Development of high expression systems for biopharmaceuticals production using genetic engineering

遺伝子工学的手法を用いた生物製剤高発現系の開発

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Contents

General Introduction

Biopharmaceuticals

Since the early 1980s biopharmaceuticals have emerged as a major new class of pharmaceuticals with over 200 marketed products that are mainly therapeutics, together with a small number of diagnostics and vaccines (Fig. 1a) (Aggarwal 2007; Aggarwal 2011; Li et al 2010; Walsh 2010). First generation biologics such as human insulin and erythropoietin were developed as therapeutics to replace deficient natural proteins (Carter 2011). Monoclonal antibodies (mAbs) have had extensive success as second generation biologics, and remain the best-selling biologic class (Fig. 1b). Because mAbs have higher affinity, decreased immunogenicity, and optimized effector functions (Buss et al. 2012).

To reduce the economic burden on the patient, next generation antibodies are presently being developed to enhance the efficacy of antibody therapeutics based on different strategies such that POTELLIGENT® and COMPLEGENT® technologies, antibody-drug conjugates (ADCs), and bi-specific antibodies (Buss et al. 2012).

The process of antibody production

Monoclonal antibodies have proven to be significantly effective in some diseases such as cancer and auto-immune disease because they can specifically bind to target molecules with high affinity and have a low side effect profile. However, they are one of the most expensive of all drugs where the annual cost per patient can reach $35,000 for antibodies treating cancer conditions (Farid 2007). The high prices result from the need for relatively high doses (>1 g per patient per year) in the clinic. Therefore

cost-effective manufacturing of antibodies is desirable for reducing cost for high doses administration and increasing market potential. Therapeutic antibodies are mainly produced in mammalian host cell lines such as NS0 murine myeloma cells and Chinese hamster ovary (CHO) cells because these can generate correctly folded and glycosylated proteins (Li et al. 2010; Wurm 2004). The process for establishment of stable cell lines starts with the construction and transfection of an expression vector bearing the heavy and light chain genes of antibody, and selectable markers. After screening of cells for high productivity, cells are usually adapted to serum-free suspension culture, and sometimes the productivity is further improved by gene amplification (if necessary). Subsequently, cells are cultivated in batch/fed-batch culture, followed by purification processes including Protein A chromatography (Beck et al. 2010; Chon and Zarbis-Papastoitsis 2011; Li et al. 2010; Shukla and Thömmes 2010).

Strategies for improving the yield of recombinant proteins;

1) Vector modification

In order to decrease manufacturing costs, the productivity of recombinant antibodies in mammalian cells which is directly linked to cost of goods has to be increased (Table 1) (Werner 2004; Zhou et al. 2008). The yield of recombinant antibodies in CHO cells has been reported to be over 1 g/L, and recent advances have enabled around 10 g/L in some cases (Birch and Racher 2006). There are two main approaches involved in the maximization of therapeutic protein yield; vector modification and host cell engineering (Fig. 2) (Barnes and Dickson 2006). The expression vector typically includes strong promoter/enhancers such as the cytomegalovirus (CMV) promoter and elongation factor alpha (EF1α) promoter to

elevate the expression levels of the heavy and light chains. Recently, innovative approaches to incorporate cis-acting DNA elements in the expression vector have been reported. The human β-globin locus control regions and the cHS4 insulator are well-known DNA elements (Bell et al. 2001; Li et al. 2002). Ubiquitous chromatin opening elements have been isolated from de-metylated CpG islands that were shown to be able to maintain a favorable chromatin conformation and to protect against gene silencing (Williams et al. 2005). In addition, stabilizing and anti-repressor elements and matrix attachment regions were identified as DNA elements which block chromatin associated repressors, and bind to the nuclear matrix, respectively (Girod et al. 2007; Otte et al. 2007). I also successfully identified novel DNA elements which could dramatically increase the expression level of proteins including antibodies in stable CHO cells through the screening based on chromatin immunoprecipitation on microarray analysis (Fig. 3) (Nishimiya and Inoue 2012). These DNA elements may specifically overcome epigenetic gene silencing in different manners, providing a high yield and the long-term expression of recombinant proteins in stable cell lines (Kwaks and Otte 2006).

2) Host cell engineering

Vector modification using cis-acting DNA elements may give a significant impact on increasing recombinant proteins production. In fact, mammalian cells have been developed by now using engineered expression vectors to give high mRNA levels. In addition, many researchers have tried to engineer cellular metabolism, apoptotic signals, cell cycle, etc., however it appears difficult to reach an enough industrial production level (Kim et al. 2012). Though these studies, Barnes and Dickson, and Dinnis and

James suggested the importance of secretion to improve the production level (Barnes and Dickson 2006; Dinnis and James 2005). However, there has been no observed correlation between the secretory ability of cells and the corresponding cellular availability of antibody mRNA levels (Mohan et al. 2008; Smales et al. 2004). There are many diverse processes in protein secretion after transcription; translation, post-translation modifications (folding, assembly, and glycosylation), and finally secretion.

The ER has a major role in protein processing (folding and assembly) and the transmission of signals to the signal transduction pathway for unfolded protein response (UPR) (Fig. 4) (Schröder and Kaufman 2005). Protein folding occurs co-translationally, and requires molecular chaperone machinery which is comprised of different molecular chaperones (e.g., GRP78/Bip, GRP94), co-chaperones (e.g., ERdj3, ERdj5) and protein foldases (e.g., PDI, ERO1α). GRP78/Bip is one of the most abundant ER chaperones that are not only implicated in folding and assembly steps but also controlling the activation of transmembrane ER stress sensors (Morris et al. 1997). Protein disulfide isomerase (PDI) works as a foldase to form disulfide bonds in the newly synthesized polypeptides (Ellgaard and Ruddock 2005). Molecular chaperones generally facilitate protein folding by binding to unfolded peptide chains, preventing of aggregation, and/or support of refolding. When misfolded or unfolded proteins are accumulated as intracellular aggregates, the UPR occurs with multiple signaling pathways, which decreases protein synthesis, up-regulates chaperone expression, enhances ER-associated degradation (ERAD) in the proteasome, and occurs ER stress-mediated apoptosis (Schröder 2006). C/EBP homologous protein (CHOP), which is also known as growth arrest and DNA damage-inducible gene 153 (Gadd153), is reported to induce ER

stress-mediated apoptosis in different cells (Oyadomari and Mori 2004). In high-producing cells, the capacities of these chaperones or UPR-related proteins may to be overloaded with recombinant proteins, leading to the induction of the UPR (Jenkins et al. 2008). Indeed, some ER-resident proteins such as GRP78/Bip and PDI were remarkably increased in cells with relatively high secretion rates of antibodies (Dinnis et al. 2006; Smales et al. 2004). Only a limited number of chaperones and UPR-related genes including GRP78/Bip, PDI and XBP-1 have been manipulated in an attempt to improve the productivity of recombinant proteins (Dinnis and James 2005; Jenkins et al. 2008). However, both positive and negative effects of manipulation have been reported, suggesting that increased secretion level cannot be attained by a simple strategy such as modification of a single protein comprising folding and secretion system.

ER chaperones or UPR-related proteins may contribute to improving protein folding and assembly, which results in increased recombinant proteins production. However, candidate proteins for host cell engineering have been so far restricted. Therefore, I aimed to investigate endogenous proteins which could be involved in enhancing recombinant antibodies in **Chapter 1** and **2**. In **Chapter 1**, I describe the identification of several ER-resident proteins including chaperones and non-ER-resident proteins that interacted with antibody fragments or whole antibodies in mammalian cells by immunoprecipitation coupled with liquid chromatography-tandem mass spectrometry (IP-MS). In **Chapter 2**, I focused on the ability of UPR-related proteins to improve protein quality and increase yields. I identified the expression of UPR-related genes, CHOP/Gadd153 and GRP78/Bip and their correlation with recombinant antibody expression. I found that endogenous genes identified in **Chapter 1** and **2** had an effect on increasing antibody expression in mammalian cells.

Transgenic chickens as an alternative host

Although recent advances in mammalian cells have enabled high productivity of recombinant proteins, that cannot cover the growing demand for protein therapeutics (as described above). Available facilities for mammalian cell culture of contract manufacturing organizations have been saturated, and recombinant proteins production for the clinical manufacturing sometimes take longer time which slow down the overall process of pharmaceutical development (Langer 2009). Additionally, cost of goods is still high despite recent progress in increasing productivity. Under this circumstance, the production of therapeutic proteins in alternative hosts such as animals or plants has been proposed as a solution. Milk is presently the most advanced system to produce recombinant proteins from transgenic livestock, and human antithrombin produced in goat milk was first approved by the European Medicines Agency in 2006 (Houdebine 2009). In addition, there has been significant progress in plant-made biologics. In 2012, the FDA approved Elelyso, a recombinant human glucocerebrosidase, produced in genetically modified carrot cells, for treating type 1 Gaucher's disease. These decisions suggest that recombinant proteins generated by transgenic animals or plants may be structurally identical to their native counterparts, and they can meet clinical and biosafety criteria for human case such as immunogenicity.

Avian species, which have been historically used in the fields of immunology, virology and vaccine development, can offer several advantages for recombinant protein production among different vertebrates: high production levels, the possibility of post-translational modifications and glycosylation, rapid maturation, low costs, and small requirements for breeding (Table 2). The different technologies to deliver a gene construct into the embryo or primordial germ cells have been sufficiently developed to

overcome the low efficiency of transgenic chicken establishment (Song and Han 2011). In **Chapter 3**, I successfully established transgenic chickens to generate human erythropoietin (hEpo), and analyzed the variation of glycosylation of produced hEpo. By now, several groups reported the production of hEpo in the milk of transgenic mammals, but its productivity was low.

In this study, to meet the increasing demand for protein-based drugs, I investigated the development of a high expression system for biopharmaceuticals production using diverse genetic engineering strategies. I will describe my results in three parts; Chapter 1, 2, and 3. In **Chapter 1** and **2,** I identified endogenous proteins including molecular chaperones and UPR-related genes which enhance recombinant antibody production in mammalian cells. In **Chapter 3**, I developed transgenic chickens producing hEpo and analyzed the quality of hEpo.

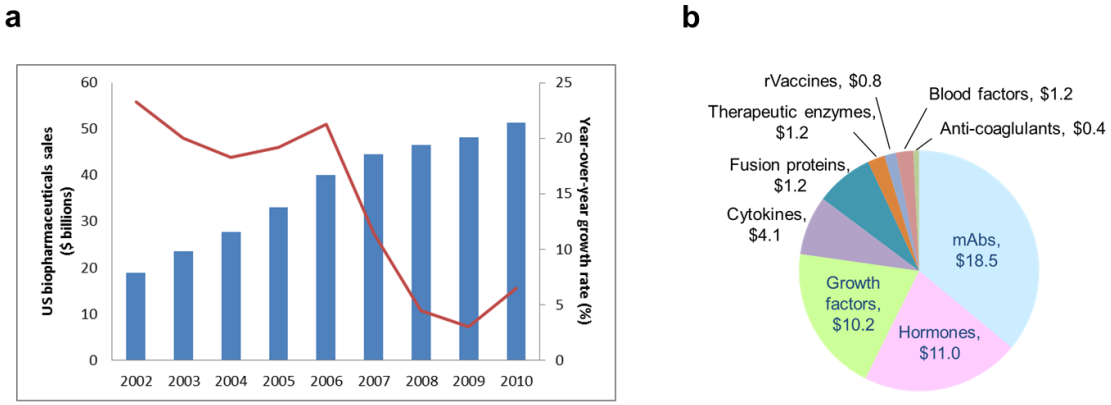

Fig. 1 Growth trends in the US biotech market for biopharmaceuticals (2002–2010)[a]. Total sales and growth rate trends (**a**). Top nine categories of biopharmaceuticals in terms of US sales in 2010 (**b**). The pie chart shows US sales of these drug categories. [a]Source: Aggarwal 2007; Aggarwal 2011

Table 1 Cost of goods/g for mammalian cell systems producing antibodies for different titers[a].

Annual production rate (kg/year)	Titer (g/L)	Capacity (L)	COG/g ($/g)	COG/year ($M/year)
250	1	20,000	260	65
250	0.1	310,000	1,500	375

[a]Source: Werner 2004; Farid 2007

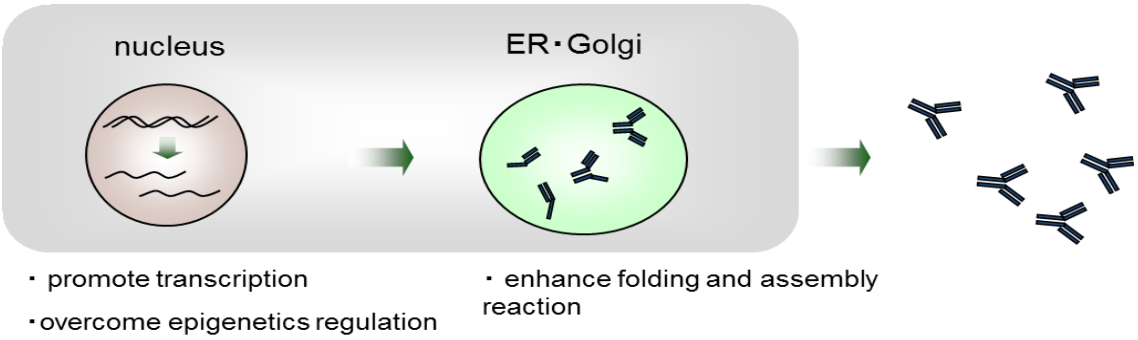

Fig. 2 Enhancement of recombinant protein production. The pathway is highly complex with multiple steps that can dictate the efficacy and stability of recombinant antibody production. To enhance antibody production, there are mainly two key approaches: vector modification that strengthens transcription and excludes epigenetic regulation, and improvement of the secretory ability such as folding and assembly.

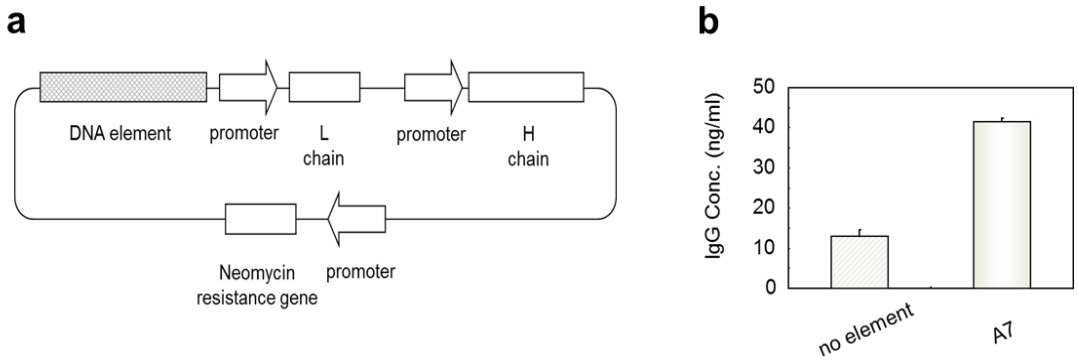

Fig. 3 Novel DNA elements identified by us can dramatically increase the production of recombinant proteins including antibodies in stable CHO cells. Vector construction introducing a DNA element to express recombinant antibody (**a**). A novel DNA element (A7) shows an increase effect on increasing antibody production in stable CHO cells (**b**).

