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Production of recombinant human erythropoietin/Fc fusion protein by genetically manipulated chickens

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Abstract

We previously reported the production of human erythropoietin (hEpo) using genetically manipulated (GM) chickens. The recombinant hEpo was produced in the serum and egg white of the GM chickens, and the oligosaccharide chain structures of the serum-derived hEpo were more favorable than those of the egg white-derived hEpo. In the present study, a retroviral vector encoding an expression cassette for a fusion protein of hEpo and the Fc region of human immunoglobulin G (hEpo/Fc) was injected into developing chicken embryos, with the aim of recovering the serum-derived hEpo from egg yolk through the yolk accumulation mechanism of maternal antibodies. The GM chickens that hatched stably produced the hEpo/Fc fusion protein not only in their serum and egg white, but also in the egg yolk as expected. Lectin blot analyses revealed that significant amounts of the oligosaccharide chains of hEpo/Fc produced in the serum and eggs of GM chickens terminated with galactose, and that the oligosaccharide chains of the serum- and yolk-derived hEpo/Fc incorporated sialic acid residues. Moreover, biological activity assessment using Epo-dependent cells revealed that the yolk-derived hEpo/Fc exhibited a comparable performance to the serum- and CHO-derived hEpo/Fc. These results indicate that transport of Fc fusion proteins from the blood circulation to the yolk in chickens represents an effective strategy for the production of pharmaceutical glycoproteins using transgenic chicken bioreactors.

Key words: transgenic avian bioreactor, human erythropoietin, Fc fusion protein, yolk transport, retroviral vector, woodchuck hepatitis virus post-transcriptional regulatory element.

Introduction

Transgenic animal bioreactors have held great promise for revolutionizing the manufacture of human biopharmaceuticals (Rudolph 1999; Kues and Niemann 2004). Standing as the most robust livestock bioreactors, the mammary glands of goat, sheep and cattle have attracted a great deal of attention with numerous reports on their use for the production of human biopharmaceuticals (Melo et al. 2007). Furthermore, successful drug approval for commercialization has recently been achieved (Houdebine et al. 2009). Despite this, mammalian bioreactors have several drawbacks, including large breeding area requirements, long periods of sexual maturation, protein purification hurdles and prion-related disease complications (Dyck et al. 2003; Whitelaw 2004). Transgenic chickens may offer several advantages as bioreactors over mammals, such as high protein productivity in eggs, straightforward scalability and similar protein glycosylation to that of humans (Raju et al. 2000; Ivarie 2003; Sang 2004). A variety of methods for generating genetically manipulated (GM) avians have been reported (Love et al. 1994; Mizuarai et al. 2001; Harvey et al. 2002; Mozdziak et al. 2003; McGrew et al. 2004; Kamihira et al. 2005; Zhu et al. 2005; Van de Lavoir et al. 2006; Lillico et al. 2007). Our group has revealed the feasibility of using GM avians for the production of various recombinant proteins, such as a single-chain antibody fragment fused with the Fc region of human immunoglobulin G (Kamihira et al. 2005; Kawabe et al. 2006a), chimeric monoclonal antibodies (Kamihira et al. 2009), Fc-fused extracellular domain of tumor necrosis factor receptor 2 (Kyogoku et al. 2008) and human erythropoietin (hEpo) (Kodama et al. 2008).

hEpo is a glycoprotein hormone associated with erythropoiesis. The protein is modified with three *N*-linked oligosaccharide chains (Asn24, Asn38 and

Asn83) and one *O*-linked oligosaccharide chain (Ser126) (Elliott et al. 2003), and the terminal sialic acid contents of the *N*-linked oligosaccharide chains affect the serum half-life and *in vivo* activity (Elliott et al. 2004). Previously, we generated GM chickens producing hEpo in their serum and egg white. The hEpo produced by the GM chickens had *N*- and *O*-linked oligosaccharide chains and retained the biological activity. This is a good example of the feasibility of using transgenic chicken bioreactors for the production of human-derived hormones, which are often difficult to produce in transgenic mammalian bioreactors because the expression of such proteins affects the physiology of the animals through the high homology with their endogenous counterparts. GM chickens expressing hEpo at relatively high levels exhibited no apparent serious effects on their health. The oligosaccharide chain structures of the serum-derived hEpo were more favorable than those of the egg white-derived hEpo (Kodama et al. 2008). We also reported that the profiles of the oligosaccharide chain structures of recombinant antibodies produced in the serum of GM chickens were very similar to those of antibodies produced in the yolk (Kamihira et al. 2009).

