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Glycoengineered Yeast as an Alternative Monoclonal Antibody Discovery and Production Platform

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1. Introduction

Biologic pharmaceuticals are gaining in both market share and clinical utility compared with small molecule therapeutics (Projan et al., 2004). Global biologic drug sales reached \$93 billion in 2009 and the sales are expected to grow at least twice as fast as those of small molecules (McCamish and Woollett, 2011). The rapid market growth and the promise of successful rate of developing biologic drugs has drawn the attention of traditional small molecule pharma into the biotechnology business. Today, more than ever, large pharmaceutical companies are venturing into the biotechnology arena with the hope that novel therapeutic proteins will augment the traditional pharmaceutical business enough to fundamentally reshape the market landscape. The acquisition/merging of pipeline and research capacity of biotech companies by big pharma is a great example of this trend. Several companies are even projecting optimistically that, within the decade, therapeutic biologics will comprise a majority of their commercial portfolios (Zhou, 2007). Among highly successful biologic products, monoclonal antibodies (mAbs) are the largest and fastest growing segment. MAbs are established as a key therapeutic modality for a range of diseases most notably rheumatoid diseases and various cancers. Due to the high degree of selectivity of these agents, in particular for cancer targets, they can be designed to selectively target tumor cells and elicit a variety of responses. These agents can kill cells directly by carrying a toxic payload to the target or can orchestrate the destruction of cells by activating immune system components, blocking receptors or sequestering growth factors (Nicolaidis et al., 2010).

It is well known that glycosylation can impact the pharmacokinetics, efficacy and tissue targeting of therapeutic proteins (Li and d'Anjou, 2009). N-glycosylation of immunoglobulin

G (IgG) at asparagine residue 297 plays a role in antibody stability and is required for immune cell-mediated Fc effector function (Correia, 2010; Kayser et al., 2011; Mimura et al., 2000). Preclinical and clinical studies have demonstrated that modulating the glycosylation of antibody and non-antibody therapeutics can be an effective means to improve the properties of biologic medicines leading to a class of drugs termed "biobetters" (Jefferis, 2009; Walsh, 2010). The strategy of engineering expression hosts to express glycoproteins with "optimized" glycosylation has been applied in both prokaryotic and eukaryotic cells (Beck et al., 2010; Hamilton et al., 2006; Hamilton and Gerngross, 2007; Jacobs and Callewaert, 2009b; Pandhal and Wright, 2010; Tomiya, 2009). Chinese Hamster Ovary (CHO) cells, a widely used host for producing therapeutic antibodies with similar human glycosylation, have been engineered in multiple ways to eliminate the core fucose on secreted mAbs. Recent preclinical and clinical studies have reported superior efficacy utilizing afucosylated mAbs (Herbst et al., 2011; Junttila et al., 2010; von Horsten et al., 2010; Ward et al., 2011; Wong et al., 2010). Non-mammalian expression hosts have also been utilized for producing afucosylated mAbs including insect cells, plants and yeast (Gasdaska et al., 2007; Barbin et al., 2006; Zhang et al., 2011).

Following marketing of the first therapeutic antibody, Muromonab-CD3 in 1986, mAbs used in the clinic have evolved over the years from entirely murine to mouse-human chimeric, and then to humanized and finally fully human antibodies in order to minimize anti-drug related immunogenicity in patients and maintain maximum potency (Li and Zhu, 2010). Several different antibody discovery technologies co-exist today in therapeutic antibody development ranging from isolating antibodies from immunized mice or engineered mice carrying human Ig-repertoire genes to flow-cytometric isolation of human antibodies from non-immune yeast display or panning a large non-immunized phage display libraries (Feldhaus et al., 2003; Vaughan et al., 1996; Weaver-Feldhaus et al., 2004). Recently *de novo* computer designed and synthetic antibody libraries based on a human Ig-repertoire and coupled with yeast or phage display have attracted considerable attention (Knappik et al., 2000). However, one key issue that is not solved by current antibody discovery technologies, is the need to switch expression hosts from antibody discovery (*E. coli*, yeast) to production host (often CHO or other mammalian cell lines). Moreover, for many current mAb discovery technologies reformatting steps from scFv/Fab to full-length antibody are required. These additional steps increase cycle times and often decrease the probabilities of success both of which have negative impacts on development timeline (Lin et al., 2012).

Yeast has been widely used for expressing proteins in research and development. There are multiple advantages to using yeast as an expression system for therapeutic glycoprotein production, including ease of genetic manipulation, stable expression, rapid cell growth, high yield of secreted protein, low-cost scalable fermentation processes and no risk of human pathogenic virus contamination. However, glycoproteins expressed in wild type yeast generally cannot be used for therapeutic applications due to fungal type high-mannose glycans which can result in immunogenicity and poor PK *in vivo* (Zhang et al., 2011). Humanization of the N-glycosylation pathways of the yeast *Pichia pastoris* has been achieved by eliminating fungal genes responsible for adding high-mannose and

concomitantly reconstituting the canonical human pathway (Hopkins et al., 2011; Jiang et al., 2011; Li et al., 2006; Liu et al., 2011; Potgieter et al., 2010; Ye et al., 2011b). This result of the engineering is a platform that allows not only yeast-based production of therapeutic glycoproteins with human glycosylation, but also provides a versatile tool for glycosylation-based structure-activity-relationship (SAR) studies to optimize therapeutic proteins for better efficacy, PK and drugability (Li et al., 2006; Nett et al., 2012; Zhang et al., 2011). In addition, development of antibody surface display on glycoengineered yeast strain as an antibody discovery tool facilitates the earliest stage of antibody discovery and development in the same expression host which is expected to have a positive impact on cycle times and probabilities of success (Lin et al., 2012).

2. Glycosylation in therapeutic proteins is important for its efficacy, PK, tissue targeting

2.1. Glycosylation on Erythropoietin affects its potency

Recombinant human Erythropoietin (rHuEPO) is a 30.4 kDa glycoprotein hormone containing multiple N-linked glycosylation sites and currently is used to treat patients with anemia. The marketed forms of recombinant erythropoietin include Epogen with three native N-glycan structures and Aranesp® (darbepoetin), an epoetin engineered to contain two additional N-glycosylation sites, conferring greater metabolic stability *in vivo*. Typical mammalian CHO cell-produced epoetin are secreted with a heterogeneous mixture of sialylated N-glycan structures. Usually the manufacturing process is controlled to enrich for the tetra-antennary sialylated glycoforms, which along with tri-antennary forms, are required for maximum *in vivo* efficacy (Egrie and Browne, 2001). However, in cell-based and receptor binding assays, tri- and tetra-sialylated erythropoietin and darbepoetin exhibit decreased potency relative to bi-antennary sialylated erythropoietin (Takeuchi et al., 1989). This paradox can be explained by the extended serum half-lives of the tetra-antennary sialylated erythropoietin and darbepoetin compared with the faster clearance rate of bi-antennary sialylated erythropoietin (Misaizu et al., 1995). Recently, glycoengineered *Pichia pastoris* has been used to produce rHuEPO with highly homogeneous bi-antennary structures. As expected, bi-antennary EPO produced by glycoengineered *Pichia* showed significantly more potent *in vitro* activity compared to predominantly tri- and tetra-antennary EPO, such as Aranesp®. The faster clearance rate of bi-antennary rHuEPO *in vivo* was compensated with Pegylation to elongate its half life in this study. The authors concluded overall *in vivo* activity of this novel glycoengineered molecule is comparable *in vitro* and *in vivo* characteristics to its counterparts produced in mammalian systems (Nett et al., 2012).