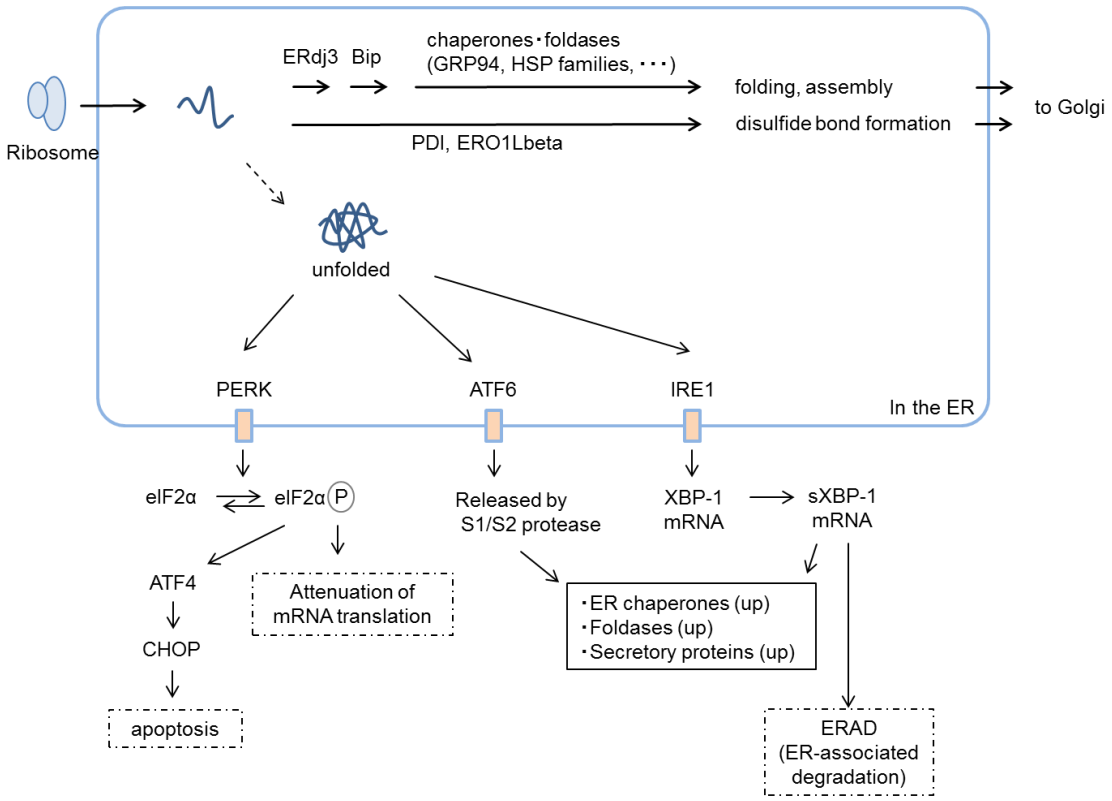

Fig. 4 Protein transport through the ER, and the unfolded protein response (UPR) which couple protein folding capacities of the ER in mammalian cells. The recognition of nascent peptide chains by co-chaperones such as ERdj3 triggers molecular chaperone machinery including Bip to activate protein folding and assembly reaction. Additionally, protein disulfide isomerase (PDI) and ERO1Lbeta catalyze disulfide bond formation, leading to correctly formed protein. However, the accumulation of unfolded or misfolded protein cause by ER stress that induces the UPR. The UPR comprises three signal transduction pathways: PERK, ATF6, and IRE1. The attenuation of mRNA translation is signaled through PERK-mediated phosphorylation of eukaryotic initiation factor 2α (eIF2α). Further, the transcription of ATF4 promoted by phosphorylation of eIF2α induces CHOP which may promote apoptosis. The cleavage of ATF6 by S1/S2 protease can induce the transcription of ER chaperones and foldases to maintain protein quality. Finally, X-box binding protein 1 (XBP-1) spliced by IRE1 regulates chaperone induction, expansion of the ER in response to ER stress, and ER-associated degradation (ERAD).

Table 2 Comparison of the different systems to produce recombinant proteins[a].

Points to consider	Production systems				
	Bacteria	Mammalian cells	Transgenic plants	Transgenic goats or cows	Transgenic chickens
Post translational modifications	+	+++	++	+++	+++
Glycosylation	−	+++	+	+++	+++
Production level	+++	++	+	++	++
Production cost	+	+++	+	++	++
Scaling up (facilities)	+++	+	+++	++	++
Time required to obtain recombinant proteins	+++	+	++	+	+++
Products on the market	++	+++	+	+	+

[a]Source: Houdebine 2009; Rudolph 1999

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Chapter 1

Identification of antibody interacting proteins that contribute to the production of recombinant antibody in mammalian cells

1-1 Summary

Protein folding and assembly processes are essential for antibody secretion; however, the endogenous proteins involved in these processes remain largely unknown. Therefore, except for some well-known endoplasmic reticulum (ER) chaperones such as GRP78/Bip and protein disulfide isomerase, enhancement of recombinant antibody expression by co-expression of these endogenous proteins has been largely elusive. Here, in addition to known ER chaperones, we identified additional endogenous proteins that interact with recombinant antibody in mammalian cells by immunoprecipitation coupled with liquid chromatography–tandem mass spectrometry. Most of our identified proteins enhanced antibody production, and furthermore, some of their combinations resulted in greater enhancement. In particular, eukaryotic initiation factor 4A combined with other proteins had approximately four-fold higher effect on antibody production. Identified proteins that could improve antibody expression contain not only ER-resident proteins like GRP78/Bip but also non-ER-resident proteins. These results suggest that this method could be effective in the investigation of novel proteins that are involved in enhancing recombinant antibody production because immunoprecipitation coupled with mass spectroscopy could identify proteins which directly interact with the antibody.

1-2 Introduction

Antibody is a complex molecule comprising two identical heavy chains and two identical light chains. Whereas the light chain can be secreted alone, the heavy chain cannot be secreted without the assembly of the light chain (Dul et al. 1996; Hendershot et al. 1987), because the CH1 domain of the heavy chain folds only by interaction with the CL domain of the light chain (Feige et al. 2009). Additionally, other posttranslational modifications including disulfide bond formation and glycosylation are also required for folding, assembly, and secretion of antibody (Srebalus Barnes and Lim 2007).

Antibody production is tightly controlled by diverse secretion processes in the cell (Helenius et al. 1992). These reactions are managed in the endoplasmic reticulum (ER) and the Golgi apparatus; however, a few proteins that play an important role in posttranslational processes prior to secretion have been characterized. For instance, the 78-kDa glucose regulated protein/BiP (GRP78/Bip) is one of the most abundant ER chaperones and contributes to the translocation of nascent chains into the ER (Dorner et al. 1987; Kassenbrock et al. 1988; Morris et al. 1997). Protein disulfide isomerase (PDI) has both chaperone and disulfide isomerase activity (Ellgaard and Ruddock 2005; Riemer et al. 2009). Because incorporation of disulfide bonds in newly synthesized proteins is dependent on conformational folding of the polypeptide chain (Welker et al. 2001), PDIs and other chaperones couple polypeptide folding to disulfide bond formation. Both chaperones seem to have essential roles for antibody formation in the ER, and thus have been targets to engineer host cells producing recombinant protein at high levels in yeast and mammalian cells until now. In fact, overexpression of Bip or PDI increases scFv expression in *Saccharomyces cerevisiae* (Shusta et al. 1998), but

they do not have a remarkable effect on increasing monoclonal antibody production in CHO cells (Borth et al. 2005; Dorner et al. 1992). These data show that their overexpression may contribute to improved protein folding or assembly in some case, but additional proteins are required for further enhancement of protein production.

Though a few ER chaperones such as GRP78/Bip and PDI have been identified, little is known with other endogenous proteins associated with antibody folding and assembly processes. Subsequently, comprehensive analyses were performed to understand intracellular phenomenon and proteins related to protein folding in greater detail. Jonikas et al. identified several genes involved in a wide range of activities such as chaperones, glycosylation, and ER-associated degradation (ERAD) which are required for protein folding in yeast (Jonikas et al. 2009). Similarly, to identify specific genes that enabled mammalian cells to achieve a high level of recombinant antibody production, proteomic method was applied to mammalian cells. Dinnis et al. observed that any single process such as protein synthesis, degradation, and processing did not greatly affect antibody production in GS-NS0 (Dinnis et al. 2006; Smales et al. 2004). In general, comprehensive analyses are important for understanding protein folding, but it is not clear whether this information can be applied to host cell engineering to obtain higher production of recombinant proteins.

Here, we attempted to identify endogenous proteins that were directly involved in antibody expression by immunoprecipitation coupled with liquid chromatography–tandem mass spectrometry (LC–MS/MS) (IP–MS). We found that most of the proteins identified by IP–MS had an effect on enhancing antibody expression, and furthermore, some of their combinations improved antibody expression. These results show that IP–MS is a useful tool for identification of proteins that

contribute to the improvement of antibody production.

1-3 Materials and Methods

1-3-1 Cell culture

FreeStyle™ 293F cells were purchased from Invitrogen and were maintained in Gibco® FreeStyle™293 Medium at 37°C and 8% CO_2. COS-1 cell line was obtained from the American Type Culture Collection. COS-1 cells were maintained in α-MEM (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) at 37°C and 5% CO_2.

1-3-2 Immunoprecipitation

For IP–MS, the heavy chain of a humanized anti-human death receptor 5 antibody (TRA-8) was amplified minus stop codon using KOD-plus- (Toyobo) with the primers 5′-accaagcttggcttgacctcaccatg-3′ and 5′-accgaattctttacccggggacag-3′. Then, the expression vector for the heavy chain with FLAG tag was constructed by cloning the PCR product into the HindIII/EcoRI site of pFLAG5.1 (Sigma). The expression vector for green fluorescent protein (GFP) with FLAG tag was similarly constructed.

Transfection of each expression vector was performed in 30 ml culture scale using FreeStyle™ MAX reagent (Invitrogen). Briefly, 30 μg of expression vectors of GFP with FLAG tag or the heavy chain with the tag was transfected into 293F cells at 1×10^6 cells/ml, and expression vectors containing the heavy chain with FLAG and the light chain were co-transfected into 293F cells at 1×10^6 cells/ml. Cells were harvested 4 days

after transfection. 293F cells were collected by centrifugation and washed with PBS several times, and then lysed in RIPA buffer (Pierce). One hundred microliters of anti-FLAG M2 affinity gel (Sigma) equilibrated with RIPA buffer was added to each lysate. After overnight incubation at 4°C, resin was washed with washing buffer (TBS containing 1% n-dodecyl-β-D-maltoside) several times and eluted with wash buffer containing 200 μg/ml of FLAG peptide (Sigma).

1-3-3 Silver staining and Western blot analysis

Aliquots of the eluted protein fractions were subjected to SDS-PAGE then detected with silver staining or Western blotting. The protein concentration of the whole cell lysates was determined using a DC protein assay (BioRad) using BSA as a standard protein. Thirty micrograms of whole cell lysates was resolved by 5–20% SDS–PAGE under non-reducing conditions. Silver staining was performed following the manufacturer's instructions (Daiichi Kagaku Yakuhin Kogyo). Each cell lysate was electrophoretically transferred onto a PVDF membrane after SDS–PAGE. Goat anti-human IgG (gamma-chain specific) Affinity Isolated Antigen Specific Antibody (Sigma: I3382) and anti-kappa chain antibody (BETYL: A80-115A) were used as primary antibodies, and an HRP-conjugated rabbit anti-goat IgG antibody (ROCKLAND: 605–4302) was used as the secondary antibody for Western blot analysis. For detection of FLAG fusion proteins, anti-FLAG M2 HRP antibody (Sigma: A8592) was used. Membranes were then developed with ECL advance Western blotting detection Kit (GE Healthcare).

1-3-4 LC–MS/MS and data analysis

Aliquots of each sample were precipitated by methanol/chloroform. The precipitate was solubilized in 8 M urea buffer (50 mM Tris–HCl (pH 8.0), 8 M urea, 10 mM EDTA, 0.005% DM). The proteins were reduced with 10 mM DTT for 20 min at 37°C, alkylated for 20 min with 20 mM iodoacetamide at room temperature under the dark, and digested overnight with trypsin (Promega) at 37°C. The resulting peptide samples were extracted and sequenced with LC–MS/MS on a DiNa nano-flow liquid chromatography system (KYA tech) coupled to LTQ-Orbitrap (Thermo Fisher Scientific). Aliquots of the peptide mixture (5 μl) were loaded onto the trap column at a flow rate of 5 μl/min. After loading, the peptides were eluted from the trap column in a counter flow direction with a linear gradient and separated on the in-house packed analytical column (40 mm× 75 μm I.D., 1.7 μm BEH C18, Waters) at a flow rate of 150 nl/min with a 60-min gradient from 5 to 35% acetonitrile in 0.1% formic acid.

The MS/MS spectra data were searched against the human IPI database (version 3.72 containing 172,784 forward and reversed protein sequences) using Mascot 2.2 (Matrix Science). Parent mass tolerance was 50 ppm, MS/MS tolerance was 0.8 Da, and fix modifications were set to carbamidomethyl cysteine and methionine oxidation and N-terminal protein acetylation. The search results were filtered and summarized using in-house developed software. In the software, we fixed the estimated false discovery rate of all peptide identifications at less than 1%, by automatically filtering on mass error and peptide score of all forward and reversed peptide identifications. Following this, gene ontology terms, which revealed an enrichment of genes related to endogenous proteins interacted with antibody fragments, were analyzed using the DAVID Bioinformatics Database (http://david.abcc.ncifcrf.gov/). *p* values for each gene ontology category were represented as a Bonferroni-correlated modified Fisher's exact

test. The Mascot score for each protein was calculated based on the ion score of the peptide identification and attributed to the respective protein, and GFP with C-terminal FLAG was used for normalization. Endogenous genes that had specific interaction with antibody fragments were defined using the following filters: significance analysis of LC–MS/MS with a false discovery rate (FDR) less than 1% and a number of identified peptides in H chain with FLAG and/or antibody with FLAG was more than threefold higher than in GFP with FLAG.

1-3-5 Co-expression of hTRA-8 antibody with candidate genes

Humanized antibody (hTRA-8) expression vector was constructed by inserting the heavy chain and the light chain of hTRA-8 into pcDNA3.1 (+) (Invitrogen). Total RNA was extracted from 1×10^7 cells of 293F cells using the RNeasy Kit (Qiagen) according to the manufacturer's instructions. Total RNA (600 ng) was reverse-transcribed into cDNA using ReverTra Ace qPCR RT Kit (Toyobo). The full length cDNAs of human GRP78/Bip, PDI, GRP94, HSP47, PDIA4, TPM3, CTPS, CCT1, CCT5, HSP90β, CALR, PRPS, Erlin-2, PHB, ATP2A2, MCM7, PCBP1, PCBP2, MRCL3, ATP5A, ELAV, eukaryotic initiation factor 4A (eIF4A), P5CS, Tu, YBX, Ran, ILF3, and MCM4 were amplified using KOD-plus- with primers as described in Table 1-1, and then each expression vector was constructed using pcDNA™ 3.1 Directional TOPO® Expression kit (Invitrogen).COS-1 cells (1×10^5) were seeded into each well of a 24-well plate and incubated for 24 h prior to transfection. Cells were transfected with recombinant humanized antibody (hTRA-8) expression vector (0.4 μg) and each expression vector (0.4 μg) in triplicate using Lipofectamine2000 (Invitrogen). The amount of DNA was kept constant by adding an empty vector. After 48 h of incubation, the supernatants

were collected and analyzed.

1-3-6 ELISA detection

Microplates (96-well) (Nalgene nunc) were coated with 1 μg/ml of anti-kappa chain antibody (BETYL) and incubated overnight at 4°C. After washing with PBST (PBS containing 0.05% (*v/v*) Tween-20), wells were then blocked with Blocker Casein in PBS (Pierce) overnight at 4°C. After washing with PBST, the supernatants were added and incubated for 1 h at 37°C. The sample solution was removed and the wells were washed as above. Peroxidase-Labeled Affinity Purified Antibody To Human IgG (Fc) (KPL) in washing buffer was then added and plate was incubated for 1 h at 37°C. After washing with PBST, a substrate solution (Nacalai Tesque) was added and the reaction proceeded at room temperature in the dark. The reaction was stopped by the addition of the stop solution and the absorbance at 405 nm was measured using SpectraMax (Molecular Devices). For IgG expression assays, multiple pairwise comparisons across groups were performed by one-way repeated measures ANOVA.

Table 1-1 List of primers for cloning identified genes.

