed [24*]. Though interesting, the relevance of producing galactosylated hybrid structures for therapeutic applications remains to be established.

The final stage in the humanization of yeast glycosylation involves the transfer of sialic acid onto terminal β-1,4 galactose sugars of complex glycoproteins. This step was viewed as the most challenging in the humanization of yeast glycosylation since it relied on four independent functions that are not present in any known wild type yeast: (i) the availability of terminal β-1,4 galactose residues on nascent glycoproteins in the Golgi, (ii) the ability to synthesize CMP-sialic acid, (iii) the ability to transport CMP-sialic acid into the Golgi and (iv) the ability to transfer sialic acid onto terminal β-1,4 galactose residues. Hamilton *et al.*, building on earlier work that demonstrated the production of terminal β-1,4 galactose in glycoengineered *P. pastoris* tackled this problem in a multi prong approach. By first simultaneously introducing the enzymes necessary to convert endogenous UDP-GlcNAc into CMP-sialic acid (Figure 2) the authors showed the production of CMP-sialic acid in vivo (data unpublished). By combining these earlier features with the expression of a CMP-sialic acid transporter and a α-2,6 sialyltransferase/leader fusion that has been pre-screened for in vivo activity, the authors were able to demonstrate the first production of complex sialylated glycoproteins in yeast [25**].

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**Figure 2**

Glycoengineering steps required for sialic acid transfer in the yeast Golgi. Endogenous UDP-GlcNAc, present in the yeast cytoplasm, is converted to CMP-sialic acid by UDP-N-acetylglucosamine-2-epimerase/N-acetylmannosamine kinase (GNE), N-acetylneuraminic acid-phosphate synthase (SPS), sialylate-9-P phosphatase (SPP) and CMP-sialic acid synthase (CSS). Subsequently, the product is translocated into the Golgi by the CMP-sialic acid transporter (CST) and sialic acid is transferred onto the acceptor glycan by sialyltransferase (ST). Enzymes are indicated by blue text and metabolic intermediates by black text. For simplicity the two GlcNAc residues present at the reducing end of all glycans have been omitted.
Conclusions
Recent advances in glycoengineering have allowed for the generation of yeast strains capable of replicating the most essential glycosylation pathways found in mammals. Not only does this provide researchers with a tool to generate homogeneous glycoproteins for structure–function analysis, but also provides an alternative platform to mammalian cell culture for the production of human therapeutic glycoproteins. To illustrate the potential of improving the therapeutic function of glycoproteins Li et al. used glycoengineered yeast strains to produce a library of anti-CD20 antibodies, each containing an identical protein sequence yet with a unique glycan structure [26**]. The resulting glycoforms showed a range of different activities depending on the particular glycan structure attached to the antibody. In addition, Hamilton et al. demonstrated that sialylated recombinant erythropoietin produced in a fully humanized yeast strain, induced a dose-dependent erythropoietic response, consistent with a biologically active form of the recombinant protein [25**]. Since the scale-up and production of recombinant proteins in yeast is a well-established technology, it is reasonable to expect the high-throughput and cost-effectiveness of the glycoengineered yeast strains to make a significant impact on the biopharmaceutical industry.

References and recommended reading
Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest


In this report the authors continue to build upon the success described in ref. [20**], by using the combinatorial library approach to demonstrate the first production of complex N-glycans in yeast.


Adopting an alternative method to achieve complex N-glycans, the authors proceed to demonstrate the production of the first galactosylated N-glycans in yeast.

This study reports the elimination of endogenous Alg3 activity in *P. pastoris*, providing the initial step in the production of an alternative method for generating complex N-glycans described in 'ref. [22]**'.


This manuscript describes a method for the production of hybrid terminally galactosylated N-glycans in *P. pastoris*.


Here the authors demonstrate the first production of sialylated complex N-glycans in yeast. By introducing three enzymes required for the synthesis of CMP-sialic acid, a CMP-sialic acid transporter and a sialyltransferase, the production of sialylated recombinant erythropoietin was demonstrated in humanized *P. pastoris*.


This report describes the use of genetically engineered yeast strains to produce antibodies possessing distinct glycoforms. In doing so, they demonstrate the ability of specific glycoforms to enhance antibody-mediated effector functions.