2.2. Glycosylation on recombinant human glucocerebrosidase is critical for its targeting in enzyme replacement therapy

Gaucher's disease is a lysosomal storage disorder caused by mutations of glucocerebrosidase (GCD), and an enzyme replacement treatment has been developed using recombinant GCD. GCD is glycoprotein and its glycosylation plays an important role in its

targeting in therapeutic setting. GCD produced in Chinese Hamster ovary (CHO) cells contains major complex glycan but it has failed to provide clinical benefit in direct infusion due to the preferential uptake of enzyme by hepatocytes rather than Kupffer cells. An *in vitro* glycan modification is required in order to expose the mannose residues on the glycans of Cerezyme (commercialized therapeutic GCD)(Bergh et al., 1990;Brumshtein et al., 2010;Pastores, 2010). Cerezyme production involves sequential *in vitro* deglycosylation, using α -neuraminidase, β -galactosidase and β -N-acetylglucosaminidase, to expose terminal mannose residues, a procedure which dramatically improves targeting and internalization. Recently the recombinant plant-derived GCD (prGCD) is targeted to the storage vacuoles, using a plant-specific C-terminal sorting signal. Notably, the recombinant human GCD expressed in the carrot cells naturally contains terminal mannose residues on its glycans, apparently as a result of the activity of a special vacuolar enzyme that modifies complex glycans(Shaaltiel et al., 2007). Hence, the plant-produced recombinant human GCD does not require exposure of mannose residues through *in vitro* enzymatic modification. Interestingly, the N-glycosylation pathway of *H. polymorpha* has been remodeled by deleting the HpALG3 gene in the Hpoch1 null mutant strain and blocked in the yeast-specific outer mannose chain synthesis and by introducing an ER-targeted *Aspergillus saitoi* α -1,2-mannosidase gene. This glycoengineered *H. polymorpha* strain produced glycoproteins mainly containing trimannosyl core N-glycan (Man₃GlcNAc₂), which is the common core backbone of various human-type N-glycans and the glycoform is the same as that of Cerezyme which was achieved through *in vitro* sequential deglycosylation of complex glycans (Oh et al., 2008). Similar glycoengineering effort was published in a glycoengineered *Pichia* with the ability of producing glycoprotein carrying similar mannose type of glycan (Davidson et al., 2004).

2.3. Afucosylated antibody has enhanced ADCC and can translate into better efficacy

Antibody Dependent Cell-Mediated Cytotoxicity (ADCC) is a mechanism of cell mediated immune defense whereby an effector cell of the immune system actively lyses a target cell that has been bound by specific antibodies. The typical ADCC involves activation of effector cells such as Natural Killer (NK) cells by antibodies. An NK cell expresses Fc receptor IIIa or CD16a (Nimmerjahn and Ravetch, 2007; Nimmerjahn and Ravetch, 2008), and this receptor recognizes and binds to the Fc portion of an antibody which it has already bound to the surface of a pathogen-infected target cell or tumor cell. The NK cell releases cytokines such as IFN- γ , and cytotoxic granules containing perforin and granzymes that enter the target cell and promote cell death by triggering apoptosis. Monoclonal antibody IgG1 Fc has single N-linked site at N-297 and its glycosylation is involved with antibody and Fc γ receptor III binding. Antibodies lacking core fucosylation show a large increase in affinity for Fc γ RIIIa leading to an improved receptor-mediated effector function. The structure study reveals that a unique type of interface consisting of carbohydrate-carbohydrate interactions between glycans of the receptor and the afucosylated Fc. In contrast, in the complex structure with fucosylated Fc, these contacts are weakened or nonexistent (Ferrara et al., 2011). Although

afucosylated IgGs exist naturally, a next generation of recombinant therapeutic, glycoengineered antibodies with enhanced ADCC activity is currently being developed for better efficacy (Mori et al., 2007; Ysebaert et al., 2010; Robak and Robak, 2011).

2.4. Glycans at Fc region impact on its pharmacokinetics

Glycosylation at mAb Fc regions not only plays a role in its effector functions, but it also can impact its pharmacokinetics. The neonatal Fc receptor (FcRn) has a major role in prolonging the exposure of therapeutic mAbs. MAbs internalized by fluid-phase or receptor-mediated endocytosis can be redirected to the cell surface by FcRn mediated recycling and released into plasma or interstitial fluids thus preventing lysosomal degradation (Chowdhury and Wu, 2005; Roopenian and Akilesh, 2007). This recycling dramatically increases systemic exposure to therapeutic mAbs by studies comparing in wild type and FcRn-deficient mice which demonstrated over an order of magnitude increase in IgG half-life as a consequence of the FcRn salvage pathway. Another mechanism for mAb clearance may be through Fc γ receptor (Fc γ R) binding on some immune effector cells. Interactions with Fc γ receptors can be influenced by the glycosylation pattern. To produce mAbs in lower eukaryotic hosts such as yeast have been reported, however, wild type yeast host expressed antibody secretes full length mAbs with hyper-mannose type glycans. MAb produced in wild type yeast exhibited 2 to 3-fold faster clearance, which might be due to the high mannose content interacting with mannose receptors. On the other hand, *in vitro* binding affinity to human FcRn or mouse FcRn was similar between the mAb produced in CHO cell line and mAbs produced in glycoengineered yeast, and the glycovariants produced in glycoengineered yeast exhibited similar PK patterns in human FcRn transgenic mice and in wild type mice (Liu et al., 2011).

3. Glycoengineered *Pichia* expresses protein with human glycosylation

3.1. Difference of glycosylation pathway between human and yeast

Yeast N-glycosylation is of the high-mannose type, which differs from human complex glycans. Fungal type of glycans confers a short half-life *in vivo* and thereby compromises efficacy of most therapeutic glycoproteins. In addition non-human types of glycans can cause immunogenicity and therefore inactivate the drug substance and even more it may cast safety concerns (Jacobs and Callewaert, 2009a; Sinclair and Elliott, 2005; Sola and Griebenow, 2009). However, the initiation of yeast N-glycosylation is similar to that of human. In ER, a core oligosaccharide (Glc3Man9GlcNAc2) is transferred onto the nascent polypeptide. Following the transfer of the core oligosaccharide to the asparagine residue within the Asn-X-Ser/Thr motif, three glucose moieties and one terminal α -1,2-mannose moiety are removed by glucosidase I and glucosidase II, and an ER-residing α -1,2-mannosidase, respectively. The resulting Man8GlcNAc2-containing glycoprotein is then transported to the Golgi apparatus where N-glycan processing differs markedly between yeast and human. In human and mammals, early Golgi N-glycan processing involves the trimming of Man8GlcNAc2 to Man5GlcNAc2 by α -1,2-mannosidase(s) (MNS1), a process that generates the substrate for *N*-acetylglucosaminyl transferase I (GNT1), which transfers

a single *N*-acetylglucosamine (GlcNAc) sugar onto the terminal 1,3-mannose of the tri-mannose core. Following this transfer, mannosidase II (MNS II) removes the two remaining α -1,3- and α -1,6 terminal mannose sugars to produce GlcNAcMan₃GlcNAc₂. This is the substrate for *N*-acetylglucosaminyl transferase II (GNT II), which adds one GlcNAc sugar to the terminal α -1,6-mannose arm of the tri-mannose core. Further processing typically involves the attachment of additional GlcNAc, galactose and sialic acid, such as *N*-acetylneuraminic acid (NANA) moieties (Wildt and Gerngross, 2005). However, a host of additional glycosyltransferases, including fucosyltransferases, GalNAc transferases and GlcNAc phosphotransferases, are known to exist, which further broadens the range of *N*-glycans found on proteins isolated from human sources. In contrast to human *N*-glycan processing, which involves the removal of mannose followed by the addition of GlcNAc, galactose, fucose and NANA, early *N*-glycan processing in yeast is limited to the addition of mannose and mannosylphosphate sugars (Hamilton et al., 2003; Wildt and Gerngross, 2005). The Golgi apparatus of *S. cerevisiae* contains α -1,2-, α -1,3- and α -1,6-mannosyltransferases as well as mannosylphosphate transferases, which produce *N*-glycan structures that are mannosylated and hypermannosylated to varying extents. In other yeast such as *K. lactis*, *H. polymorpha* and *P. pastoris*, a similar set of mannosyltransferases exists, resulting in the production of mostly high-mannose structures that resemble those produced in *S. cerevisiae* (Wildt and Gerngross, 2005).