GRP78/Bip	5’-caccatgaagctctccctggtggc-3’, 5’- ctacaactcatctttttctgctgta-3’
PDI	5’-caccatgcgcctccgccgcctagcgctg-3’, 5’- ttagagatcctcctgtgccttcttc-3’
GRP94	5’-caccatgagggccctgtgggtgc-3’, 5’-ttacaattcatctttttcagctgtagattc-3’
HSP47	5’caccatgcgctccctcctgcttctcagcg-3’, 5’-ctataactcgtctcgcatcttgtcacc-3’
PDIA4	5’-caccatgaggccccggaaagccttcctgctc-3’, 5’-tcaaagctcttccttggtcctgctc-3’
TPM3	5’-caccatggctaccatcaccaccatcgag-3’, 5’-ctacatctcattcaggtcaagcagggt-3’
CTPS	5’-caccatgaagtacattctggttactggtgg-3’, 5’-tcagtcatgatttattgatggaaac-3’
CCT1	5’-caccatggaggggcctttgtccgtgttcg-3’, 5’-tcaatcattaagggctccagagtg-3’
CCT5	5’-caccatggcgtccatggggaccctcgccttc-3’, 5’-tcattcttcagattctccaggcttac-3’
HSP90β	5’-caccatgcctgaggaagtgcaccatg-3’, 5’-ctaatcgacttcttccatgcgagac-3’
CALR	5’-caccatgctgctatccgtgccgctgctgc-3’, 5’-ctacagctcgtccttggcctggcc-3’
PRPS	5’-caccatgccgaatatcaaaatcttcagcg-3’, 5’-ttataaagggacatggctgaataggta-3’
Erlin-2	5’-caccatggctcagttgggagcagttgtggc-3’, 5’-tcaattctccttagtggccgtctcc-3’
PHB	5’-caccatggctgccaaagtgtttgagtcca-3’, 5’-tcactggggcagctggaggagcacg-3’
ATP2A2	5’-caccatggagaacgcgcacaccaagacg-3’, 5’-tcaagaccagaacatatcgctaaag-3’
MCM7	5’-caccatggcactgaaggactacgcgctag-3’, 5’-tcagacaaaagtgatccgtgtccgg-3’
PCBP1	5’-caccatggatgccggtgtgactgaaagtg-3’, 5’-ctagctgcaccccatgcccttctc-3’
PCBP2	5’-caccatggacaccggtgtgattgaaggtg-3’, 5’-ctagctgctccccatgccaccc-3’
MRCL3	5’-caccatgtcgagcaaaagaacaaagac-3’, 5’-tcagtcatctttgtctttggctccatgtttc-3’
ATP5A	5’-caccatgctgtccgtgcgcgttgc-3’, 5’-ttaagcttcaaatccagccaagaaatttgttac-3’
ELAV	5’-caccatgtctaatggttatgaagaccac-3’, 5’-ttatttgtgggacttgttggttttgaagga-3’
eIF4A	5’-caccatgtctgcgagccaggattcccg-3’, 5’-tcagatgaggtcagcaacattgaggg-3’
P5CS	5’-caccatgttgagtcaagtttaccgctgtg-3’, 5’-tcagttggtgtttctctgaggaatagg-3’
Tu	5’-caccatgaccacaatggcggccg-3’, 5’-tcaaccccatttgatattcttctcctcctc-3’
YBX	5’-caccatgagcagcgaggccgagacccagc-3’, 5’-ttactcagccccgccctgctcagcc-3’
Ran	5’-caccatggctgcgcagggagagc-3’, 5’-tcacaggtcatcatcctcatccgg-3’
ILF3	5’-caccatgcgtccaatgcgaatttttgtg-3’, 5’-ttatctgtactggtagttcatgctgtggtc-3’
MCM4	5’-caccatgtcgtccccggcgtcgac-3’, 5’-tcagagcaagcgcacggtcttcc-3’

1-4 Results

1-4-1 Expression of recombinant antibody with FLAG

To understand how antibody assembly and folding were regulated, we analyzed antibody-associated proteins by IP–MS. In this study, we used the humanized TRA-8 anti-DR5 antibody (IgG1) as a model. TRA-8 heavy chain with C-terminal FLAG tag and antibody complex with C-terminal FLAG tag were expressed in 293F cells and then each cell lysate was purified with anti-FLAG agarose. Purified H chain and antibody were subjected to non-reducing SDS–PAGE, silver staining, and immunoblotting using anti-gamma and anti-kappa antibodies to confirm assembly and conformation. As shown in Fig. 1-1, cells expressing H chain-FLAG produced dimeric and monomeric H chain that were mainly observed at approximately 100 and 50 kDa, respectively. Cells expressing antibody-FLAG contained mostly H2L2 at 150 kDa. Additionally, H monomer, HL heterodimer, H dimer, and H2L were also observed at about 50, 75, 100, and 125 kDa, respectively. This result shows that H chain-FLAG tag were assembled and folded similarly to whole antibody molecule (H2L2).

1-4-2 Identification of endogenous proteins interacting with antibody fragments or antibody

All purified proteins were subjected to LC–MS/MS analysis to identify endogenous interacting proteins. With LC–MS/MS and the analysis by the DAVID Bioinformatics Database, 284 proteins with peptide FDR <1 % were identified, and 205 of them interacted with the heavy chain and/or antibody with FLAG. Using gene ontology term enrichment, some gene terms involved in protein expression pathways

(translation, cellular macromolecular complex subunit organization, cellular protein complex assembly, protein folding, and cellular macromolecular complex assembly) were significantly enriched at the top of this gene ontology annotation list (Table 1-2). Data *p* values were for less than 0.001 that ensure a high confidence in the IP–MS results.

1-4-3 Effect of IP-MS identified genes on antibody production

To ensure the identified endogenous interaction partners were indeed involved in the antibody expression process, we performed co-expression of antibody with these identified proteins. First, we selected and cloned 28 human genes shown in Table 1-3. Proteins which were involved in protein expression process such as protein folding and assembly were mainly selected because overexpressed recombinant antibody accumulated in intracellular fraction should be in immature form. We also selected GRP78/Bip, HSP90β, and eIF4A because their number of identified peptides in H chain or antibody data set was much higher than with GFP even though they were detected in all samples. In particular, GRP78/Bip and GRP94 are reported to be ER chaperones that interact with immunoglobulin chains (Knarr et al. 1995; Melnick et al. 1994).

We have carried out a transient expression assay for evaluating the effect of identified proteins on antibody expression in COS-1 cells. Cells have been transformed with the large T antigen of SV40, allowing episomal replication of their plasmids containing the SV40 origin. Therefore, the high yield of transgene expression has been achieved in transient experiments. In a similar way, COS-1 cells were the most suitable for transient overexpression in our preliminary experiment among cell lines checked. COS-1 cells were then co-transfected with antibody expression vector and expression

vectors for interacting proteins. Antibody expression level was measured as an indicator to confirm the efficacy of interacting proteins on the antibody expression processes. As the result of co-expression with our identified proteins, antibody expression was mostly increased, and in particular, 19 proteins including GRP78/Bip, GRP94, and ATP2A2 enhanced antibody expression about twofold (Fig. 1-2). However, HSP47 and CALR had no effect on antibody expression even though they are known as ER chaperones. Overexpression of each identified protein was confirmed by quantitative real-time PCR of cell lysates from transfected COS-1 cells (data not shown). Next, in order to further increase antibody expression, we examined the effect of combinations of identified proteins. GRP78/Bip, PHB, ATP2A2, and eIF4A (which had the evident effect on antibody expression in the co-transfection experiment) were selected as representative genes from each category (ER chaperone, translation, and others). In addition to either one of these proteins, identified protein was co-expressed to detect additive effects of proteins. Overall, either one of these proteins except for PHB showed additive effect on antibody expression with the other protein (Fig. 1-3). Among them, the combination of eIF4A with the other identified protein gave the highest enhancement at around four-folds.

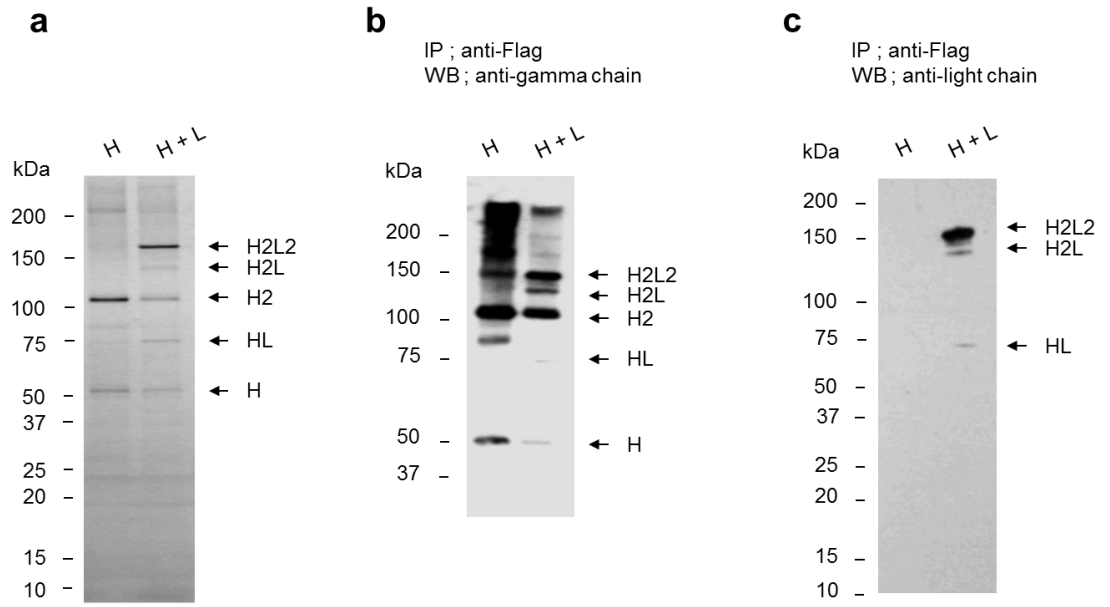

Fig. 1-1 Purification of heavy chain and antibody with FLAG. 293F cells were transfected with each expression vector (H chain-FLAG, or H-FLAG plus L chain). Each fusion protein was purified by anti-FLAG affinity gel, and was resolved by SDS-PAGE without DTT followed by **a** silver staining, **b** immunoblotting analyses with anti-gamma chain antibody and **c** anti-kappa chain antibody.

Table 1-2 *p* values for gene ontology category by a Bonferroni-correlated modified Fisher's exact test.

Gene Term	Term	Count	%	P-value	Fold Enrichment	Bonferroni	Benjamini	FDR
translation	GO:0006412~translation	22	11.80	1.17E-09	5.23	1.42E-06	1.42E-06	1.90E-06
cellular macromolecular complex subunit organization	GO:0034621~cellular macromolecular complex subunit organization	20	10.80	1.27E-07	4.41	1.54E-04	7.68E-05	2.06E-04
cellular protein complex assembly	GO:0043623~cellular protein complex assembly	14	7.53	1.35E-07	6.80	1.63E-04	5.45E-05	2.19E-04
protein folding	GO:0006457~protein folding	14	7.53	3.79E-07	6.22	4.59E-04	1.15E-04	6.14E-04
cellular macromolecular complex assembly	GO:0034622~cellular macromolecular complex assembly	18	9.68	5.71E-07	4.45	6.91E-04	1.38E-04	9.24E-04
RNA stabilization	GO:0043489~RNA stabilization	6	3.23	8.25E-07	31.46	9.98E-04	1.66E-04	1.34E-03
mRNA stabilization	GO:0048255~mRNA stabilization	6	3.23	8.25E-07	31.46	9.98E-04	1.66E-04	1.34E-03
protein polymerization	GO:0051258~protein polymerization	8	4.30	2.50E-06	12.84	3.03E-03	4.33E-04	4.05E-03
RNA splicing, via transesterification reactions	GO:0000375~RNA splicing, via transesterification reactions	12	6.45	3.78E-06	6.17	4.56E-03	5.71E-04	6.11E-03
nuclear mRNA splicing, via spliceosome	GO:0000398~nuclear mRNA splicing, via spliceosome	12	6.45	3.78E-06	6.17	4.56E-03	5.71E-04	6.11E-03
RNA splicing, via transesterification reactions with bulged adenosine as nucleophile	GO:0000377~RNA splicing, via transesterification reactions with bulged adenosine as nucleophile	12	6.45	3.78E-06	6.17	4.56E-03	5.71E-04	6.11E-03

Table 1-3 Antibody interacting proteins identified by IP-MS. These endogenous proteins were identified as interacting with the heavy chain and/or antibody (heavy plus light chains) significantly (a false discovery rate less than 1%, and/or antibody with FLAG >2 in GFP with FLAG).

IPI ID	Gene	Description	Mascot score		
			GFP	H	H + L
IPI00003362	GRP78/Bip	glucose-regulated protein 78kDa	324	1364	1607
IPI00010796	PDI (P4HB)	protein disulfide isomerase		62	187
IPI00027230	GRP94	glucose regulated protein 94kDa		230	867
IPI00032140	HSP47	heat shock protein 47kDa		237	417
IPI00009904	PDIA4	protein disulfide isomerase family A member 4		273	502
IPI00183968	TPM3	tropomyosin 3		37	481
IPI00290142	CTPS	CTP synthase		319	68
IPI00005747	CCT1	t-complex 1		303	216
IPI00010720	CCT5	t-complex 1 subunit epsilon		117	98
IPI00411633	HSP90 beta	heat shock 90kD protein 1, beta	60	250	166
IPI00020599	CALR	calreticulin		278	351
IPI00218371	PRPS	phosphoribosyl pyrophosphate synthetase 1		60	209
IPI00026942	ERLIN-2	ER lipid raft associated 2		19	144
IPI00005222	PHB	prohibitin		229	189
IPI00177817	ATP2A2	SR Ca(2+)-ATPase 2		140	35
IPI00219740	MCM7	minichromosome maintenance complex component 7		185	114
IPI00016610	PCBP1	poly(rC) binding protein 1		163	217
IPI00012066	PCBP2	poly(rC) binding protein 2		169	213
IPI00220573	MRCL3	myosin regulatory light chain 3		24	200
IPI00440493	ATP5A	ATP sythase (F1-ATPase) alpha subunit		272	166
IPI00301936	ELAV	(embryonic lethal, abnormal vision, Drosophila)-like 1		138	79
IPI00025491	eIF4A	eukaryotic translation	31	322	179

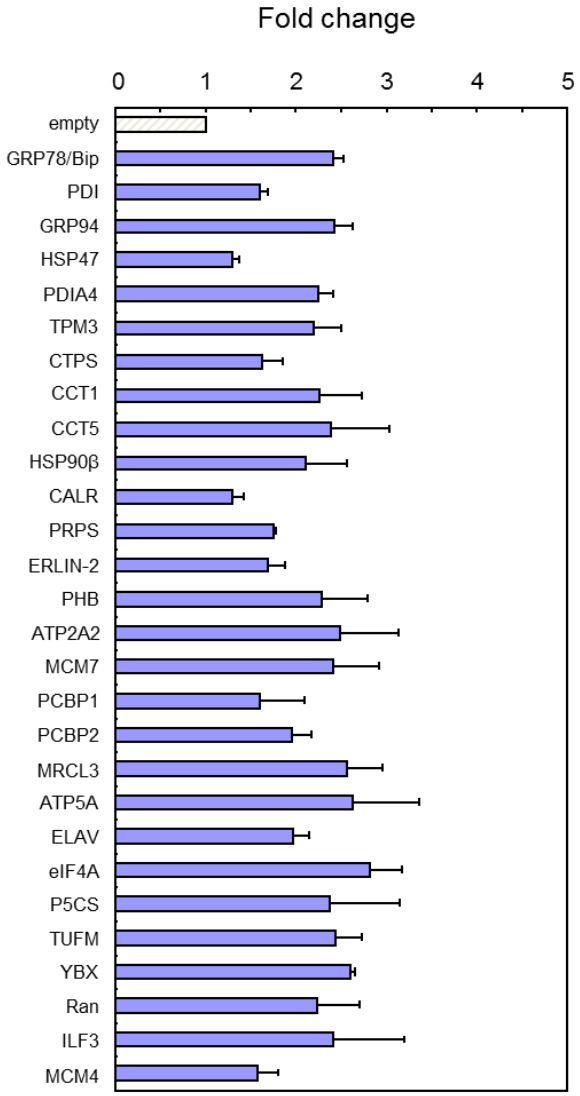

Fig. 1-2 Overexpression of each identified protein increased antibody expression**.** COS-1 cells were co-transfected with antibody expression vector and endogenous protein expression vectors shown in Table 1-1. Control cells were co-transfected with antibody expression vector and empty vector. The concentration of recombinant antibody was measured by ELISA. IgG expression level was normalized to control which was co-transfected with antibody expression vector and the empty vector and was calculated as fold change. *Bars* represent mean and SD from three independent experiments (one-way repeated measures ANOVA); $p<0.05$. Statistical comparisons were made among all groups using raw data.