In the present study, hEpo was molecularly fused to the Fc region of human immunoglobulin G (hEpo/Fc). By this modification, hEpo produced in the serum can be expected to be recovered from the egg yolk via the mechanism of maternal antibody transport from the blood circulation into the yolk (Loeken et al. 1983; Mohammed et al. 1998; Morrison et al. 2000; Kawabe et al. 2006b). GM chickens were generated by injecting a retroviral vector encoding an expression cassette for hEpo/Fc under the control of a constitutive chicken β-actin promoter into developing chicken embryos. The production levels and biological activities of hEpo/Fc in the serum and eggs of GM chickens were measured, and the oligosaccharide structures of the hEpo/Fc proteins produced were

characterized by lectin blotting.

Materials and methods

Vector construction

Schematic drawings of the retroviral vectors used in this study are shown in Fig. 1. The retroviral plasmid pMSCVneo (Clontech, Palo Alto, CA, USA) derived from the murine stem cell virus was used for the construction of pMSCV/GΔAhEpo/Fc and pMSCV/GΔAhEpo/FcW. An hEpo cDNA sequence was amplified from pMSCV/GΔAhEpoW (Kodama et al. 2008) using the primers 5′-GCAGCCAAGCTTACCATGGG-3′ and 5′-AAAAGCATGCCTCCCCTGTGTACAGCTTCA-3′ to append *Hin*dIII and *Sph*I digestion sites (underlined) onto either end of the PCR product. The PCR was initiated with a DNA polymerase (KOD plus; Toyobo, Osaka, Japan) at 94°C for 2 min, followed by 30 cycles of amplification at 94°C for 15 s, 59°C for 30 s and 68°C for 30 s. The Fc fragment derived from human immunoglobulin G2 (IgG2), which was added to a papain cleavage site (Wenig et al. 2004), was amplified by PCR from the plasmid pMSCV/GΔAscFv-Fc2 (Kawabe et al. 2006b) using the primers 5′-TACTGCATGCAGGACAGGGGACAGAGAGCGCAAATGTTGTGTCG-3′ and 5′-TTTTATCGATAAGCTTTCATTTACCC-3′ to append *Sph*I and *Cla*I sites (underlined) onto both ends of the PCR product. The PCR was initiated using the KOD plus DNA polymerase at 94°C for 2 min, followed by 30 cycles of amplification at 94°C for 15 s, 57°C for 30 s and 68°C for 40 s. The amplified DNA fragments were digested with the respective restriction enzymes and ligated into *Hin*dIII and *Cla*I-digested pMSCV/GΔAhEpo or pMSCV/GΔAhEpoW to generate pMSCV/GΔAhEpo/Fc or

pMSCV/GΔAhEpo/FcW, respectively. The DNA sequences were confirmed using a DNA sequencer (Prism 3130 Genetic Analyzer; Applied Biosystems, Foster City, CA, USA).

Retroviral vector production and microinjection into chicken embryos

VSV-G-pseudotyped pantropic retroviral vectors were produced as reported previously (Kamihira et al. 2005). The viral titer was determined using NIH-3T3 cells based on the expression of GFP residing in the viral vectors. Microinjection of the viral solution and embryo cultures were performed as reported previously (Kamihira et al. 2005).

All animal experiments in this study were approved by the Ethics Committee for Animal Experiments of the Faculty of Engineering, Kyushu University (18-003-1).

Measurement of hEpo/Fc concentration

The hEpo/Fc concentration was determined by an enzyme-linked immunosorbent assay (ELISA). The amounts of hEpo and Fc were measured using a commercially available kit (Epo ELISA kit; Roche Diagnostics, Basel, Switzerland) and an in-house established Fc-based ELISA, respectively. The quantification of the Fc-fused protein was performed as reported previously (Kamihira et al. 2009). Rabbit IgG fraction of anti-human IgG (Fc) (Rockland Immunochemicals, Philadelphia, PA, USA) and rabbit peroxidase (POD)-conjugated anti-human IgG antibodies (Rockland Immunochemicals) were used as the primary and secondary antibodies, respectively. Calibration curves were created using a dilution series of purified hEpo/Fc produced by CHO-K1 cells or the human Fc fragment (Jackson ImmunoResearch, West

Grove, PA, USA). The hEpo concentrations in the samples were also determined using the Epo ELISA kit according to the manufacturer's instructions.