3.2. Genetic manipulation of *Pichia* glycosylation pathway

The process involved eliminating endogenous yeast glycosylation pathways, and we introduced more than 14 heterologous genes into *Pichia pastoris*, allowing us to replicate the sequential steps of human glycosylation. The enzyme OCH1, an α -1,6 mannosyltransferase adds the first mannose onto the α -1,3 branch of the trimannose core leading to an α -1, 6 extension to initiate the outer-chain, then additional mannoses are transferred into the new created mannose substrate by other mannose transferases and results in hypermannosylation. To knockout of the OCH1 gene in yeast is the critical step to prevent hypermannosylation and then generate a predominately single glycoform Man₈GlcNAc₂. Besides OCH1 gene, other mannosylphosphate transferases and β -mannosyltransferases can further modify the glycans at secreted proteins into fungal type of glycosylation, and to eliminate these genes is an essential step of engineering the yeast glycosylation pathways into human like. After eliminating yeast enzymes specific for fungal glycosylation modification, an efficient high throughput approach was developed by screening combinatorial libraries of fusing catalytic domain of modification enzymes with localization leader sequence (Choi et al., 2003; Nett et al., 2011). With this approach, α -1,2 mannosidase (MNS1), *N*-acetylglucosaminyltransferase I (GNT1), mannosidase II (MNS2) and *N*-acetylglucosaminyl transferase II (GNT2) were engineered into *Pichia pastoris* and localized from early to later golgi in a sequential order. The engineered *Pichia* strain with these fungal mannosyltransferases knockouts and human or mammalian modification genes knockins is able to express glycoproteins with human type of biantennary complex glycan with terminal GlcNAc (Gerngross, 2005; Hamilton et al., 2003). Humanization of the glycoengineered *Pichia*

glycosylation pathway was continued by introducing a -1,4-galactosyl transferase (GalT) with its selected leader through the library approach mentioned above. But *Pichia pastoris* does not contain activated sugar nucleotide precursor UDP-galactose as the substrate for galactosyl transferase to add galactose to GlcNAc terminated glycans, and the problem was solved by introducing UDP-galactose epimerase. Up to this step, the glycoengineered *Pichia* is able to produce glycoproteins with terminal galactose at its glycans. These strains would be suitable to be used for producing therapeutic monoclonal antibodies (mAbs) since most commercial mAbs produced by mammalian cells with galactose topping on its glycans (Bobrowicz et al., 2004). However, sialic acid containing glycan in therapeutic glycoproteins plays a critical role in its pharmacokinetics and efficacy, to maximal the glycoengineered *Pichia* platform to produce therapeutic biologics, the efforts was advanced to the last stage by further humanization of *Pichia* glycosylation by introducing sialylation pathway. In addition properly localize sialyltransferase at the late golgi, to produce the sugar nucleotide precursor CMP-sialic acid and translocate into the late golgi is a must-have. Four genes including UDP-N-acetylglucosamine-2-epimerase/N-acetylmannosamine Kinase (GNE), N-acetylneuraminate-9-phosphate synthase (SPS), sialylate-9-P phosphatase (SPP) and CMP-sialic acid synthase (CSS) is used to convert UDP-GlcNAc to CMP-Sialic Acid in the cytosol. CMP-Sialic Acid is transported into the golgi by engineered CMP-sialic acid transporter and then transferred onto the acceptor glycan by sialyltransferase. Sialylated glycoprotein exits the secretory pathway into culture supernatant similar to wild type yeast (Hamilton et al., 2003; Hamilton et al., 2006).

4. Alternative monoclonal antibody production in glycoengineered *Pichia pastoris*

4.1. Selection of glycoengineered *Pichia* expressing mAbs

Conventional mammalian cell lines as expression host secrete glycoprotein usually containing heterogeneous glycans. Mammalian cells, such as Chinese Hamster Ovary (CHO) cells maintain the capability of adding sialic acid at its galactose terminal glycans. However, mAbs expressed in CHO have little or no sialylation at its Fc region due to the steric hindrance of the Fc structure (Nimmerjahn and Ravetch, 2010). On the other hand, glycoengineered *Pichia* provides the possibility of expressing antibody with different glycoforms in relatively more homogenous fashion (Potgieter et al., 2009). Since the engineering of the glycosylation pathway of *Pichia* was a sequential process, which makes it possible to generate a panel of hosts which express glycoprotein with different glycoforms. Each host carries one major glycan, such as mannose type glycan of Man5GlcNAc2 or hybrid type of glycan (Potgieter et al., 2009), or GlcNAcMan5GlcNAc2 (Choi et al., 2003), or complex glycans of GlcNAc2Man5GlcNAc2, Gal1GlcNAcMan3GlcNAc2 or Gal2GlcNAc2Man3GlcNAc2 (Hamilton et al., 2003). In addition, hosts with capability of transferring sialic acid onto bi-antennary glycans with terminal galactoses were also available (Bobrowicz et al., 2004; Hamilton et al., 2006). With these hosts bearing different glycosylation, it actually offers a unique tool to produce the same monoclonal antibody or other non-mAb containing various glycoforms at its Fc region, which allows us to study the

glycan structure and its activity relationship (SAR) for efficacy, tissue distribution and pharmacokinetics. In consideration of pharmacokinetics (PK), pharmacodynamics (PD) and reducing potential immunogenicity, glycoengineered *Pichia* provides great potential to generate mAbs with more homogeneous glycans and desired efficacy.

4.2. Difference in glycosylation profile of antibodies from CHO and glycoengineered *Pichia*

Mammalian cells, e.g. CHO produced mAbs carry N-linked carbohydrate structures with predominantly core-fucosylated asial-biantennary types with varying degrees of galactosylation. Within a given product, there are lot-to-lot variations even though manufacturing processes are tightly controlled and ensure a high degree of product consistency. Besides complex glycoforms, CHO cells expressed monoclonal antibodies still contains some percentage of Man5 type of glycans. While production of consistent and reproducible mAb glycoform profiles still remains a considerable challenge for CHO cells, variations in cell culture processes play a role in the mAb glycosylation profile. Potential variables in the cell culture physicochemical environment including culture pH, cell culture media composition, raw materials lot-to-lot variations, equipment, and process control differences are just a few examples that can potentially alter glycosylation profiles. In a case study, glycoengineered *Pichia* with the capability of generating human complex glycans has been chosen to express anti-Her2 mAb using amino acid sequence identical to Trastuzumab. The N-glycan composition of anti-HER2 mAb produced in glycoengineered *Pichia* differed from that of CHO produced counterpart primarily in the proportion of GlcNAc2Man3GlcNAc2 (G0), GlcNAc2Man3GlcNAc2Gal(G1) and GlcNAc2Man3GlcNAc2Gal2 (G2) and it was entirely devoid of fucose in the glycan structure. The mAb produced in glycoengineered *Pichia* contains a small number of O-linked single mannose glycan but O-linked glycans were rare in CHO produced Trastuzumab. In another study, using an early stage glycoengineered *Pichia* host, the glycoengineered yeast produced antibody has similar motilities on SDS-PAGE, comparable size exclusion chromatograms and antigen binding affinities compared to its CHO produced comparator but with highly uniform N-linked glycans of that type.