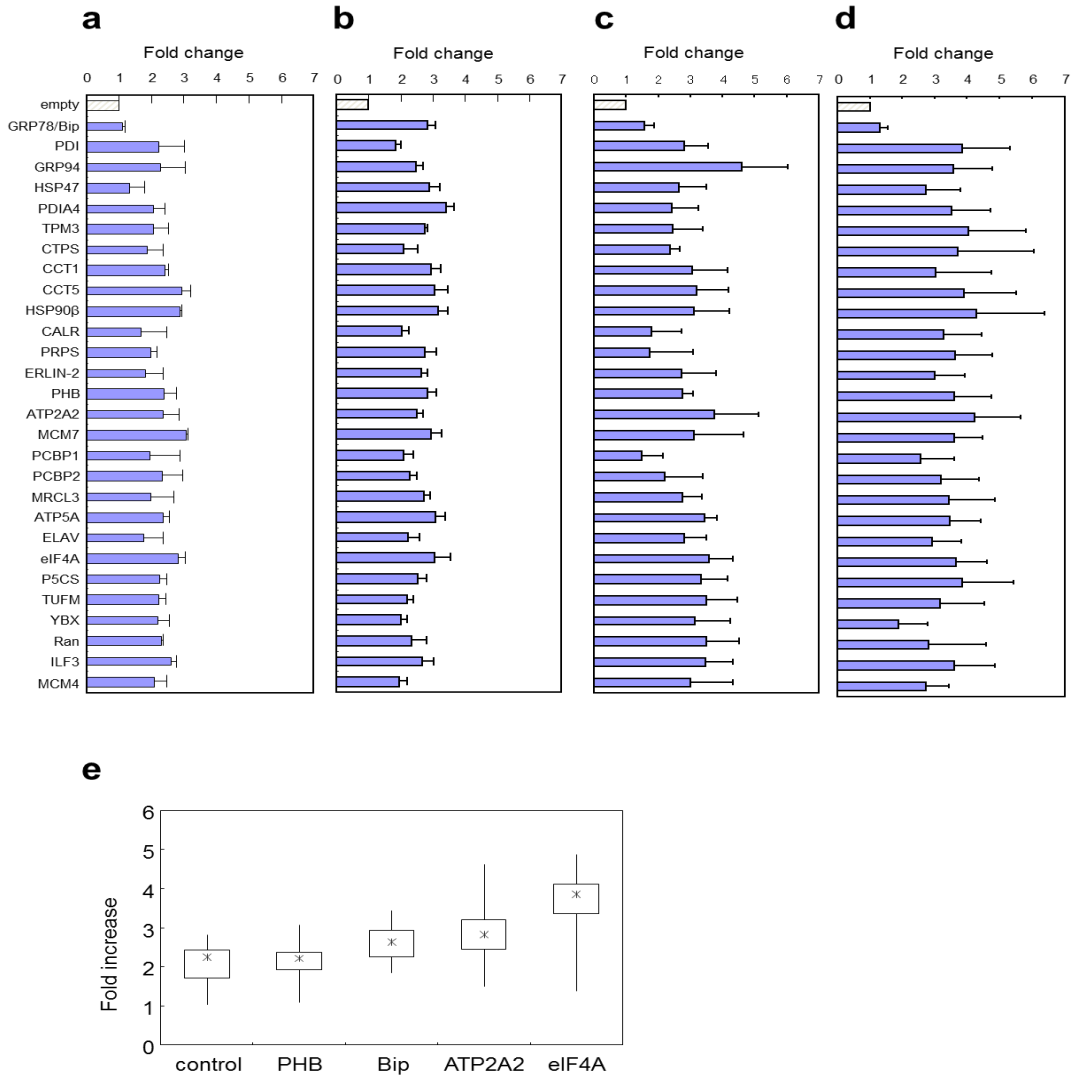

Fig. 1-3 The combination of identified proteins enhanced antibody expression. COS-1 cells were transfected with antibody expression vector, and vectors expressing either one of the identified proteins shown in Table 1-3, as well as expression vectors for **a** PHB, **b** GRP78/Bip, **c** ATP2A2, and **d** eIF4A. The concentration of recombinant antibody was measured by ELISA. Each IgG expression level for normalization was calculated as fold change. *Bars* represent mean-value and SD from three independent experiments. Statistical comparisons were carried out among all groups using one-way repeated measures ANOVA; $p<0.05$. The effects of each gene (PHB, GRP78/Bip, ATP2A2, and eIF4A) in combination with identified 28 genes were analyzed by box-and whisker plot (**e)**. The *lines of the box* in this figure are at the lower quartile and the upper quartile values, and *the asterisk in the box* represents the median value. The *whisker lines* extend from each end of the box to the most extreme data value.

1-5 Discussion

Few endogenous proteins that could affect recombinant antibody expression in mammalian cells have been so far elucidated. In this study, by IP–MS based on gene ontology terms, we could identify endogenous proteins interacting with antibody fragments or whole antibodies in mammalian cells. The list includes both non-ER-resident proteins and some ER chaperones (GRP78/Bip and GRP94) that were shown to interact with antibody (Dorner et al. 1987; Feige et al. 2009; Hendershot et al. 1987; Knarr et al. 1995; Melnick et al. 1994). Interestingly, not only ER chaperones but also non-ER-resident proteins improved antibody expression. Most of the proteins that we identified were previously unknown to have any effect on antibody expression.

First, our identified proteins included many ER-resident proteins: GRP78, GRP94, PDI family members, HSP90AB1, ATP2A2, Erlin-2, HSP47, and CALR (Table 1-3). ER chaperones are related to protein folding or assembly, and therefore, most of them enhanced antibody production (Fig. 1-2). Known ER chaperones such as GRP78 and PDI enhanced antibody productivity, though GRP78 did not have a positive effect in CHO cells (Borth et al. 2005; Dorner et al. 1992; Shusta et al. 1998; Khan and Schröder 2008). We suppose that different chaperones or combination of chaperones enhance the production of recombinant protein depending on the nature of cell line. Among identified proteins, HSP90AB1 is also a known ER chaperone (Chen et al. 2005; Wandinger et al. 2008), and HSP90 proteins normally associate with other co-chaperones and play important roles in folding of newly synthesized proteins or stabilizing and refolding of denatured proteins after stress. Likewise, ATP2A2, also known as the sarco/endoplasmic reticulum calcium-ATPase, is a calcium pump in the

ER (Caspersen et al. 2000; Manjarrés et al. 2010). In mammalian cells, the ER is a key player in the stress signaling pathway, regulating Ca^{2+}-dependent chaperones that are essential for protein folding (Meldolesi and Pozzan 1998). Therefore, ATP2A2 might help antibody expression indirectly unlike the above-described ER chaperones. Erlin-2 (also known as SPFH2) falls in the family of prohibitin domain-containing (PHB) proteins and, unlike other PHB family members, that is localized to the ER (Browman et al. 2006). Erlin-2 is a member of the ER membrane complex that mediates ERAD (Pearce et al. 2009). As degradation of unfolded proteins is mediated by ERAD (Meusser et al. 2005; Tsai et al. 2002), Erlin-2 might be also important for the enhancement of antibody secretion with the correct conformation. On the other hand, HSP47 and CALR which are described as ER chaperones did not have any enhancing effect on antibody expression in our study. However, it is reported that HSP47 is a molecular chaperone in collagen maturation (Hendershot and Bulleid 2000) and CALR controls protein quality by interacting with newly synthesized glycoprotein (Michalak et al. 2009). Thus, we cannot exclude the possibility that HSP47 or CALR may have selectivity and stabilizing effect for heterologous proteins developing on host cell lines.

Apart from ER-resident proteins, we also found that some non-ER-resident proteins could enhance antibody expression. However, their functional involvement in increasing antibody expression has not been elucidated, and we consider that most of them might affect antibody expression indirectly. For instance, CCT1 and 5 are cytosolic proteins and belong to the cytosolic chaperonins containing t-complex polypeptide (CCT) family that can fold actin, tubulin, and several other proteins (Plath and Rapoport 2000; Yokota et al. 2001). Some of our identified non-ER-resident proteins might have unknown chaperone activities or be involved in transport pathways

of protein. In combinatorial expression experiments, we observed further enhancement of antibody expression (Fig. 1-3). These results suggested that each protein might cooperate in different pathways in antibody expression. Compared to GRP78/Bip, PHB, or ATP2A2, eIF4A had the higher combinatorial effect on antibody expression in combinations with ER chaperones. eIF4A catalyzes the recruitment of mRNA to the ribosome (Linder et al. 1989; Pestova et al. 2001). Thus, acceleration of translation in combination of improvement of protein folding and assembly led to the higher antibody expression. Overall, we propose that it is important to enhance translation, protein folding and assembly simultaneously to get maximum expression of recombinant protein.

Unlike other comprehensive methods such as microarray, IP–MS can characterize and identify protein–protein interaction networks. Here, we analyzed the human cell line as a model case to identify proteins that specifically interact with an antibody, because mass spectrometry analysis needs the protein database. Our method might be also applied to other cell lines including CHO cell line as the genome of CHO cells was recently exhibited in the future (Xu et al. 2011).

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Chapter 2

Overexpression of C/EBP homologous protein (CHOP) alone and in combination with chaperones is effective in improving antibody production in mammalian cells

2-1 Summary

Secretory capacities including folding and assembly are believed to be limiting factors in the establishment of mammalian cell lines producing high levels of recombinant therapeutic proteins. To achieve industrial success, it is thus important to improve protein folding, assembly, and secretory processes in combination with increasing transcription and translation. Here, we identified the expression of CHOP/Gadd153 and GRP78, which are unfolded protein response (UPR)-related genes, correlated with recombinant antibody production in stable CHO cells. Subsequently, CHOP overexpression resulted in increasing recombinant antibody production in some mammalian cell lines, and a threefold enhancement was obtained by combining expression with UPR-related genes or ER chaperones such as GRP94 and ATP2A2 in transient assays. Overexpression of CHOP had no effect on the biochemical characteristics of the product. These results suggest overexpression of CHOP and its combinations may be an effective to develop cell lines with a high level of antibody production in the manufacturing.

2-2 Introduction

Although mammalian cells are the promising hosts for recombinant antibodies, significant effort and time are required to achieve yields of more than 1 g/L (Huang et al. 2010; Li et al. 2010). In order to develop a cell bank for manufacture, it is necessary to isolate a single clone that shows a higher production from a transfected cell pool (Birch and Racher 2006). After acquiring a high-producing cell line, cultivation condition such as culture media and temperature must be optimized (Al-Fageeh et al. 2006; Luo et al. 2012).

To obtain higher productivities more effectively and reduce the effort required to select a good cell line, conventional methods such as a selection of (spontaneous) mutation could be improved by genetic engineering. Previous reports have shown that vector modifications employing strong promoters, enhancers, or cis-acting elements resulted in increased protein production (Benton et al. 2002; Girod et al. 2007; Li et al. 2007). Vector modifications could have a positive effect on increasing antibody production, but transcription is probably not the sole limiting factor. For instance, there was no correlation between the secretory ability of the cell and mRNA levels in GS-NS0 murine myeloma cells (Birch and Racher 2006; Smales et al. 2004). This example suggests that the bottleneck in the productivity is likely to be in post-translational steps such as folding, assembly, and the unfolded protein response (UPR) (Harraghy et al. 2008; Kim et al. 2012).

Eukaryotic cells have several quality control systems against stress caused by environmental alteration in the ER (Jenkins et al. 2008; Kleizen and Braakman 2004; Schröder and Kaufman 2005). For instance, GRP78/Bip functions as a chaperone and UPR sensor. The UPR introduces signal transduction through UPR-genes such as PERK,

ATF6 and XBP-1. It is particularly relevant to understand how cells such as CHO cells perceive the environmental situations that trigger apoptosis or UPR. C/EBP homologous protein (CHOP) is one of UPR-related genes, and is also known as growth arrest and DNA damage-inducible gene 153. CHOP is reported to indicate ER stress-mediated apoptosis in a wide of variety of cells (Oyadomari and Mori 2004; Wang et al. 1996). The introduction of UPR-related genes or chaperones has a positive effect on heterologous protein productions (Barnes and Dickson 2006; Dinnis and James 2005; Khan and Schröder 2008). For instance, in yeast cells, Hac1 which is an XBP-1 homolog was shown to have beneficial effects on recombinant proteins production by enlarging the capacity of protein production in the ER (Valkonen et al. 2003). Moreover, ATF4, which is downstream from eIF2α phosphorylation, has been shown to enhance production of recombinant human antithrombin III in CHO cells (Ohya et al. 2008). However, knowledge concerning the available genes is limited though some UPR genes or chaperones have been reported (Dorner et al. 1990; Hayes et al. 2010; Kim et al. 2012).

In this work, we developed a CHO cell line which stably produced an antibody at a high level, and we then investigated UPR-related genes which were involved in antibody production by comparing recombinant CHO cells with wild-type CHO cells. As a result, we identified the expression of CHOP and GRP78 correlated with antibody expression in recombinant CHO cells. Moreover, the introduction of CHOP combined with UPR genes or chaperones strongly enhanced antibody production in mammalian cells, indicating that the combination expression of these would facilitate the efficient selection of a cell line with a high level of antibody production.

2-3 Materials and Methods

2-3-1 Plasmids

An expression vector for a humanized antibody (hTRA-8) was constructed by inserting both its heavy and light chains into expression vector pcDNA3.1 (+) (Invitrogen).

Full-length cDNAs of GRP78, CHOP and PDI were cloned from CHO-K1 cells, and GRP78, CHOP, ERO1Lbeta, ERdj3, and DRdj5 were cloned from 293F cells. In brief, total RNA was isolated from each cell line using the RNeasy Mini Kit (Qiagen) and then reverse-transcribed with oligo-dT using ReverTra Ace qPCR RT Kit (TOYOBO). DNA fragments were amplified from resulting cDNAs using KOD-plus- (TOYOBO) with the following primers for each genes: GRP78 (CHO), 5’-accgaattcatgaagttccctatggtggc-3’ and 5’-accctcgagctacaactcatcttttc-3’; hGRP78, 5’-accgaattcatgaagctctccctggtggc-3’ and 5’-accctcgagctacaactcatcttttctg-3’; CHOP (CHO), 5’-accgaattcatggcagctgagtccctgcc-3’ and 5’-accctcgagtcatacttgttgcagattta-3’; hCHOP, 5’-accgaattcatggcagctgagtcattgcc-3’ and 5’-accctcgagtcatgcttggtgcagatt-3’; PDI (CHO), 5’-accgaattcatgctgagccgttctctgctg-3’ and 5’-accctcgagctacaattcgtcctttaca-3’; hERO1Lbeta, 5’-accgaattcatgagccaaggggtccg-3’ and 5’-accctcgagttacctactgtgttgtaataagac-3’; hERdj3, 5’-accgaattcatggctccgcagaacctg-3’ and 5’-accctcgagtcaatatccttgcagtccattg-3’; hDRdj5, 5’-accgaattcatgggagtctggttaaataa-3’ and 5’-accctcgagtcaaagttcatccttattc-3’. Expression vectors for these genes were constructed by cloning into pcDNA3.1 (+).

The expression vectors for GRP78/Bip, PDI, GRP94, HSP47, PDIA4, TPM3, CTPS, CCT1, CCT5, HSP90β, CALR, PRPS, Erlin-2, PHB, ATP2A2, MCM7, PCBP1,

PCBP2, MRCL3, ATP5A, ELAV, eIF4A, P5CS, Tu, YBX, Ran, ILF3, and MCM4 were constructed using the pcDNA™ 3.1 Directional TOPO® Expression kit (Invitrogen) (Nishimiya et al. 2012).

2-3-2 Cell culture, expression, and purification of recombinant antibody

CHO-K1 and COS-1 cell lines were obtained from the American Type Culture Collection. CHO-K1 cells were cultured in suspension in CD-CHO medium (Invitrogen) at 37°C with 5% CO_2; 1×10^6 cells/ml of suspended CHO-K1 cells were then transfected with a recombinant antibody (hTRA-8) expression vector using Lipofectamine 2000 (Invitrogen). Following selection in G418 for about two weeks, several stable cell lines were established. CHO cell line #1 was subsequently selected from these, since it showed the highest expression level of the recombinant antibody (hTRA-8) in fed-batch culture for over one week. For evaluation of cells in fed-batch culture, the suspended CHO-K1 cells (wild type) and CHO-K1 cell line #1 stably producing hTRA-8 antibody were seeded into 125-ml erlenmeyer flasks, and cells were cultured and fed on designated days with the medium.