4.3. Bioanalytical characterization of glycoengineered *Pichia* produced antibody

A glycoengineered *Pichia* strain chosen for producing monoclonal antibody is capable of transferring terminal β -1,4 galactose onto biantennary N-linked glycan, which yields antibody entirely devoid of fucose at its glycan structure. MAb was purified through affinity capture using protein A beads and further purified by ion exchange chromatography. Antibody purity by SDS-PAGE and its spectrum from size exclusion chromatography HPLC were compared with CHO produced comparator. Purified mAb was composed of more than 99% fully assembled antibody including double heavy and light chain, and the quality of the antibody profile was comparable to that of mammalian cell expressed antibody. Unlike mammalian cells, glycoengineered *Pichia* derived mAb contains no sialic acid and

fructose because *Pichia* inherently lacks these pathways and was not intentionally engineered in. However, due to the steric hindrance of Fc, the normal IgGs produced in glycoengineered *Pichia* with the capability of transferring terminal β 1,4 galactose maintain the glycan heterogeneity. The major glycoform is agalactosylated G0 and various degree of single or two terminal galactosylated glycans. Interestingly when antibody was expressed in another glycoengineered strain, it delivered relatively homogenous type of glycans with more than 90% antibody carrying Man5GlcNAc2 (Potgieter et al., 2009).

4.4. Glycoengineered *Pichia* produced antibody is comparable to CHO derived antibody *in vitro* assays

Glycoengineered *Pichia pastoris* not only provides the capacity of secreted glycoprotein with human like glycans, but it is also capable to assemble heterodimer large molecule like antibody with 16 pairs of disulfide bonds. With the exception of the difference in glycosylation between CHO and glycoengineered *Pichia* produced antibody, they share similar purity based on SDS-PAGE and size exclusion chromatography HPLC analysis. The difference of glycosylation at Fc region has little or no impact on Fab dependant antigen binding in both ELISA based assay and cell based FACS analysis using cell line with antigen expressed on its surface. The antibody and antigen engagement can lead to proper biology function in cell based assays e.g. glycoengineered *Pichia* produced anti-HER2 has demonstrated comparable potencies in receptor inhibition assays *in vitro* compared to CHO derived trastuzumab. They have very similar HER2 and AKT phosphorylation inhibition and therefore both antibodies inhibit tumor cell proliferation at similar levels (Jiang et al., 2011; Liu et al., 2011; Zhang et al., 2011; Potgieter et al., 2009).

4.5. Glycoengineered *Pichia* produced mAb has similar *in vivo* efficacy and serum half-life

As stated before, fungal type hypermannosylated glycans at the antibody Fc region could have detrimental effects on its pharmacokinetics and would result in fast clearance in the blood stream (Liu et al., 2011). These non-human glycans can trigger the human immune response and causes immunogenicity. To ensure antibody produced from glycoengineered *Pichia* is suitable for therapeutic purpose, its PK needs to be monitored and compared with its counterpart from traditional mAb production platform. Comparing to a humanized antibody produced in CHO cell (Trastuzumab) with predominately human complex glycans at its Fc, in both rodent and non human primates, blood time concentration curve of glycoengineered *Pichia* produced mAb (with identical amino acid sequence to Trastuzumab) was almost super-imposable on that of CHO produced Trastuzumab. As a result, the key PK parameters (CL, t_{1/2}, AUC and V_{ss}) were comparable between these two mAbs. Moreover, the same antibody was studied and compared its *in vivo* efficacy in a xenograft nude mice model. With highly expressed receptor antigen on the tumor surface, glycoengineered *Pichia* produced antibody could engage and inhibit the target and therefore prevent the progression of tumor growth. Based on the time course for the average tumor growth, at

low, intermediate and high dosage range, glycoengineered *Pichia* produced antibody with humanized glycan shows comparable tumor inhibitory efficacy (Zhang et al., 2011).

4.6. Antibody produced by glycoengineered *Pichia* has better efficacy

Preclinical studies have shown that antibody dependant cell-mediated cytotoxicity (ADCC) is an important part of mechanism of action of therapeutic monoclonal antibodies, especially anti-cancer antibodies, such as Trastumab and Rituximab against tumors (Mori et al., 2007). Some clinical evidence based on genetic analysis of leukocyte receptor (Fc γ R) polymorphisms of cancer patients treated with anti-CD20 IgG1 Rituximab and anti-HER2 IgG1 Trastuzumab therapies has revealed that ADCC is one of the critical mechanisms responsible for the clinical efficacy of these therapeutic antibodies (Musolino et al., 2008; Kim et al., 2006; Cartron et al., 2002). ADCC enhancement technology is expected to be excised in development of "biobetter" therapeutic antibodies with improved clinical efficacy. A strong correlation with Fc γ receptor affinity and antibody binding to Fc γ RIIIA in particular has shown to positively correlate with ADCC activity. Trastuzumab produced as Fc engineered or afucosylated mAb showed increased ADCC and improved tumor inhibition in a mouse xenograft model with human immuno-effector cells. A large number of studies with Fc engineered antibodies have firmly established that increased affinity for Fc γ RIIIA leads to increased NK cell or PBMC-mediated ADCC *in vitro*, and can result in better efficacy *in vivo* in models dependent on immune effector functions. Modulation of the Fc region by utilizing protein and glyco-engineering are two main ways to increase antibody and Fc γ receptor IIIa binding and then to enhance ADCC activity. But conventional therapeutic antibody production cell line such as CHO secrete monoclonal antibody with predominately fucosylated glycans which binds to Fc γ IIIa with lower affinity compared with afucosylated antibody. There are several technologies capable of generating afucosylated mAbs. This includes Kyowa Hakko/Biowa FUT8 (α -1,6-fucosyltransferase) gene knockout CHO line. Glycart (now part of Roche) utilizes a CHO line with inducible expression of the enzymes β (1,4)-N-acetylglucosaminyltransferase III (GnTIII) and wild-type golgi α -mannosidase II, and this line has been used to produce their GA101 antibody (Heinrich et al., 2009; Friess et al., 2007; Umana et al., 2007), an enhanced anti-CD20 that is currently in late-stage clinical trials for the treatment of Non-Hodgkin lymphoma (NHL). On the other hand, we reconstitute entire human glycosylation pathway in yeast *Pichia de novo* which inherently lacks of fucose transferase and its substrate. We also purposely leave out the fucosylation pathway through engineering, thus glycoengineered *Pichia* naturally produce antibody carrying zero percent fucosylated glycans. Afucosylated antibody from glycoengineered *Pichia* showed 6- and 8-fold increases in affinity for Fc γ RIIIA polymorphism F158 and V158, respectively compared to the CHO produced counterpart. This increased antibody affinity with Fc gamma receptor IIIa has translated 6-fold increase in NK cell-mediated ADCC activity and 4-fold and 3- fold increase in ADCC with PBMC and monocyte effector cells, respectively. Afucosylated antibodies from glycoengineered *Picha* platform and CHO FUT8 knockout platform have consistently increased affinity for human Fc γ RIIIa, which results in enhanced ADCC (Zhang et al., 2011). As such glycoengineered *Pichia* can be applied to

produce afucosylated antibody where ADCC is part of mechanism of action, this could lead to develop "bio-better" therapeutic antibodies compared to conventional CHO fucosylated platform.

5. One stop shop for antibody development with human glycosylation

5.1. Antibody surface display on glycoengineered *Pichia*

Yeast surface display has been a widely used tool for protein engineering and for antibody engineering in particular (Boder and Wittrup, 1997; Boder and Wittrup, 2000; Wittrup, 2009). However, displaying Fabs or full-length antibodies on the surface of *Pichia pastoris* requires post-translational assembly of the heavy and the light chain. Moreover, the glycans of glycosylphosphatidylinositol (GPI) proteins, most often used as a cell wall anchor for display, play an important role in stabilizing its anchoring on the cell surface. Engineering the glycosylation pathway of *Pichia pastoris* can profoundly modify the glycosylation profile of the endogenous cell surface glycoproteins and recombinant GPI proteins (Bobrowicz et al., 2004; Sethuraman and Stadheim, 2006; Wang et al., 2007; Zhou et al., 2007). Thus the cell wall GPI protein anchors suitable for wild type *Pichia pastoris* strains may not necessarily work well in glycoengineered *Pichia* strains due to the difference of glycosylation profile of cell wall and cell wall anchoring proteins. Using combinatorial approach, we successfully developed a glycoengineered *Pichia* Fab display using a pair of coiled-coil peptides as the linker and *Saccharomyces cerevisiae* Sed1p GPI anchored cell surface protein as an anchoring domain in a host with mammalian mannose type Man5GlcNAc2 N-linked glycans. The system was validated by displaying multiple Fab molecules and sorting mixed Fab-displaying strains based on both expression and affinity. The results demonstrate a high level of concordance in expression and affinity between the displayed Fab and its secreted Fab as well as its full length IgG, which supports the coiled-coil/ScSed1p-based Fab display system as a platform for antibody affinity and expression maturation in glycoengineered *Pichia* strains (Lin et al., 2012).

5.2. Antibody expression platform in glycoengineered *Pichia pastoris*

Glycoengineered *Pichia* strain GFI5.0 was chosen as the expression host for producing mAb. GFI5.0 is capable of transferring terminal β -1,4 galactose onto biantennary complex N-linked glycan, which yields antibody entirely devoid of fucose at its glycan structure (Zhang et al., 2011). DNA sequences encoding the heavy and light chain are under methanol inducible AOX1 promoter. A gene encoding for drug Zeocin® resistance enable selection of transformants and a fragment of a *Pichia pastoris* gene such as the AOX2 promoter and terminator to enable integration of the vector onto the chromosome are part of the expression vector. The expression vector is integrated into *Pichia* chromosome by recombination after linearization followed by electroporation. Transformants are isolated as single colonies from agar plates containing Zeocin® for selection resistance marker (Li et al., 2006). Several strategies could be used through host strain modifications or expression vector optimization could be used to improve the expression level of full length monoclonal

antibodies in yeast, for example (a) to increase copy number of heavy and light chain expression cassettes, (b) to improve mRNA stability (c) to optimize codon usage, (d) to screen best signal peptides (e) to engineer folding chaperones, such as immunoglobulin binding protein (BiP) and protein disulfide isomerase (PDI), (f) to delete protease genes and (g) to fuse heavy and light chain with a fusion partner.

5.3. Develop a robust and scalable monoclonal antibody production platform using glycoengineered *Pichia*

Glycoengineered *Pichia pastoris* strain which is capable of producing humanized glycoprotein with terminal galactose for monoclonal antibody production. Like mammalian cell lines, fermentation process plays a critical role to achieve the highest titer as well as maintain good quality glycans. A design of experiments (DoE) approach is often used to optimize the process parameters. In one case study followed by further optimization of the specific methanol feed rate, induction duration, and the initial induction biomass, the resulting process yielded up to 1.6 g/L of for one monoclonal antibody. Even more this process was also scaled-up to 1,200-L scale, and the process profiles, productivity, and product quality were comparable with 30-L scale. The successful scale-up demonstrated that this glycoengineered *Pichia pastoris* fermentation process is a robust and commercially viable process (Berdichevsky et al., 2011). In another case study, an oxygen-limited process was developed and optimized with the use of DoE for the production of monoclonal antibodies in glycoengineered *Pichia pastoris*. The process relied on pulsed feeding of methanol and its productivity was found to depend on biomass concentration and oxygen availability. Oxygen uptake rate was used as a scale-up parameter in demonstrating consistency of the process between the 3 L laboratory scale and the 1200 L pilot manufacturing scale. Scalability and productivity were improved by reducing oxygen consumption and cell growth, allowing extension of the induction phase without the associated mAb fragmentation characteristic of methanol limitation. The final mAb concentration was increased from 1.2 to 1.9 g L⁻¹. Oxygen limitation also improved N-glycan quality in terms of percentage of complex glycans (Ye et al., 2011a).

6. Summary

Glycosylation on therapeutic glycoproteins plays a critical role on its pharmacokinetics, efficacy and tissue targeting. By eliminating pathways responsible for fungal glycosylation and engineering in human glycosylation pathways, glycoengineered *Pichia* expresses glycoprotein with human type of glycans. Glycoengineered *Pichia* was applied as an alternative monoclonal antibody production platform. Comparing glycoengineered *Pichia* produced antibody with traditional mammalian cell CHO produced counterpart, antibody from glycoengineered *Pichia* has comparable *in vitro* functions and *in vivo* efficacy and serum half life. Moreover, by developing antibody yeast surface display in glycoengineered *Pichia* coupled with matured monoclonal antibody production, it becomes possible to integrate antibody discovery and manufacture into a single united platform, and it could

increase the probability of success in therapeutic antibody development. Furthermore, glycoengineered *Pichia* antibody producing strain is a scalable and robust antibody production host.

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7. References

- Barbin, K., Stieglmaier, J., Saul, D., Stieglmaier, K., Stockmeyer, B., Pfeiffer, M., Lang, P. and Fey, G.H., 2006. Influence of variable N-glycosylation on the cytolytic potential of chimeric CD19 antibodies. *Journal of Immunotherapy* 29, 122.
- Beck, A., Cochet, O. and Wurch, T., 2010. GlycoFi's technology to control the glycosylation of recombinant therapeutic proteins. *Expert Opinion on Drug Discovery* 5, 95.
- Berdichevsky, M., d'Anjou, M., Mallem, M.R., Shaikh, S.S. and Potgieter, T.I., 2011. Improved production of monoclonal antibodies through oxygen-limited cultivation of glycoengineered yeast. *J Biotechnol.* 155, 217.
- Bergh, M.L.E., Friedman, B., Bamgrover, D., Hayes, M. and Kutzko, J., 1990. Processing and Glycosylation of Human Glucocerebrosidase Expressed in Insect Cells and in Chinese Hamster Ovary Cells. *Abstracts of Papers of the American Chemical Society* 200, 109-BIOT.
- Bobrowicz, P., Davidson, R.C., Li, H., Potgieter, T.I., Nett, J.H., Hamilton, S.R., Stadheim, T.A., Miele, R.G., Bobrowicz, B., Mitchell, T., Rausch, S., Renfer, E. and Wildt, S., 2004. Engineering of an artificial glycosylation pathway blocked in core oligosaccharide assembly in the yeast *Pichia pastoris*: production of complex humanized glycoproteins with terminal galactose. *Glycobiology* 14, 757.
- Boder, E.T. and Wittrup, K.D., 1997. Yeast surface display for screening combinatorial polypeptide libraries. *Nature Biotechnology* 15, 553.
- Boder, E.T. and Wittrup, K.D., 2000. Yeast surface display for directed evolution of protein expression, affinity, and stability. *Applications of Chimeric Genes and Hybrid Proteins*, Pt C 328, 430.
- Brumshtein, B., Salinas, P., Peterson, B., Chan, V., Silman, I., Sussman, J.L., Savickas, P.J., Robinson, G.S. and Futerman, A.H., 2010. Characterization of gene-activated human acid-beta-glucosidase: Crystal structure, glycan composition, and internalization into macrophages. *Glycobiology* 20, 24.
- Cartron, G., Dacheux, L., Salles, G., Solal-Celigny, P., Bardos, P., Colombat, P. and Watier, H., 2002. Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor Fc gamma RIIIa gene. *Blood* 99, 754.
- Choi, B.K., Bobrowicz, P., Davidson, R.C., Hamilton, S.R., Kung, D.H., Li, H., Miele, R.G., Nett, J.H., Wildt, S. and Gerngross, T.U., 2003. Use of combinatorial genetic libraries to humanize N-linked glycosylation in the yeast *Pichia pastoris*. *Proc. Natl. Acad. Sci. U. S. A* 100, 5022.
- Chowdhury, P.S. and Wu, H., 2005. Tailor-made antibody therapeutics. *Methods* 36, 11.
- Correia, I.R., 2010. Stability of IgG isotypes in serum. *Mabs* 2, 221.