COS-1 cells were maintained in α-MEM (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) at 37°C with 5% CO_2; 1×10^5 COS-1 cells were seeded into a 24-well plate and incubated for 24 h prior to transfection. Cells were transfected with the recombinant antibody (hTRA-8) expression vector and each UPR-related gene expression vector using Lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection, the supernatants were collected and analyzed by ELISA.

FreeStyle™ CHO-S cells were purchased from Invitrogen, and were maintained in GIBCO® FreeStyle™ CHO medium at 37°C with 5% CO_2; 1×10^6 cells/ml of CHO-S

cells were seeded into 125-ml erlenmeyer flasks, and then co-transfected with 18 μg of the hTRA-8 expression vector and 18 μg of each expression vector. After 5 days of cultivation, the supernatants were collected and analyzed. Recombinant antibody was affinity purified from CHO-S cell culture supernatant using a Protein A column by AKTA FPLC chromatography (GE Healthcare).

2-3-3 Quantitative real-time PCR analysis

Total RNA was extracted from 1×10^7 cells using the RNeasy Kit (Qiagen) according to the manufacturer's instructions. Total RNA (600 ng) was reverse transcribed into cDNA using ReverTra Ace qPCR RT Kit (TOYOBO). Expressions of GRP78 (CHO) and CHOP (CHO) were quantified by real-time qPCR with SYBR Premix Ex Taq (TAKARA) using the specific primers as follows: GRP78 (CHO), 5'-ggatggttaatgatgcagagaagtttgc-3' and 5'-ggtcttcaatgtctgcatcctggtgg-3'; CHOP (CHO), 5'-cactaccctagaccctgcatccctagc-3' and 5'-gagtggcctctacctctctggtcagg-3'; Gene expression levels were normalized to that of β-actin by using primers 5'-gtgcccatctatgagggctac-3' and 5'-gtcacgcacaatttccctctc-3'.

2-3-4 ELISA detection of recombinant antibody

Ninety-six well microplates (Nalgene Nunc) were coated with anti-kappa chain antibody (BETYL) and incubated overnight at 4°C. After washing with PBST (PBS containing 0.05 % (*v/v*) Tween-20), wells were then blocked in Blocker Casein in PBS (PIERCE) for overnight at 4°C. After washing with PBST, cell supernatants were added and incubated for 1 h at 37°C. Samples were removed and the wells were washed.

Peroxidase-labeled affinity-purified antibody to human IgG (Fc) (KPL) was then

added in washing buffer and incubated for 1 h at 37°C. After washing with PBST, substrate solution (Nacalai Tesque) was added and the reaction was allowed to proceed at room temperature in the dark. The reaction was stopped by the addition of stop solution and the absorbance at 405 nm was measured using SpectraMax (Molecular Devices). For IgG expression assays, multiple pairwise comparisons across groups were performed by one-way repeated measurement ANOVA.

For the binding analysis, competitive ELISA was performed. Ninety-six well microplates were coated with DR5-Fc and incubated overnight at 4°C. After washing with PBST, biotinylated hTRA-8 and purified hTRA-8 which had been expressed in CHO-S were added and incubated for 2 h at 37°C. After washing with PBST, streptavidin-HRP was added and incubated for 1 h at 37°C. After washing with PBST, substrate solution (Sumilon) was added and the reaction was allowed to proceed at room temperature. The reaction was stopped by the addition of stop solution and the absorbance at 490 nm was measured using Mithras LB940 (Berthold Technologies).

2-3-5 Detection of cytotoxic activity

Highly purified hTRA-8, which comprised a monomeric fractions of >99% purity, was used as a positive control. Human TRA-8 that had been expressed in CHO-S and purified, were diluted with the goat anti-human IgG antibody solution and added into each well of a 96-well plate. Then, 1×10^3 Jurkat cells were plated per well and incubated at 37°C with 5% CO_2 for 72 h. The cell viabilities of each well were determined by detection of ATP using CellTiter-Glo™ Luminescent Cell Viability Assay (Promega) according to the manufacturer's instruction.

2-4 Results

2-4-1 Identification of CHOP in stable CHO cells producing high levels of recombinant antibody

To examine whether unfolded protein response related genes correlates to antibody expression in mammalian cells, CHO-K1 cells which stably expressed antibody were analyzed. CHO-K1 cells were transfected with hTRA-8 expression vector, and then a CHO-K1 cell line #1 which was a high producer of the humanized death receptor 5 antibody (hTRA-8) was established. Antibody-expressing cells and wild-type CHO-K1 cells were cultivated in fed-batch culture. As shown in Fig. 2-1a, both cell lines exhibited similar growth curves, and the antibody production in CHO-K1 cell line #1 was greater than 300 mg/L for 5 days. Through the cultivation, the mRNA expression levels of XBP-1, PDI, GRP78, and CHOP, which were well-known ER chaperones or UPR-related proteins, were compared (Fig. 2-1b). GRP78 was found to be highly expressed in the CHO cell line #1, and CHOP was found to be expressed in #1 at a higher level comparing to wild-type CHO-K1 at the late stage of cell culture, whereas there were no differences in mRNA levels of XBP-1 and PDI between wild-type CHO-K1 cells and #1 (data not shown). This result indicates that the expressions of both GRP78 and CHOP were positively correlated with antibody production in CHO cells.

2-4-2 Effects of ER chaperones and UPR-related proteins on antibody expression

Next, we addressed how GRP78 or CHOP was involved in recombinant antibody production. GRP78 and CHOP cDNAs were cloned. PDI and ERO1Lβ which are

essential for protein disulfide formation were also cloned from CHO-K1 or 293F cells. A transient expression assay was performed using COS-1 cells to assess their effects on antibody production conveniently. COS-1 cells were co-transfected with the antibody expression vector and either one of expression vector for the cloned genes. In each expression experiment, antibody expression levels were measured to confirm the influence on antibody expression processes. The amount of GRP78 and CHOP mRNA were increased in each transfectant compared to the endogenous expression level (data not shown). No differences were observed in the effect of orthologous GRP78 or CHOP between human and CHO cells due to the high conservation of amino acid homology (>88%) between humans and hamsters (Fig. 2-2a). Overexpression of CHOP accelerated antibody expression equal to or higher than that of GRP78, PDI, or ERO1Lβ (Fig. 2-2b).

2-4-3 Combination of CHOP with UPR genes or ER chaperones on antibody expression

Although several UPR genes such as XBP-1 and ATF4 have been reported to enhance the production of therapeutic proteins, a master regulator which increases the production of transgenes has not yet been identified (Dinnis and James 2005; Khan and Schröder 2008). In an attempt to further increase antibody production, combinations of UPR-related proteins were assessed. It has been reported that ERdj3 serves as a cofactor for the interaction of GRP78's interactions with unfolded proteins, and GRP78-associated ERdj5 acts on misfolded proteins as a disulfide reductase in the ER (Jin et al. 2008; Shen and Hendershot 2005; Ushida et al. 2008). We therefore hypothesized that overexpression of ERdj3 or ERdj5 would further enhance antibody

expression through its association with GRP78. Unfortunately, the combined overexpression of these proteins with GRP78 did not improve antibody production (Fig. 2-3a) On the other hand, CHOP enhanced antibody expression more markedly in combination with GRP78, ERdj3, or ERdj5. Next, to investigate whether CHOP could further improve antibody production when combined with ER chaperones, the combinations of GRP78, PDI and ERO1Lβ with CHOP were examined. As expected, the combination of CHOP with ER chaperones improved antibody production (Fig. 2-3b).

Similarly, combinations with antibody interacting proteins such as GRP78/Bip, PDI, GRP94, HSP47, PDIA4, TPM3, CTPS, CCT1, CCT5, HSP90β, CALR, PRPS, Erlin-2, PHB, ATP2A2, MCM7, PCBP1, PCBP2, MRCL3, ATP5A, ELAV, eIF4A, P5CS, Tu, YBX, Ran, ILF3, and MCM4, which we had previously identified by IP-MS (Nishimiya et al. 2012), were examined (Fig. 2-4). These proteins were also found to further promote antibody expression when combined with CHOP expression.

2-4-4 Biochemical characterization

It has been observed in some cases that efforts to enhance productivity through genetic modification or optimization of cell culture conditions lead to alterations in the quality of product (Reid et al. 2010; Werner et al. 2007). To confirm whether the overexpression of CHOP affects product quality, the binding activity of the resultant hTRA-8 antibody to its antigen DR5 was investigated together with its cytotoxic activity. Both products (hTRA-8) from CHO-S cells or CHO-S cells overexpressing CHOP were purified by Protein A chromatography, and binding activity and cytotoxic activity were analyzed (Fig. 2-5). Between these proteins, there were no differences both in binding

activity to the antigen DR5 and in cytotoxic activity. Taken together, our results indicate that CHOP overexpression does not affect these biochemical characteristics of the product.

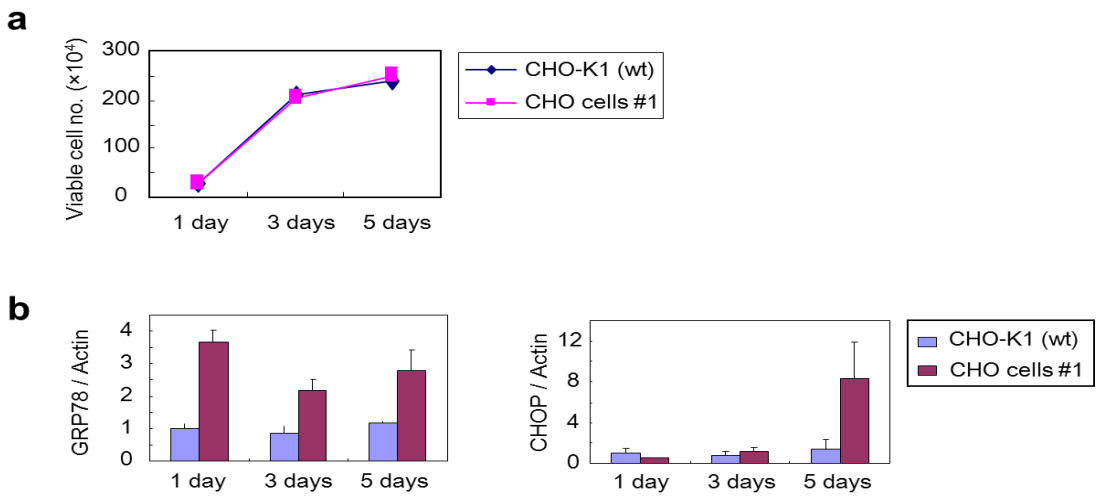

Fig. 2-1 Quantitative real-time PCR analysis of UPR genes in CHO cells (wild type) and CHO cell line #1 stably expressing antibody. CHO cells were transfected with antibody hTRA-8 expression vector, and cell line #1 stably expressed antibody was established. CHO-K1 wild type and #1 were cultivated in fed-batch culture (**a**). Subsequently, GRP78 and CHOP mRNA were quantified by real-time PCR, and normalized to β-actin (**b**). The values represent the mean ± SD (*n*=3).

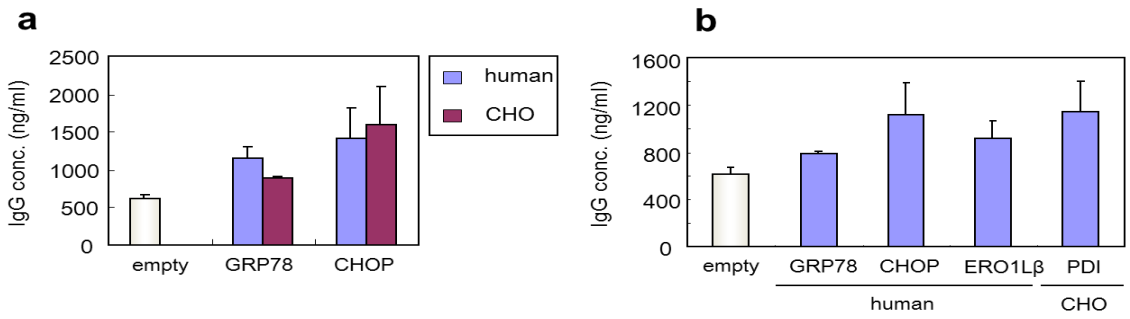

Fig. 2-2 Overexpression of CHOP and ER chaperones increases antibody expression. COS-1 cells were co-transfected with antibody expression vector and the following vectors: **a** GRP78 or CHOP from human and CHO cells, **b** GRP78, CHOP, and ERO1Lβ from human, and PDI from CHO cells. Control cells were co-transfected with antibody expression vector and empty vector. The concentration of recombinant antibody was measured by ELISA. The values represent the mean ± SD (n=3). The significant differences from the control (one-way repeated measures ANOVA) was $p<0.05$. Statistical comparisons were made among all groups using raw data.

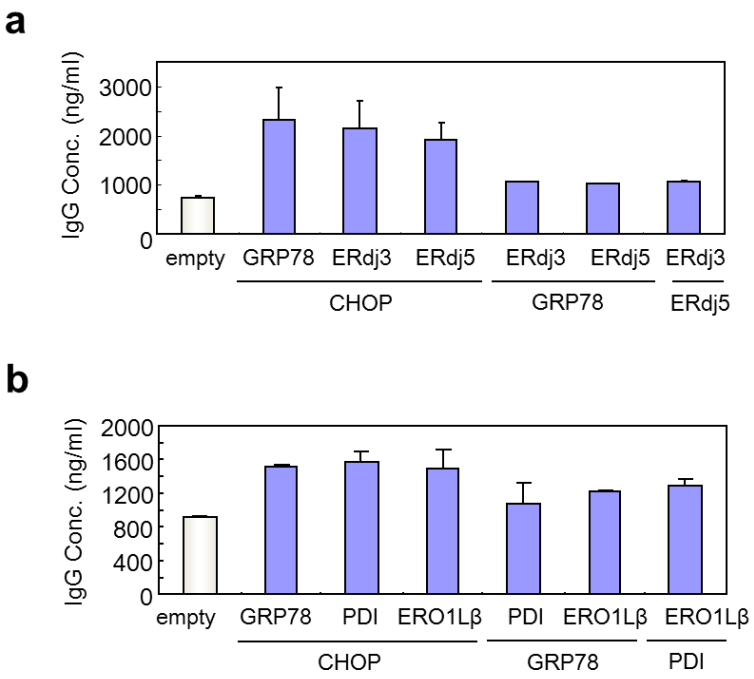

Fig. 2-3 Combination of CHOP with UPR-related genes or ER chaperones enhanced antibody expression. COS-1 cells were transfected with antibody expression vector in combination with the following vectors: **a** GRP78, CHOP, ERdj3, and ERdj5; **b** GRP78, CHOP, PDI, and ERO1Lβ. Control cells were co-transfected with antibody expression vector and empty vector. The concentration of recombinant antibody was measured by ELISA. The values represent the mean ± SD (n=3). The significant differences from the control (one-way repeated measures ANOVA) was p<0.0005. Statistical comparisons were made among all groups using raw data.