- Davidson, R.C., Nett, J.H., Renfer, E., Li, H., Stadheim, T.A., Miller, B.J., Miele, R.G., Hamilton, S.R., Choi, B.K., Mitchell, T.I. and Wildt, S., 2004. Functional analysis of the ALG3 gene encoding the Dol-P-Man: Man(5)GlcNAc(2)-PP-Dol mannosyltransferase enzyme of *P-pastoris*. *Glycobiology* 14, 399.
- Egrie, J.C. and Browne, J.K., 2001. Development and characterization of novel erythropoiesis stimulating protein (NESP). *British Journal of Cancer* 84, 3.
- Feldhaus, M.J., Siegel, R.W., Opresko, L.K., Coleman, J.R., Feldhaus, J.M.W., Yeung, Y.A., Cochran, J.R., Heinzelman, P., Colby, D., Swers, J., Graff, C., Wiley, H.S. and Wittrup, K.D., 2003. Flow-cytometric isolation of human antibodies from a nonimmune *Saccharomyces cerevisiae* surface display library. *Nature Biotechnology* 21, 163.
- Ferrara, C., Grau, S., Jager, C., Sondermann, P., Brunner, P., Waldhauer, I., Hennig, M., Ruf, A., Rufer, A.C., Stihle, M., Umana, P. and Benz, J., 2011. Unique carbohydrate-carbohydrate interactions are required for high affinity binding between Fc gamma RIII and antibodies lacking core fucose. *Proceedings of the National Academy of Sciences of the United States of America* 108, 12669.
- Friess, T., Gerdes, C., Nopora, A., Patre, M., Preiss, S., van Puijenbroek, E., Schnell, C., Bauer, S., Umana, P. and Klein, C., 2007. GA101, a novel humanized type IICD20 antibody with glycoengineered Fc and enhanced cell death induction, mediates superior efficacy in a variety of NHL xenograft models in comparison to rituximab. *Blood* 110, 691A.
- Gasdaska, J.R., Sterling, J.D., Regan, J.T., Cox, K.M., Sherwood, S.W. and Dickey, L.F., 2007. Expression of a glyco-optimized anti-CD20 antibody in the aquatic plant *lemna* with enhanced ADCC activity. *Blood* 110, 697A.
- Gerngross, T., 2005. Production of complex human glycoproteins in yeast. *Adv. Exp. Med. Biol.* 564, 139.
- Hamilton, S.R., Bobrowicz, P., Bobrowicz, B., Davidson, R.C., Li, H.J., Mitchell, T., Nett, J.H., Rausch, S., Stadheim, T.A., Wischnewski, H., Wildt, S. and Gerngross, T.U., 2003. Production of complex human glycoproteins in yeast. *Science* 301, 1244.
- Hamilton, S.R., Davidson, R.C., Sethuraman, N., Nett, J.H., Jiang, Y.W., Rios, S., Bobrowicz, P., Stadheim, T.A., Li, H.J., Choi, B.K., Hopkins, D., Wischnewski, H., Roser, J., Mitchell, T., Strawbridge, R.R., Hoopes, J., Wildt, S. and Gerngross, T.U., 2006. Humanization of yeast to produce complex terminally sialylated glycoproteins. *Science* 313, 1441.
- Hamilton, S.R. and Gerngross, T.U., 2007. Glycosylation engineering in yeast: the advent of fully humanized yeast. *Current Opinion in Biotechnology* 18, 387.
- Heinrich, D.A., Klein, C., Decheva, K., Weinkauff, M., Hutter, G., Zimmermann, Y., Weiglein, T., Hiddemann, W. and Dreyling, M.H., 2009. Anti-CD20 Antibody GA101 Shows Higher Cytotoxicity but Is Competitively Displaced by Rituximab in Mantle Cell Lymphoma. *Blood* 114, 1059.
- Herbst, R., Wang, Y., Gallagher, S., Mittereder, N., Kuta, E., Damschroder, M., Woods, R., Rowe, D.C., Cheng, L., Cook, K., Evans, K., Sims, G.P., Pfarr, D.S., Bowen, M.A., Dall'Acqua, W., Shlomchik, M., Tedder, T.F., Kiener, P., Jallal, B., Wu, H. and Coyle, A.J., 2011. B-Cell Depletion In Vitro and In Vivo with an Afucosylated Anti-CD19 Antibody (vol 335, pg 213, 2010). *Journal of Pharmacology and Experimental Therapeutics* 336, 294.