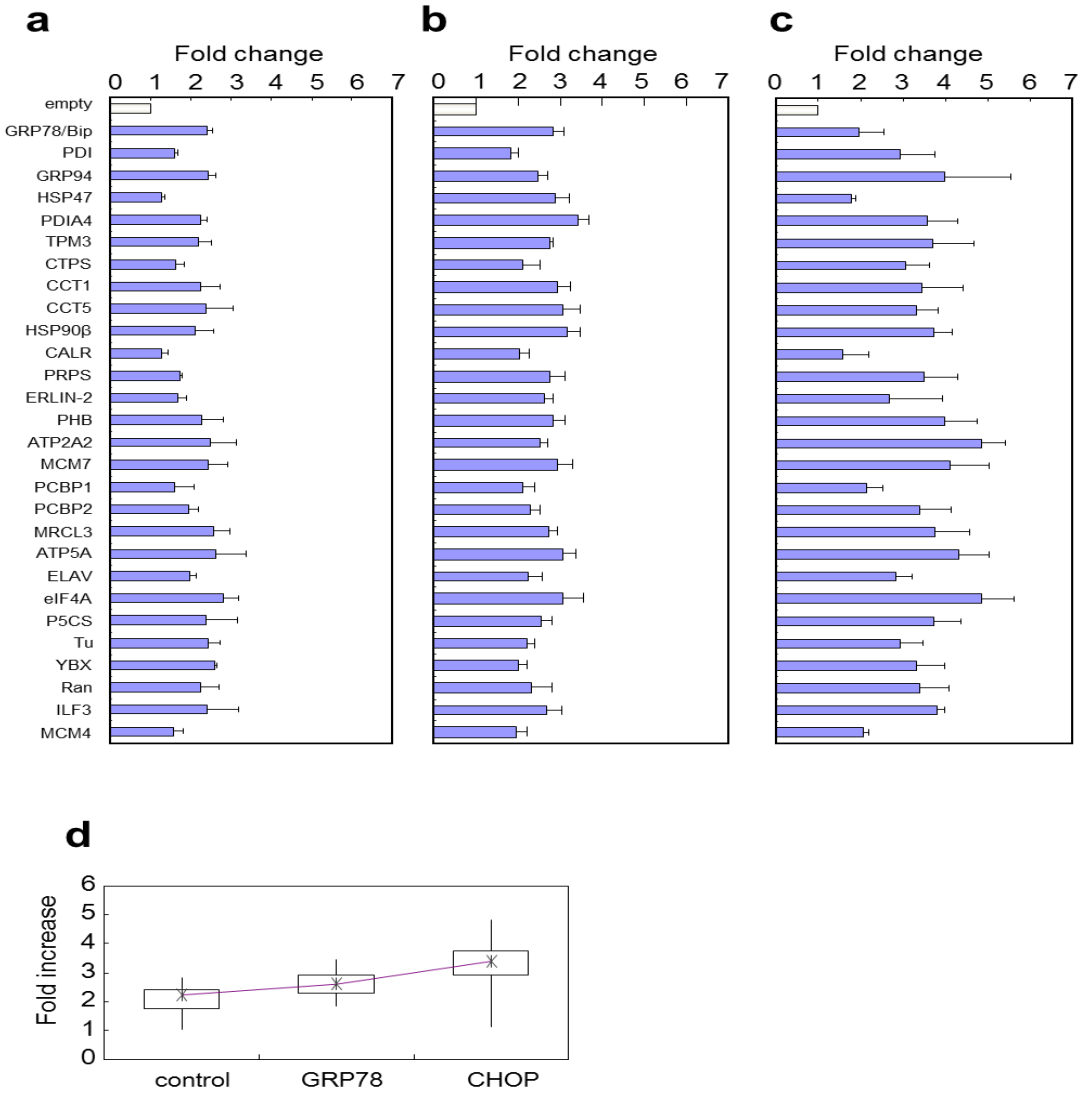

Fig. 2-4 CHOP combined with identified proteins enhanced antibody expression. COS-1 cells were transfected with antibody expression vector and vectors expressing either of identified proteins, combined with expression vectors for **a** empty vector, **b** GRP78/Bip, and **c** CHOP. The concentration of recombinant antibody was measured by ELISA. IgG expression level used for normalization was calculated as fold change. The values represent the mean ± SD (n=3). Statistical comparisons were carried out among all groups using one-way repeated measures ANOVA; p<0.0005. The effects of each gene (empty, GRP78/Bip, and CHOP) in combination with 28 identified genes were analyzed by box-and whisker plot (**d**). The *lines* of the box in this figure are at the lower quartile and the upper quartile values, and the *asterisk* in the box represents the median value. The *whisker lines* extend from each end of the box to the most extreme data value.

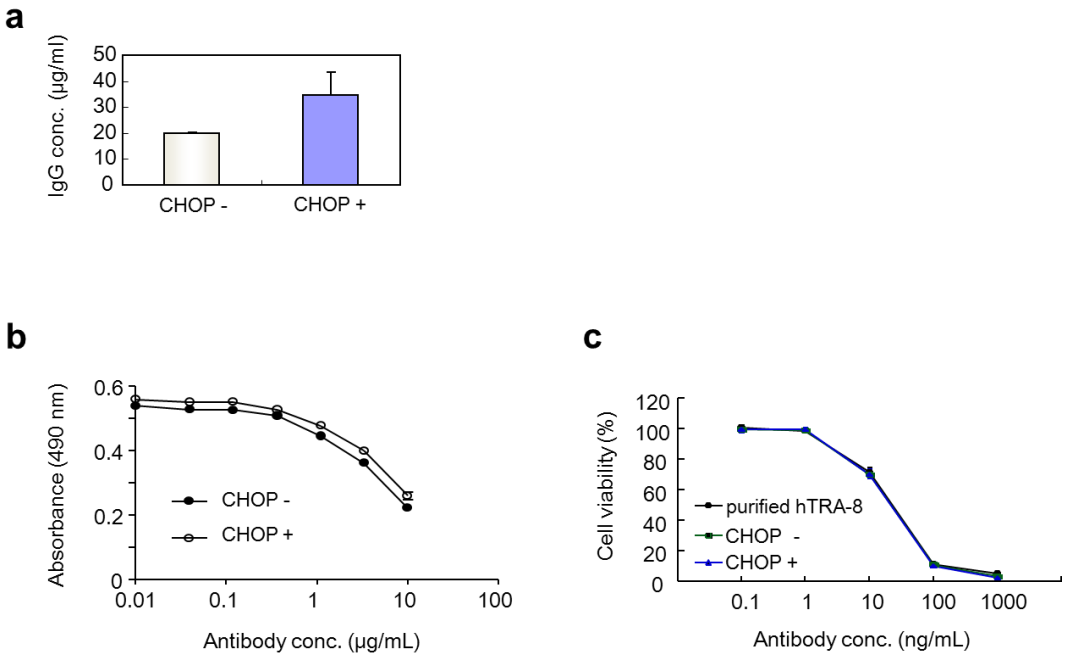

Fig. 2-5 Biochemical characterization of antibody produced in CHO-S cells expressing CHOP –/+. CHO-S cells were transiently co-transfected with hTRA-8 antibody expression vector and empty vector (CHOP –) or CHOP expression vector (CHOP +). The concentration of recombinant antibody was measured by ELISA (**a**). The values represent the mean ± SD (n=3). Statistical comparisons were carried out using two-sided un-paired t test; p<0.05. Subsequently, 5 days after transfection, the supernatants were collected and purified using Protein A chromatography. The binding activity of purified antibodies to DR5 was examined by competitive ELISA (**b**). Cytotoxic activity was detected using Jurkat cells (**c**). In addition to antibodies produced in CHO-S cells (–/+ CHOP), highly purified hTRA-8 (>99% purity) was used as positive control.

2-5 Discussion

Most recombinant proteins are likely to pass through the ER and golgi, and post-translational capacities including folding and assembly are known to be limited (Bibila and Flickinger 1992; Feige et al. 2010; Hooker et al. 1999). To achieve industrial success in the production of therapeutic proteins, it is necessary to engineer secretory pathways in combination with the vector modifications which enhance transcription or translation (Barnes and Dickson 2006; Omasa et al. 2010; Rita et al. 2010). ER chaperones or UPR-related proteins are considered to help newly synthesized peptides to fold and enhance the degradation of misfolded proteins (Borth et al. 2005; Harraghy et al. 2008; Kim et al. 2012). Recently, proteomic analysis and transcriptome analysis was carried out on recombinant cells to facilitate genetic engineering (Dinnis et al. 2006; Wlaschin et al. 2005). However, the master regulators have not been identified although these comprehensive strategies may be useful in understanding the cellular functional phenotype.

In the present study, we hypothesized that the expression of ER chaperones or UPR-related proteins would correlate with recombinant antibody expression. Our data shows that expression of CHOP as well as GRP78 was clearly enhanced in stable CHO cells producing recombinant antibody when compared with wild-type CHO cells (Fig. 2-1). GRP78 is known to be part of a multi-protein complex associated with nascent Ig heavy chains in the ER, and participates in folding and assembly (Feige et al. 2009; Knarr et al. 2002; Melnick et al. 1994). CHOP functions as a member of the UPR-related proteins to improve the environment in the ER (Oyadomari and Mori 2004; Wang et al. 1996). It is possible that this function of CHOP may contribute to

increased recombinant antibody production. On the other hand, CHOP is also known as an ER stress-mediated apoptosis indicator in a wide variety of cells (Oyadomari and Mori 2004; Wang et al. 1996). Nevertheless, CHOP overexpressing cells have been cultivated without observing cell death. It has been reported that CHOP expression is not sufficient for apoptosis in NS0 myeloma cells (Cudna and Dickson 2006). Therefore, it seems that CHOP expression alone is not sufficient to induce the apoptosis that arises from UPR activation in mammalian cells expressing therapeutic proteins. Moreover, CHOP expression is also noted to be induced by various conditions such as nutrient depletion (Murphy et al. 2001). Our results indicate that there is some relationship between CHOP and antibody expression, suggesting that CHOP could be induced not only by nutrient depletion but also by transgene expression.

Next, we elucidated which genes improve antibody production. As expected, we observed that ER chaperones such as GRP78 and PDI contributed to the enhancement of antibody production (Figs. 2-3 and -5a) in accordance with the results shown in Chapter 1 (Figs. 1-2 and -3) and other reports (Shusta et al. 1998). It is known that proteins related to protein folding, assembly and secretion work cooperatively. For instance, ERdj3 or ERdj5 are associated with GRP78, and ERO1Lβ facilitates disulfide bond formation in immunoglobulin by selectively oxidizing PDI (Appenzeller-Herzog et al. 2010; Jin et al. 2008; Mezghrani et al. 2001; Shen and Hendershot 2005; Ushida et al. 2008). We examined combinations of these proteins increase the protein expression. Although various combinations improved antibody secretion, a greater effect was observed with combinations containing CHOP plus one of these proteins (Figs. 2-3 and -4). Indeed, these results indicate that CHOP may regulate some ER chaperones or UPR-related proteins in downstream genes, although downstream genes of CHOP have

not been studied in detail (Wang et al. 1998).

In this study, our goal was to determine a means to enhance the expression level of recombinant antibody by the host cell line engineering. We found that expressions of both CHOP and GRP78 were induced in response to antibody production in stable CHO cells. In addition, CHOP combined with UPR-related genes or ER chaperones increased antibody expression without affecting the biological properties of the product in transient assays. We could not rule out the possibility that different results could be obtained between stable cell lines and transient cells. However, we used a transient assay to evaluate effective combinations with CHOP. Overall, our results showed that the introduction of CHOP together in combinations with other genes showed a positive effect on antibody production. Further works to optimize these combinations will be required in the future, since the optimal gene combination with CHOP is dependent on the kinds of antibody produced or strain of CHO cells (e.g., CHO-K1, DG44, etc).

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Chapter 3

Production of human erythropoietin by chimeric chickens

3-1 Summary

The use of transgenic avian allows cost effective and safe production of pharmaceutical proteins. Here, we report the successful production of chimeric chickens expressing human erythropoietin (hEpo) using a high-titer retroviral vector. The hEpo expressed by transgenic hens accumulated abundantly in egg white and had *N*- and *O*-linked carbohydrates. While attachment of terminal sialic acid and galactose was incomplete, portions of *N*- and *O*-linked carbohydrates were present. In vitro biological activity of egg white-hEpo was comparable to that produced by recombinant CHO cells.

3-2 Introduction

As an alternative method for producing large amounts of proteins, various livestock species have been used to generate transgenic animals (Houdebine 2000; Kues and Niemann 2004; Rudolph 1999). Transgenic chickens have been established by gene transfer to embryo mediated by lentivirus (McGrew et al. 2004) and retroviruses (Harvey et al. 2002; Kamihira et al. 2005), and by transfection of non-viral DNA to ES cells (Zhu et al. 2005). Although several methods have been developed to manipulate chicken embryos or embryonic cells for establishment of transgenic chicken, model proteins such as GFP have been used in these experiments, with only a few reports on

the production of pharmaceutical proteins directly applicable to diagnostics (Kamihira et al. 2005; Zhu et al. 2005).

N-glycans affects the physiological function and/or stability of protein. In the *N*-linked carbohydrate of IgG, the terminal sialic acid of the sugar moiety is *N*-acetyl-neuraminic acid in human, while almost all other vertebrates have *N*-glycoyl-neuraminic acid as terminal sialic acids (Raju et al. 2000). Among the livestock that has been established as transgenic animals, only chickens contain solely the human-relevant *N*-acetyl-neuraminic acid as a terminal residue in *N*-linked carbohydrate (Raju et al. 2000). In general, this fact gives an advantage to chicken as transgenic livestock. However, it was reported recently that *N*-linked carbohydrate of an antibody produced and deposited in egg white by transgenic chicken does not contain terminal sialic acids (Zhu et al. 2005). Since the addition of terminal sialic acid profoundly affects the half-life of proteins in serum, incomplete sugar modification presently limits the usefulness of chicken transgenic technology.

In this regard, we examined the variation of the sugar moiety in human erythropoietin (hEpo) produced and accumulated in egg white of transgenic chicken in the present study. hEpo is a glycoprotein consisting of 166 amino acids with a calculated molecular weight of 18,399 that stimulates the proliferation and maturation of erythroid precursor cells (Jelkmann 1992). In the molecule, there are three potential *N*-glycosylation and one *O*-glycosylation sites. Carbohydrate, especially terminal sialic acid, considerably affects the serum half-life and *in vivo* activity of hEpo (Elliott et al. 2004). To date, several groups have explored the possibility of producing hEpo in the milk of transgenic mammals (Aguirre et al. 1998; Park et al. 2006; Toledo et al. 2005), but the production level has been low. In the present study, we successfully used

chimeric hens to produce hEpo in milligram quantities in egg white and analyzed the nature of sugar modification.

3-3 Materials and Methods

3-3-1 Vector construction

hEpo cDNA sequence was amplified by PCR with the primers summarized in Table 3-1. hEpo cDNA was ligated to the HindIII-digested retroviral plasmid vector pMSCV/GΔAscFvFc, which contains a single-chain FvFc cDNA (Ono et al. 2003). Instead of the single-chain FvFc gene, hEpo cDNA was inserted in this plasmid construct to generate the vector plasmid pMSCV/GΔAhEpo(c). The plasmid pWHV8, which contains the WPRE sequence of Woodchuck hepatitis virus, was obtained from the ATCC (GenBank Accession No. J04514). This sequence stabilizes mRNA (Zufferey et al. 1999). The amplified DNA fragment containing WPRE sequence and pMSCV/GΔAhEpo(c) were digested with ClaI and ligated. This plasmid was designated as pMSCV/GΔAhEpo(c)W and is shown in Fig. 3-1a.

3-3-2 Infection into chicken embryos with retroviral vector

The packaging cell line GP293/MSCV/GΔAhEpo(c)W was established as reported previously (Kamihira et al. 2005). Packaging cells were transfected with pVSV-G (Clontech), and virus particles were concentrated by centrifugation. Microinjection and culture of embryos were performed as reported (Kamihira et al. 2005).

3-3-3 Determination of transgene copy number by real-time PCR

Genomic DNA was isolated from organs, erythrocytes, and sperm using Mag extractor -genome- (Toyobo). Purified DNA (20 ng) was used for real-time PCR using LightCycler Faststart DNA Master Hybprobe (Roche). Primers and probes were designed inside a packaging signal region of mouse stem cell virus (Table 3-1), which allows direct comparison of data with other transgenic chickens for calculation of copy number. As a copy number standard, erythrocytes from a G2 transgenic chicken producing scFvFc (Kamihira et al. 2005) (one copy) and from non-transgenic chickens (zero copy) were used.

3-3-4 Detection and measurement of hEpo produced by chimeric chickens

hEpo samples from serum and egg white of chimeric chickens were separated on a 10% SDS–PAGE. After transfer to a PVDF membrane (GE Healthcare), hEpo was detected with rabbit anti-human Epo antibody (R&D Systems) and goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology) with recombinant hEpo produced by CHO cells (Epoetin Beta; EPOGIN, Chugai Pharmaceutical) as a control. For ELISA, anti-hEpo monoclonal antibody, purified from culture supernatant of a hybridoma (HB-8209, ATCC) using a protein A column, and rabbit anti-hEpo antibody were used. As a standard, egg white-hEpo, whose quantity was determined by a Recombigen EPO radioimmunoassay kit (Mitsubishi Kagaku Iatron) was used.

3-3-5 Partial purification of hEpo from serum and egg white

Egg white was mixed using a magnetic stirrer for 15 min at room temperature, and

five volumes of deionized water were then added. After adjusting the pH of the solution to 5.0, the sample was mixed for 15 min followed by centrifugation at 5,000 rpm at 4 °C for 10 min. The supernatant was recovered, pH was adjusted to 7.1, and the sample was stored at –80 °C until required. Serum and egg white samples were absorbed to Blue-sepharose (GE Healthcare) equilibrated with 50 mM Tris–HCl (pH 7.0), the resin was washed with the same buffer, and hEpo was eluted with 50 mM Tris–HCl (pH 7.0) containing 1 M NaCl.

3-3-6 Enzymatic release of carbohydrates

For removal of *N*-linked carbohydrate, CHO-, serum-, and egg white-hEpo samples were digested with 0.5 U of PNGase F (*N*-glycosidase from Elizabethkingia meningoseptica; Sigma–Aldrich) in 50 mM of sodium phosphate buffer (pH 7.5) containing 0.75% (*v/v*) Triton X-100 in a total volume of 50 μl at 37°C for 90 min. For partial digestion, 0.05 U of PNGase F was used. For enzymatic removal of *O*-linked carbohydrate, hEpo samples were initially digested with 3 mU of Neuraminidase F (Marukin Bio) at 37°C for 1.5 h, with 2.5 mU of *O*-glycosidase from *Streptococcus pneumoniae* (Sigma–Aldrich) in 50 mM of sodium phosphate buffer (pH 5.0) in a total volume of 20 μl and finally with PNGase F. For enzymatic removal of the terminal sialic acid, hEpo samples were digested with 3 mU of Neuraminidase F in 8 mM of sodium phosphate buffer (pH 7.0) in a total volume of 50 μl at 37°C for 16 h. For enzymatic removal of the terminal galactose, hEpo samples were first digested with 2 mU of Neuraminidase F in 50 mM of sodium phosphate buffer (pH 5.5) in a total volume of 48 μl at 37°C for 3 h, and then with 1 mU of β-galactosidase from *Streptococcus* 6646K (Seikagaku Kogyo) for 16 h. For all the experiments, samples

corresponding to 24 IU of hEpo were used.

3-3-7 Lectin blotting

Untreated samples and samples treated with Neuraminidase F and β-galactosidase were electrophoresed, blotted to a PVDF membrane, and the terminal sugar residue was detected with a suitable lectin. α2-6 linked sialic acid was detected by a combination of *Sambucus sieboldiana* (SSA) lectin (Seikagaku Kogyo), mouse anti-SSA lectin antiserum, and goat anti-mouse IgG-HRP (Santa Cruz Biotechnology). For detection of α2-3 linked sialic acid, *Maackia amurensis* (MAM) lectin conjugated with biotin (Seikagaku Kogyo) was used. Terminal galactose was detected with RCA-120 lectin from *Ricinus communis* (Seikagaku Kogyo) and mouse anti-RCA lectin serum, as with the SSA lectin. For detection of terminal *N*-acetylglucosamine, the membrane was reacted to *Griffonia simplicifolia* (GS-II) lectin conjugated with biotin (EY Laboratories). Antisera against SSA and RCA lectins were obtained by immunization of BALB/c mice. After the detection, lectins were removed by treatment with a stripping buffer (250 mM glycine–HCl, pH 2.5 containing 1% SDS), and membranes were reprobed with anti-hEpo antibody.

3-3-8 Isoelectric focusing

An Ampholine PAG plate (pH 3.5–9.5, T = 5%, C = 3%; GE Healthcare) with an anode buffer (1 M H_3PO_4) and a cathode buffer (1 M NaOH) was used. After electrophoresis (500 V, 8 mA, 90 min, 10 °C), proteins were blotted onto a PVDF membrane and analyzed by Western blotting.

3-3-9 *In vitro* assay

Ba/F3 cells expressing murine erythropoietin receptor (kindly provided from Dr. Nagamune, The University of Tokyo) (Ueda et al. 2000) were seeded at 1×10^4 cells/well in 96 well plates (IWAKI), and a series of diluted samples was added. After cultivation for 24 h, 18.5 KBq/well of [^{3}H-methyl] thymidine (GE Healthcare) was added, cells were cultured for an additional 24 h, and DNA was recovered on a glass microfiber filter (Whatman) using a cell harvester (Nunc). Isotope incorporation was measured by a liquid scintillation counter. Half maximal effective concentration (EC_{50}) was determined using GraphPad Prism (GraphPad Software).

Table 3-1 Sequences of primers and probes.

Name	sequence
Cloning for hEpo cDNA, forward	5'-AATTAAGCTTACCATGGGGGTGCACG-3' (underline indicates *Hin*dIII site)
reverse	5'-AATTAAGCTTACGCGT-3' (underline indicates *Hin*dIII site)
Cloning for WPRE, forward	5'-CCATCGATAATCAACCTCTGGATTACAAAATTTGTGA-3' (underline indicates *Cla*I site)
reverse	5'-CCATCGATCAGGCGGGGAGGCG-3' (underline indicates *Cla*I site)
Packaging signal, forward	5'-CAAGAAGAGACGTTGGGTTAC-3'
reverse	5'-CTTCCCAGGTCACGATGT-3'
Packaging signal probe, 5' LCRed	5'-GGTGATGGAGGTCTCGGTTAAAGGTGCC-3'
3' FITC	5'-GGCCAGGTGAAAAGACCTTGATCTTAACCTT-3'
GFP for G_1 screen, forward	5'-GGTCACTACCTTCACCTATGGCGTGC -3'
reverse	5'-TTGATGCCATTCTTTTGCTTGTC -3'
hEpo for G_1 screen, forward	5'-GCAGCAGGCCGTAGAAGTCTGGCA -3'
reverse	5'-AGTGATTGTTCGGAGTGGAGCA -3'
GAPDH as a PCR control, forward	5'-GGGCACGCCATCACTATC -3'
reverse	5'-GTGAAGACACCAGTGGACTCC -3'

3-4 Results

3-4-1 Production of chimeric chickens

The retroviral vector containing the hEpo expression cassette under control of the chicken β-actin promoter (Fig. 3-1a) was introduced into the heart of each developing chicken embryo after incubation for 55 h at stages 13–15 of the staging of Hamburger and Hamilton (Hamburger and Hamilton 1951). Virus injection was attempted separately five times. In total, 90 embryos were injected with the virus preparations (titers of 3×10^8 to 5×10^{10} cfu/ml), and 24 embryos hatched (27%; Table 3-2).

Specificity of organs in gene delivery was studied with one male (#103) and one female (#117) chickens (Fig. 3-1b). While the transgene was detected in a number of organs, the copy number differed between organs and between individuals. Since the virus was injected into the embryonic heart, the heart and erythrocytes showed a higher mosaic rate of 2.5–4.5 DNA copies per cell. It was noteworthy that at the stage of infection, primordial germ cells are migrating in blood stream (D'Costa et al. 2001), but the copy number in sperm was very low (Table 3-2). Male chickens with copy numbers exceeding 0.04 (theoretically one out of 50 descendants is transgenic) mated with wild-type (wt) or chimeric hens. Although more than the theoretically predicted number of off-springs (282 chicks; 36 for #103-wt, 80 for #106-wt, 4 for #106-#117, and 162 for #208-wt) were checked by PCR analysis, no transgenic descendants have been obtained.

3-4-2 Productivity of hEpo in chimeric chicken

Among female chickens, three individuals (#111, #117, and #528) were selected

to study the long-term production of hEpo. One of them (#117) produced a relatively high concentration of hEpo (4,000–7,000 IU/ml) for almost two years (Fig. 3-2). The other two individuals produced 1,000–4,000 IU/ml on average in egg white through the experimental period. The average hEpo serum levels (IU/ml) were 121 (#111), 118 (#117), and 111 (#528), and the production level showed long-term stability (Fig. 3-2).

In all cases, hEpo accumulated abundantly in egg white, less so in serum, and was barely detected in yolk. It is likely that hEpo produced in various organs was transported in serum, and hEpo produced in tubular gland cells of magnum was deposited in egg white, as with the case in ovalbumin. Thus, higher production of hEpo in egg white depends on the transgene copy number in tubular gland cells of magnum, which secrete the major part of proteins deposited in egg white. To verify this point, the magnum portion of the oviduct was isolated and the transgene copy number was assessed by real-time PCR. The transgene copy number in the magnum of #111 and #117 was 1.3 and 5.1, respectively (Fig. 3-3). Although our tubular gland cell sample was not completely pure and contained certain percentages of other cell types that did not secrete proteins accumulated in egg white, the difference of egg white-hEpo production level between #111 and #117 can be at least partially explained by the difference in the transgene copy number of the magnum.

3-4-3 Sugar moiety of hEpo produced by chimeric chicken

hEpo production in serum and egg white of G0-chimeric chickens was analyzed by Western blotting. All chickens produced hEpo in serum, and female chickens produced much more hEpo in egg white. However, the apparent molecular weight of hEpo in serum and egg white was different, as revealed by Western blotting (Fig. 3-4a).

hEpo derived from serum and egg white of chimeric chicken was partially purified, and *N*-linked carbohydrates were then removed enzymatically using PNGase F. After complete digestion, both serum- and egg white-hEpo produced two bands, possibly corresponding to *O*-glycosylated and non-*O*-glycosylated molecules, as previously reported (Skibeli et al. 2001). On the other hand, the major product of CHO-hEpo after the complete digestion was the *O*-glycosylated form, and trace amounts of non-glycosylated molecule could be detected (Fig. 3-4a). We then analyzed *O*-glycosylation of the hEpo derived from chimeric chicken by successive enzymatic digestion of *N*- and *O*-linked carbohydrates (Fig. 3-4b). By complete removal of sugars, all samples produced a single band, indicating that the relatively slow migrating bands of about 21 kDa, observed by PNGase F digestion in Fig. 3-4a, were due to *O*-glycosylated molecules.

By partial digestion of *N*-linked carbohydrate, two bands in addition to the original non-digested band were observed with CHO-, serum-, and egg white-hEpo; these possibly corresponded to mono-, di-, and tri-*N*-glycosylated molecules (Fig. 3-4a). However, the mobility of the corresponding bands was slightly different between samples; egg white-hEpo exhibited faster-migrating bands and CHO-hEpo exhibited slower-migrating bands. Mobility of serum-hEpo sample was intermediate between these two samples. This suggested that the structure of *N*-linked carbohydrate is different among each sample; egg white-hEpo possibly has a shorter sugar side-chain.

Fig. 3-4c shows the results of lectin blotting. CHO-hEpo strongly reacted with the RCA-120 lectin after Neuraminidase F treatment. Although egg white-hEpo treated with Neuraminidase F reacted with RCA-120 lectin, the band intensity was only approximately 35% of that of CHO-hEpo. Since RCA-120 is specific for β-galactose

and lactose, a certain percentage of egg white-hEpo contains terminal galactose on *N*- and/or *O*-linked carbohydrates. Use of GS-II lectin, which specifically reacts with terminal α- or β-linked *N*-acetylglucosamine, determined that 70% egg white-hEpo double-digested with Neuraminidase F and β-galactosidase were reactive, suggesting that a majority of hEpo derived from egg white contained *N*-acetylglucosamine. Since *N*-acetylglucosamine is present on *N*-linked but not on *O*-linked carbohydrate, this result suggests that the core portion of *N*-linked carbohydrate is attached to egg white-hEpo. α2-6 linked sialic acid-specific SSA lectin failed to react with any hEpo species, and α2-3 linked sialic acid-specific MAM lectin detected CHO-hEpo but reacted markedly less with egg white-hEpo.

For further analysis of the sugar moiety, isoelectric points of each hEpo were determined (Fig. 3-4d). CHO-hEpo produced a band corresponding to an isoelectric point of less than 4.5, which is consistent with previous reports (Skibeli et al. 2001; Caldini et al. 2003). The isoelectric point of serum-hEpo was greater than 4.5, but only slightly higher than CHO-hEpo. On the other hand, egg white-hEpo exhibited several bands in alkaline sides, suggesting that considerably less sialic acid residues were attached to the molecule. Incubating the egg white-hEpo with Neuraminidase F induced an alkaline shift of the band (Fig. 3-4d), supporting the weak sialylation.

3-4-4 *In vitro* biological activity of hEpo produced by chimeric chicken

In vitro biological activity of serum- and egg white-hEpo was measured as shown in Fig. 3-5. Egg white- and serum-hEpo showed similar or little higher *in vitro* activities compared with CHO-hEpo, judging from their EC_{50}, and produced a similar dosage curve. For treatment with Neuraminidase F alone or in combination with

β-galactosidase, the activity of CHO- and serum-hEpo increased but that of egg white-hEpo was unchanged or slightly decreased. PNGase F treatment decreased the activity of all samples. Previously, it was reported that removal of terminal sialic acid from *N*-linked carbohydrate increases *in vitro* activity through increases in binding affinity, and complete removal of *N*-linked carbohydrate reduces *in vitro* activity (Takeuchi et al. 1990; Takeuchi and Kobata 1991; Tsuda et al. 1990). Since CHO- and serum-hEpo have many sialic acids (Fig. 3-4d), our results agree with these reports.

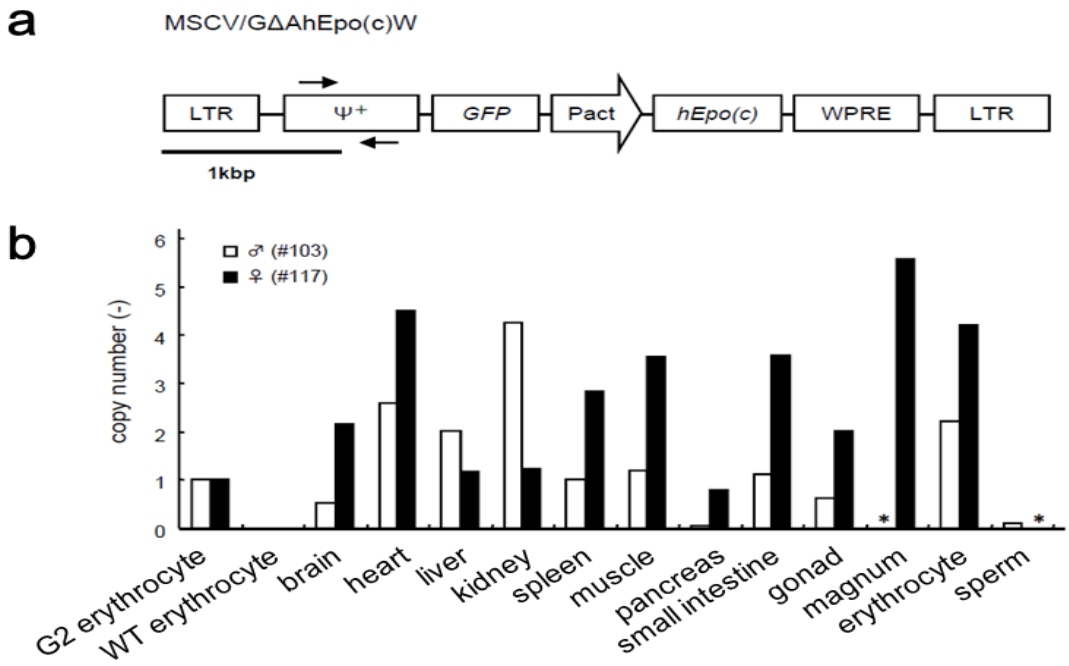

Fig. 3-1 Structure of hEpo expression vector. LTR, long terminal repeat from mouse stem cell virus (**a**); Ψ+, virus packaging signal sequence; GFP, green fluorescent protein; Pact, chicken β-actin promoter; hEpo(c), human erythropoietin cDNA; WPRE, Woodchuck hepatitis virus posttranscriptional regulatory element. Arrows indicate primers for real-time PCR analysis. Detection of transgene in various tissues of chimeric chicken. Genomic DNA was extracted from various tissues of a male (#103) and a female (#117) and real-time PCR was performed to evaluate the transgene copy number using scFvFc G2 genomic DNA as a standard (**b**).*, not applicable

Table 3-2 Retroviral vector injection into chicken embryos.