- Hopkins, D., Gomathinayagam, S., Rittenhour, A.M., Du, M., Hoyt, E., Karaveg, K., Mitchell, T., Nett, J.H., Sharkey, N.J., Stadheim, T.A., Li, H.J. and Hamilton, S.R., 2011. Elimination of beta-mannose glycan structures in *Pichia pastoris*. *Glycobiology* 21, 1616.
- Jacobs, P.P. and Callewaert, N., 2009a. N-glycosylation Engineering of Biopharmaceutical Expression Systems. *Current Molecular Medicine* 9, 774.
- Jacobs, P.P. and Callewaert, N., 2009b. N-glycosylation Engineering of Biopharmaceutical Expression Systems. *Current Molecular Medicine* 9, 774.
- Jefferis, R., 2009. Recombinant antibody therapeutics: the impact of glycosylation on mechanisms of action. *Trends in Pharmacological Sciences* 30, 356.
- Jiang, Y.W., Li, F., Zha, D.X., Potgieter, T.I., Mitchell, T., Moore, R., Cukan, M., Houston-Cummings, N.R., Nylen, A., Drummond, J.E., McKelvey, T.W., d'Anjou, M., Stadheim, T.A., Sethuraman, N. and Li, H.J., 2011. Purification process development of a recombinant monoclonal antibody expressed in glycoengineered *Pichia pastoris*. *Protein Expression and Purification* 76, 7.
- Junttila, T.T., Parsons, K., Olsson, C., Lu, Y.M., Xin, Y., Theriault, J., Crocker, L., Pabonan, O., Baginski, T., Meng, G., Totpal, K., Kelley, R.F. and Sliwkowski, M.X., 2010. Superior In vivo Efficacy of Afucosylated Trastuzumab in the Treatment of HER2-Amplified Breast Cancer. *Cancer Research* 70, 4481.
- Kayser, V., Chennamsetty, N., Voynov, V., Forrer, K., Helk, B. and Trout, B.L., 2011. Glycosylation influences on the aggregation propensity of therapeutic monoclonal antibodies. *Biotechnology Journal* 6, 38.
- Kim, D.H., Du Jung, H., Kim, J.G., Lee, J.J., Yang, D.H., Park, Y.H., Do, Y.R., Shin, H.J., Kim, M.K., Hyun, M.S. and Sohn, S.K., 2006. FCGR3A gene polymorphisms may correlate with response to frontline R-CHOP therapy for diffuse large B-cell lymphoma. *Blood* 108, 2720.
- Knappik, A., Ge, L.M., Honegger, A., Pack, P., Fischer, M., Wellnhofer, G., Hoess, A., Wolle, J., Pluckthun, A. and Virnekas, B., 2000. Fully synthetic human combinatorial antibody libraries (HuCAL) based on modular consensus frameworks and CDRs randomized with trinucleotides. *Journal of Molecular Biology* 296, 57.
- Li, H.J. and d'Anjou, M., 2009. Pharmacological significance of glycosylation in therapeutic proteins. *Current Opinion in Biotechnology* 20, 678.
- Li, H.J., Sethuraman, N., Stadheim, T.A., Zha, D.X., Prinz, B., Ballew, N., Bobrowicz, P., Choi, B.K., Cook, W.J., Cukan, M., Houston-Cummings, N.R., Davidson, R., Gong, B., Hamilton, S.R., Hoopes, J.P., Jiang, Y.W., Kim, N., Mansfield, R., Nett, J.H., Rios, S., Strawbridge, R., Wildt, S. and Gerngross, T.U., 2006. Optimization of humanized IgGs in glycoengineered *Pichia pastoris*. *Nature Biotechnology* 24, 210.
- Li, J. and Zhu, Z.P., 2010. Research and development of next generation of antibody-based therapeutics. *Acta Pharmacologica Sinica* 31, 1198.
- Lin, S., Houston-Cummings, N.R., Prinz, B., Moore, R., Bobrowicz, B., Davidson, R.C., Wildt, S., Stadheim, T.A. and Zha, D.X., 2012. A novel fragment of antigen binding (Fab) surface display platform using glycoengineered *Pichia pastoris*. *Journal of Immunological Methods* 375, 159.
- Liu, L.M., Stadheim, A., Hamuro, L., Pittman, T., Wang, W.R., Zha, D.X., Hochman, J. and Prueksaritanont, T., 2011. Pharmacokinetics of IgG1 monoclonal antibodies produced in

- humanized *Pichia pastoris* with specific glycoforms: A comparative study with CHO produced materials. *Biologicals* 39, 205.
- McCamish, M. and Woollett, G., 2011. Worldwide experience with biosimilar development. *Mabs* 3, 209.
- Mimura, Y., Church, S., Ghirlando, R., Ashton, P.R., Dong, S., Goodall, M., Lund, J. and Jefferis, R., 2000. The influence of glycosylation on the thermal stability and effector function expression of human IgG1-Fc: properties of a series of truncated glycoforms. *Molecular Immunology* 37, 697.
- Misaizu, T., Matsuki, S., Strickland, T.W., Takeuchi, M., Kobata, A. and Takasaki, S., 1995. Role of Antennary Structure of N-Linked Sugar Chains in Renal Handling of Recombinant-Human-Erythropoietin. *Blood* 86, 4097.
- Mori, K., Iida, S., Yamane-Ohnuki, N., Kanda, Y., Kuni-Kamochi, R., Nakano, R., Imai-Nishiya, H., Okazaki, A., Shinkawa, T., Natsume, A., Niwa, R., Shitara, K. and Satoh, M., 2007. Non-fucosylated therapeutic antibodies: the next generation of therapeutic antibodies. *Cytotechnology* 55, 109.
- Musolino, A., Naldi, N., Bortesi, B., Pezzuolo, D., Capelletti, M., Missale, G., Laccabue, D., Zerbini, A., Camisa, R., Bisagni, G., Neri, T.M. and Ardizzoni, A., 2008. Evidence for Linkage Disequilibrium Between Fc gamma RIIa-V158F and Fc gamma RIIa-H131R Polymorphisms in White Patients, and for an Fc gamma RIIa-Restricted Influence on the Response to Therapeutic Antibodies IN REPLY. *Journal of Clinical Oncology* 26, 5491.
- Nett, J.H., Gomathinayagam, S., Hamilton, S.R., Gong, B., Davidson, R.C., Du, M., Hopkins, D., Mitchell, T., Mallem, M.R., Nylen, A., Shaikh, S.S., Sharkey, N., Barnard, G.C., Copeland, V., Liu, L.M., Evers, R., Li, Y., Gray, P.M., Lingham, R.B., Visco, D., Forrest, G., DeMartino, J., Linden, T., Potgieter, T.I., Wildt, S., Stadheim, T.A., d'Anjou, M., Li, H.J. and Sethuraman, N., 2012. Optimization of erythropoietin production with controlled glycosylation-PEGylated erythropoietin produced in glycoengineered *Pichia pastoris*. *Journal of Biotechnology* 157, 198.
- Nett, J.H., Stadheim, T.A., Li, H., Bobrowicz, P., Hamilton, S.R., Davidson, R.C., Choi, B.K., Mitchell, T., Bobrowicz, B., Rittenhour, A., Wildt, S. and Gerngross, T.U., 2011. A combinatorial genetic library approach to target heterologous glycosylation enzymes to the endoplasmic reticulum or the Golgi apparatus of *Pichia pastoris*. *Yeast* 28, 237.
- Nicolaides, N.C., Sass, P.M. and Grasso, L., 2010. Advances in targeted therapeutic agents. *Expert Opinion on Drug Discovery* 5, 1123.
- Nimmerjahn, F. and Ravetch, J.V., 2007. Fc-receptors as regulators of immunity. *Advances in Immunology*, Vol 96 96, 179.
- Nimmerjahn, F. and Ravetch, J.V., 2008. Fc gamma receptors as regulators of immune responses. *Nature Reviews Immunology* 8, 34.
- Nimmerjahn, F. and Ravetch, J.V., 2010. Antibody-mediated modulation of immune responses. *Immunol. Rev.* 236, 265.
- Oh, D.B., Park, J.S., Kim, M.W., Cheon, S.A., Kim, E.J., Moon, H.Y., Kwon, O., Rhee, S.K. and Kang, H.A., 2008. Glycoengineering of the methylotrophic yeast *Hansenula polymorpha* for the production of glycoproteins with trimannosyl core N-glycan by blocking core oligosaccharide assembly. *Biotechnol. J* 3, 659.