Run No.	Viral titer (cfu/ml)	Number of embryo		Chicken No.	Sex (M:male, F:female)	Average of hEpo concentration (IU/ml)		Copy number in sperm (-)
		injected	hatched (%)			serum	egg white	
1	3×10^{8}	22	12 (55)	#103	M	102.3	-	0.044
				#106	M	116.5	-	0.066
				#108	M	70.0	-	-
				#109	M	104.5	-	-
				#111	F	120.8	1249	-
				#117	F	117.9	5667	-
2	1.8×10^{9}	21	5 (24)	#208	M	132.6	-	0.043
				#215	M	95.71	-	0.070
3	7.5×10^{9}	19	1 (5.3)	#316	M	121.7	-	0.025
4	3.3×10^{9}	10	1 (10)	#404	M	44.41	-	-
	5.0×10^{10}	9	2 (22)	#411	M	119.0	-	0.004
				#413	M	40.0	-	0.005
5	5.0×10^{9}	9	3 (33)	#528	F	111.1	3505	-
Total		90	24 (27)					

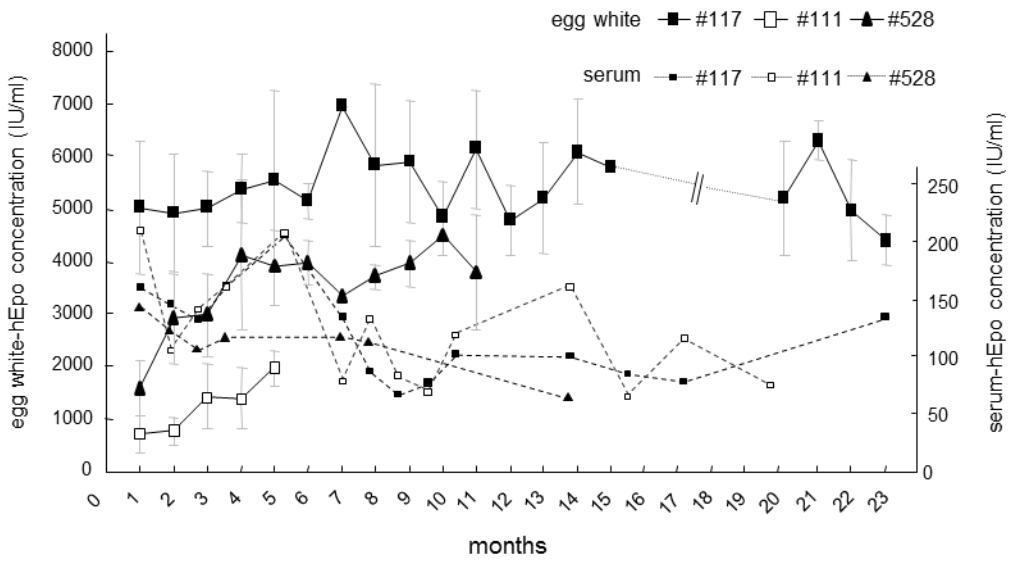

Fig. 3-2 Productivity of hEpo produced by chimeric chickens was determined by ELISA. Long-term hEpo expression in egg white (left axis) and serum (right axis).

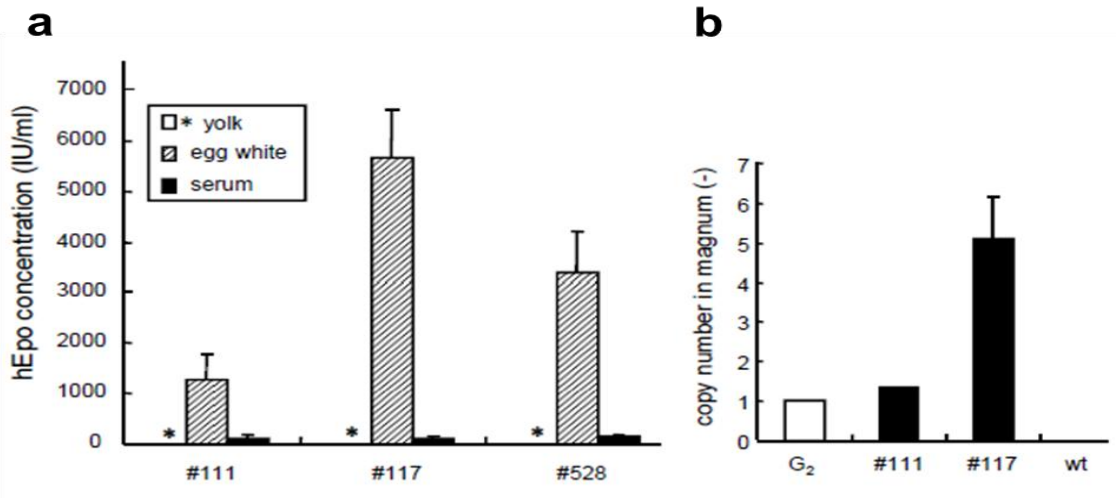

Fig. 3-3 Productivity of hEpo in serum and egg white of chimeric chickens. Average concentrations of hEpo produced in yolk, egg white, and serum of each female chimera throughout breeding (**a**). Transgene copy number in the magnum portion of the oviduct. Chimera #528 is still alive, and is therefore not applicable (**b**).

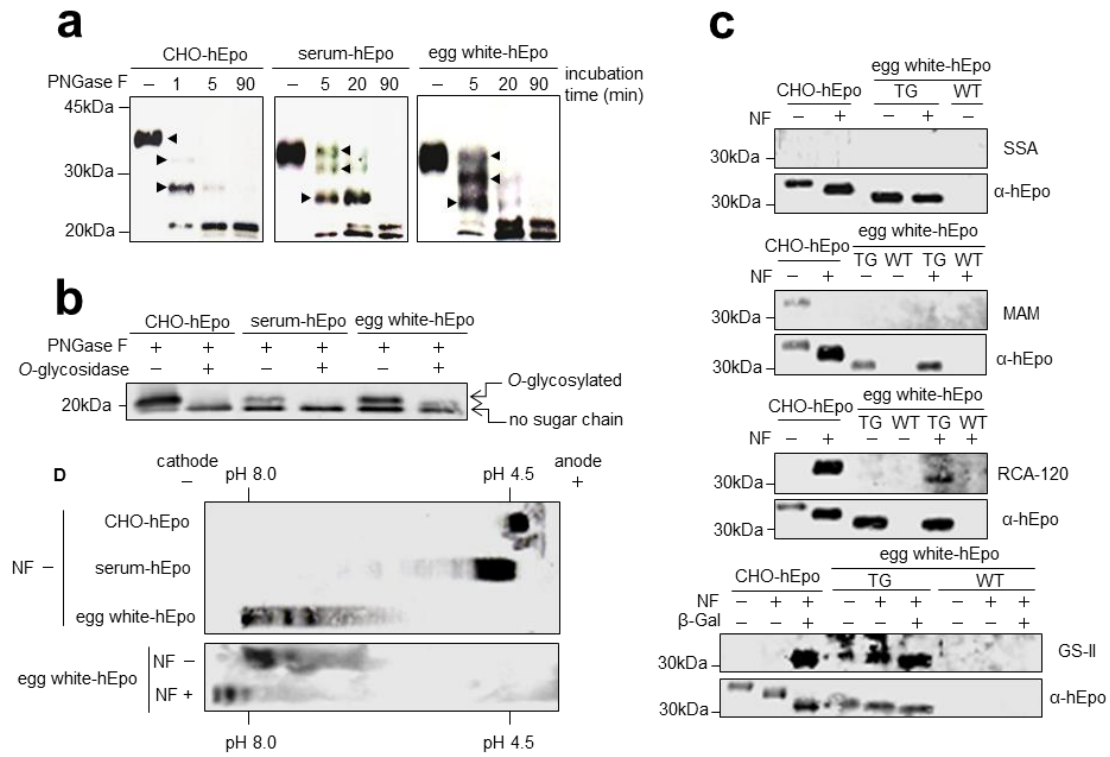

Fig. 3-4 Analysis of sugar moiety of hEpo produced by chimeric chickens. The *N*-linked carbohydrate of each sample was partially or completely removed by digestion with PNGase F and analyzed by Western blotting (**a**). Arrowheads show the band positions of hEpo containing *N*-linked carbohydrate. Each sample was treated with a combination of Neuraminidase F, *O*-glycanase, and PNGase F (**b**). Terminal sialic acid (SSA and MAM), galactose (RCA-120), and *N*-acetylglucosamine (GS-II) were detected by suitable lectins after Neuraminidase F (NF) and β-galactosidase (β-Gal) digestion. Isoelectric focusing analysis of chicken-derived hEpo (**d**).

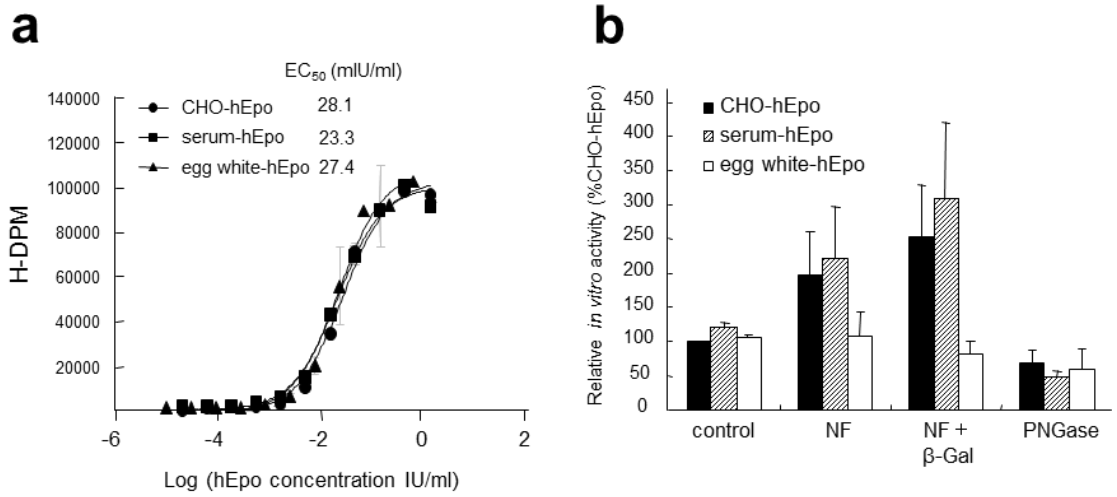

Fig. 3-5 *In vitro* biological activity of hEpo produced by chimeric chicken. *In vitro* biological activity of partially purified hEpo was measured by ^{3}H-thymidine uptake into Ba/F3 cells expressing murine erythropoietin receptor (**a**). Relative *in vitro* activity of enzymatically treated hEpo (**b**).

3-5 Discussion

In the present paper, we established chimeric chickens producing hEpo by retroviral-mediated gene transfer. The chimeric chickens produced high levels of hEpo in egg white (1,000–6,000 IU/ml corresponding to roughly 7–40 μg/ml, based on the specific activity of CHO-hEpo), but serum concentration was low. This concentration in egg white is corresponding to the amount of 0.2–1.2 mg/egg given that an egg contains about 30 ml of egg white. Furthermore, hEpo did not accumulate in yolk. In the viral vector construct, we did not use a cell type-specific expression system, which enables the specific deposition of hEpo in egg white; instead, ubiquitous chicken β-actin promoter was used. Nevertheless, all the chimeric hens accumulated hEpo in egg white. This suggests that hens displaying this type of deposition may survive, because ectopic expression and subsequent release of hEpo in blood stream causes severe polycythemia to host animals, given that Epo is structurally conserved in many animals (Wen et al. 1993). This is also in accordance with the fact that a transgenic offspring ubiquitously expressing hEpo could not presently be obtained.

Judging from the results of lectin blotting and isoelectric focusing, roughly less than 10 and 35% of egg white-hEpo contained terminal sialic acid and galactose, respectively, in *N*- and *O*-linked carbohydrate. However, approximately 70% of egg white-hEpo contained *N*-acetylglucosamine in *N*-linked carbohydrate. This result suggests that egg white-hEpo possesses a shorter half-life in serum and lesser *in vivo* activity than CHO-hEpo, although *in vitro* activity was almost similar. The lack of terminal sialic acid has also been observed with an antibody deposited in egg white (Zhu et al. 2005; our unpublished results). Thus, it is possible that tubular gland cells of

magnum, which secrete a majority of egg white proteins, lack a sugar modification system or have one that is insufficient. Quantitative real-time PCR has demonstrated that magnum cells express certain amount of α2,3-sialyltransferase IV and VI, and β1,4-galactosyltransferase III (Kodama, unpublished results). Now, we are studying whether these enzymes are expressed in the Golgi of magnum cells to form an active glycosyltransferase system.

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Conclusion

Mammalian cells such as CHO cells have been widely utilized to produce therapeutic proteins including antibodies (Shukla and Thömmes 2010). In order to meet the increasing demand for biopharmaceuticals, it is necessary to create innovative drugs with enhanced efficacy and/or to improve their productivities resulting in reduction in cost of goods, and give a solution to problems such as limited manufacturing facilities (Farid 2007). The goal of this study was to develop high expression systems for therapeutic proteins production including recombinant antibodies with high quality.

I addressed the improvement of recombinant antibody production by host cell engineering employing proteins related to protein folding, assembly, secretory pathways, and the UPR in **Chapter 1** and **2**, in parallel with vector modification such as DNA elements. In **Chapter 1**, I identified several endogenous proteins that interact with antibody fragments or whole antibodies in mammalian cells by immunoprecipitation coupled with liquid chromatography-tandem mass spectrometry (IP-MS) based on gene ontology terms. The list includes both non-ER-resident proteins and novel ER chaperones, in addition to known ER chaperones such as GRP78/Bip and GRP94 that were shown to interact with antibodies (Feige et al. 2009; Hendershot et al. 1987; Melnick et al. 1994). Most of the identified proteins increased antibody production in mammalian cells, and further, some of their combinations resulted in greater enhancement. I demonstrated that this method could be effective in the investigation of novel proteins that are involved in enhancing recombinant antibody production because IP-MS could identify proteins which directly interact with the antibodies.

In **Chapter 2,** I focused on the unfolded protein response (UPR) which is one of

the major regulators of the secretory pathway involved in protein quality controls as well as ER chaperones (Schröder and Kaufman 2005). First of all, stable CHO cells which showed high expression level of recombinant antibody were established (>300 mg/L for 5 days in fed-batch culture). Compared with wild-type CHO cells, I found that expressions of both CHOP/Gadd153 and GRP78/Bip, which are UPR-related genes, were induced in response to recombinant antibody production in stable CHO cells. CHOP overexpression resulted in increasing recombinant antibody production in some mammalian cells including CHO cells without affecting the biological properties of the product. Here, I showed that several chaperones, non-ER-resident proteins, and UPR-related proteins identified in **Chapter 1** and **2** enhanced antibody productions in some mammalian cells. Further optimization will be needed for the industrial success, since optimal genes for host cell engineering depend on the kind of antibody or strains of cells.

In **Chapter 3**, I established transgenic chickens as a next generation alternative host to CHO cells. Transgenic chickens developed by retroviral vector infection into chicken embryos could produce high level of human erythropoietin (hEpo) in egg white with long term stability. However, small amounts of terminal sialic acid and galactose were detected with N-glycan of egg white-hEpo. Furthermore, for treatment of with Neuraminidase F alone or in combination with β-galactosidase, *in vitro* activity of egg white-hEpo was almost unchanged though that of CHO-hEpo was increased, suggesting that tubular gland cells which secrete egg white proteins would have insufficient sugar modification systems. This study proposes that further genetic modifications of glycosylation should be required to produce fully glycosylated recombinant proteins produced by transgenic chickens.

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1 Kodama D, Nishimiya D, Iwata K, Yamaguchi K, Yoshida K, Kawabe Y, Motono M, Watanabe H, Yamashita T, Nishijima K, Kamihira M, Iijima S (2008) Production of human erythropoietin by chimeric chickens. Biochem Biophys Res Commun 367(4):834-9

2 Nishimiya D, Ogura Y, Sakurai H, Takahashi T (2012) Identification of antibody-interacting proteins that contribute to the production of recombinant antibody in mammalian cells. Appl Microbiol Biotechnol doi: 10.1007/s00253-012-4132-y

3 Nishimiya D, Mano T, Miyadai K, Yoshida H, Takahashi T (2012) Overexpression of CHOP alone and in combination with chaperones is effective in improving antibody production in mammalian cells. Appl Microbiol Biotechnol doi: 10.1007/s00253-012-4365-9

4 Nishimiya D (2010) METHOD FOR PRODUCTION OF ANTIBODY IN CULTURED MAMMALIAN CELL BY USING MOLECULAR CHAPERONE. WO/2010/010848

5 Nishimiya D, Inoue T (2012) DNA ELEMENT HAVING THE ACTIVITY OF ENHANCING FOREIGN GENE EXPRESSION. WO/2012/005378

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