- Pandhal, J. and Wright, P.C., 2010. N-Linked glycoengineering for human therapeutic proteins in bacteria. *Biotechnology Letters* 32, 1189.
- Pastores, G.M., 2010. Recombinant Glucocerebrosidase (Imiglucerase) as a Therapy for Gaucher Disease. *Biodrugs* 24, 41.
- Potgieter, T.I., Cukan, M., Drummond, J.E., Houston-Cummings, N.R., Jiang, Y.W., Li, F., Lynaugh, H., Mallem, M., McKelvey, T.W., Mitchell, T., Nylen, A., Rittenhour, A., Stadheim, T.A., Zha, D.X. and d'Anjou, M., 2009. Production of monoclonal antibodies by glycoengineered *Pichia pastoris*. *Journal of Biotechnology* 139, 318.
- Potgieter, T.I., Kersey, S.D., Mallem, M.R., Nylen, A.C. and d'Anjou, M., 2010. Antibody Expression Kinetics in Glycoengineered *Pichia Pastoris*. *Biotechnology and Bioengineering* 106, 918.
- Projan, S.J., Gill, D., Lu, Z.J. and Herrmann, S.H., 2004. Small molecules for small minds? The case for biologic pharmaceuticals. *Expert Opinion on Biological Therapy* 4, 1345.
- Robak, T. and Robak, E., 2011. New Anti-CD20 Monoclonal Antibodies for the Treatment of B-Cell Lymphoid Malignancies. *Biodrugs* 25, 13.
- Roopenian, D.C. and Akilesh, S., 2007. FcRn: the neonatal Fc receptor comes of age. *Nature Reviews Immunology* 7, 715.
- Sethuraman, N. and Stadheim, T.A., 2006. Challenges in therapeutic glycoprotein production. *Curr. Opin. Biotechnol.* 17, 341.
- Shaaltiel, Y., Bartfeld, D., Hashmueli, S., Baum, G., Brill-Almon, E., Galili, G., Dym, O., Boldin-Adamsky, S.A., Silman, I., Sussman, J.L., Futerman, A.H. and Aviezer, D., 2007. Production of glucocerebrosidase with terminal mannose glycans for enzyme replacement therapy of Gaucher's disease using a plant cell system. *Plant Biotechnology Journal* 5, 579.
- Sinclair, A.M. and Elliott, S., 2005. Glycoengineering: The effect of glycosylation on the properties of therapeutic proteins. *Journal of Pharmaceutical Sciences* 94, 1626.
- Sola, R.J. and Griebenow, K., 2009. Effects of Glycosylation on the Stability of Protein Pharmaceuticals. *Journal of Pharmaceutical Sciences* 98, 1223.
- Takeuchi, M., Inoue, N., Strickland, T.W., Kubota, M., Wada, M., Shimizu, R., Hoshi, S., Kozutsumi, H., Takasaki, S. and Kobata, A., 1989. Relationship Between Sugar Chain Structure and Biological-Activity of Recombinant Human Erythropoietin Produced in Chinese-Hamster Ovary Cells. *Proceedings of the National Academy of Sciences of the United States of America* 86, 7819.
- Tomiya, N., 2009. Humanization of recombinant glycoproteins expressed in insect cells. *Trends in Glycoscience and Glycotechnology* 21, 71.
- Umana, P., Ekkehard, M., Peter, B., Gabriele, K., Ursula, P., Suter, T., Grau, R., Schmidt, C., Herter, S., Gerdes, C., Nopora, A., Patre, M., Moser, S., Sondermann, P., Wheat, L., Dyer, M.J.S., Poppema, S., Bauer, S., Kubbies, M., Strein, P., Fertig, G., Friess, T., Dabbagh, K., DalPorto, J. and Klein, C., 2007. GA101, a novel humanized type II CD20 Antibody with glycoengineered Fc and enhanced cell death induction, exhibits superior anti-tumor efficacy and superior tissue B cell depletion in vivo. *Blood* 110, 694A.
- Vaughan, T.J., Williams, A.J., Pritchard, K., Osbourn, J.K., Pope, A.R., Earnshaw, J.C., McCafferty, J., Hodits, R.A., Wilton, J. and Johnson, K.S., 1996. Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library. *Nature Biotechnology* 14, 309.

- von Horsten, H.H., Ogorek, C., Blanchard, V., Demmler, C., Giese, C., Winkler, K., Kaup, M., Berger, M., Jordan, I. and Sandig, V., 2010. Production of non-fucosylated antibodies by co-expression of heterologous GDP-6-deoxy-D-lyxo-4-hexulose reductase. *Glycobiology* 20, 1607.
- Walsh, G., 2010. Post-translational modifications of protein biopharmaceuticals. *Drug Discovery Today* 15, 773.
- Wang, Q.J., Li, L., Chen, M., Qi, Q.Q. and Wang, P.G., 2007. Construction of a novel system for cell surface display of heterologous proteins on *Pichia pastoris*. *Biotechnology Letters* 29, 1561.
- Ward, E., Mittereder, N., Kuta, E., Sims, G.P., Bowen, M.A., Dall'Acqua, W., Tedder, T., Kiener, P., Coyle, A.J., Wu, H., Jallal, B. and Herbst, R., 2011. A glycoengineered anti-CD19 antibody with potent antibody-dependent cellular cytotoxicity activity in vitro and lymphoma growth inhibition in vivo. *British Journal of Haematology* 155, 426.
- Weaver-Feldhaus, J.M., Lou, J.L., Coleman, J.R., Siegel, R.W., Marks, J.D. and Feldhaus, M.J., 2004. Yeast mating for combinatorial Fab library generation and surface display. *Febs Letters* 564, 24.
- Wildt, S. and Gerngross, T.U., 2005. The humanization of N-glycosylation pathways in yeast. *Nature Reviews Microbiology* 3, 119.
- Wittrup, K., 2009. Protein and Antibody Engineering by Yeast Surface Display. *Journal of Biomolecular Screening* 14, 883.
- Wong, A.W., Baginski, T.K. and Reilly, D.E., 2010. Enhancement of DNA Uptake in FUT8-Deleted CHO Cells for Transient Production of Afucosylated Antibodies. *Biotechnology and Bioengineering* 106, 751.
- Ye, J., Ly, J., Watts, K., Hsu, A., Walker, A., McLaughlin, K., Berdichevsky, M., Prinz, B., Sean, K.D., d'Anjou, M., Pollard, D. and Potgieter, T., 2011a. Optimization of a glycoengineered *Pichia pastoris* cultivation process for commercial antibody production. *Biotechnol. Prog.* 27, 1744.
- Ye, J.X., Ly, J., Watts, K., Hsu, A., Walker, A., McLaughlin, K., Berdichevsky, M., Prinz, B., Kersey, D.S., d'Anjou, M., Pollard, D. and Potgieter, T., 2011b. Optimization of a glycoengineered *Pichia pastoris* cultivation process for commercial antibody production. *Biotechnology Progress* 27, 1744.
- Ysebaert, L., Laprevotte, E., Gross, E., Klein, C., Fournie, J., Laurent, G. and Ouillet-Mary, A., 2010. Ga101 Displays Higher Anti-Leukemic Activity Mainly Through Enhanced Adcc in Chronic Lymphocytic Leukemia. *Haematologica-the Hematology Journal* 95, 46.
- Zhang, N.Y., Liu, L.M., Dumitru, C.D., Cummings, N.R.H., Cukan, M., Jiang, Y.W., Li, Y., Li, F., Mitchell, T., Mallem, M.R., Ou, Y.S., Patel, R.N., Vo, K., Wang, H., Burnina, I., Choi, B.K., Huber, H., Stadheim, T.A. and Zha, D.X., 2011. Glycoengineered *Pichia* produced anti-HER2 is comparable to trastuzumab in preclinical study. *Mabs* 3, 289.
- Zhou, H.H., 2007. Biologics in the pipeline: Large molecules with high hopes or bigger risks? *Journal of Clinical Pharmacology* 47, 550.
- Zhou, J.G., Zhang, H.C., Liu, X.W., Wang, P.G. and Qi, Q.S., 2007. Influence of N-glycosylation on *Saccharomyces cerevisiae* morphology: A golgi glycosylation mutant shows cell division defects. *Current Microbiology* 55, 198.