Purification of hEpo/Fc from serum, egg white and yolk samples

hEpo/Fc produced by GM chickens was purified using staphylococcal protein A-Sepharose beads (rProtein A-Sepharose™ Fast Flow; GE Healthcare, Fairfield, CT, USA) as described previously (Kamihira et al. 2009). For purification from egg white and yolk, the samples were diluted 5-fold with phosphate-buffered saline (PBS) and exhaustively cleared by centrifugation (20,000 × *g*, 5 min, 4°C). After adjustment of the pH to 8.5 using 1 M Tris-HCl (pH 9.0), the samples were incubated with the beads at 4°C for 3 h under constant stirring to allow capture of the hEpo/Fc protein. After packing the beads into a column (cat. no. 732-1010; Bio-Rad Laboratories) and equilibration with 20 mM sodium phosphate (pH 7.4), hEpo/Fc protein was eluted using 100 mM glycine-HCl buffer (pH 3.5). The eluate was immediately neutralized by addition of 1 M Tris-HCl (pH 8.0). The purified samples were dialyzed against PBS and stored at -80°C until use.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

Reducing and non-reducing SDS-PAGE followed by Western blot analysis were performed to detect hEpo/Fc, as described previously (Kamihira et al. 2009). Purified hEpo/Fc produced by CHO-K1 cells and the human Fc fragment (Jackson ImmunoResearch) were used as positive controls. Samples were boiled in SDS-PAGE sample buffer with or without 2-mercaptoethanol, separated in 10% or 7.5% polyacrylamide gels (reducing and non-reducing, respectively) and transferred onto PVDF membranes (GE Healthcare). hEpo/Fc protein on the

membranes was detected using a rabbit anti-hEpo monoclonal primary antibody (R&D Systems, Minneapolis, MN, USA) and a POD-conjugated goat anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The specific antibody-antigen complexes were detected using an ECL detection system (GE Healthcare). hEpo/Fc protein was also detected using a rabbit POD-conjugated anti-human IgG antibody (Rockland Immunochemicals) after treatment of the membranes with a stripping solution (0.2 M NaOH) at 37°C for 5 min.

Lectin blotting

Purified samples were electrophoresed, blotted onto PVDF membranes and analyzed for their terminal carbohydrate residues by lectin blotting. Briefly, the membranes were immersed in a blocking solution (TBS containing 0.05% Tween-20 and 1% bovine serum albumin) for 1 h at room temperature. After extensive washing, the membranes were incubated with biotin-conjugated *Erythrina cristagalli* (ECA) lectin (Seikagaku Kogyo, Tokyo, Japan), *Maackia amurensis* (MAM) lectin (Seikagaku Kogyo) or *Sambucus sieboldiana* (SSA) lectin (Seikagaku Kogyo) to detect terminal galactose, α2-3-linked sialic acid and α2-6-linked sialic acid residues, respectively, followed by incubation with POD-conjugated streptavidin (Wako). Streptavidin-biotin complexes were detected using the ECL detection system. The bound lectins were then removed by treatment with a stripping buffer (250 mM glycine-HCl pH 2.5, 1% SDS) at room temperature for 30 min, and the membranes were reprobed with anti-hEpo or anti-human IgG antibodies.

***In vitro* bioassay**

The *in vitro* biological activity of hEpo/Fc was assessed by a proliferation-based assay involving Ba/F3 cells expressing the murine Epo receptor (Ueda et al. 2000). Briefly, the cells were seeded in 96-well plates (Asahi Glass) at 5000 cells/well, followed by the addition of a series of diluted samples. After culture for 48 h, cell proliferation was measured using a kit (Cell Counting Kit-8; Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Data analysis and calculation of EC_{50} values were performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Statistical significance was assessed by an unpaired Student's *t*-test. Values of $p<0.05$ were considered to be significant.

Results and discussion

Enhancement of retroviral titer and hEpo expression by the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE)

For the generation of transgenic avians using retroviral vectors, the preparation of high-titer viral solutions is crucial (Mizuarai et al. 2001; Kamihira et al. 2005; Kawabe et al. 2006a). It has been reported that the WPRE enhances both the viral titer and transgene expression from a variety of viral vectors (Zufferey et al. 1999; Ramezani et al. 2000; Xu et al. 2003). In our preliminary experiment, the hEpo expression from the cDNA sequence was lower than that from the genomic DNA (data not shown). Therefore, we examined the effects of the WPRE on the viral titer and hEpo expression from retroviral vectors encoding an hEpo cDNA expression cassette. Insertion of the WPRE downstream of the *hEpo* or *hEpo/Fc* gene under the control of the chicken β-actin promoter (Fig. 1) led to increases in the viral titer by up to 10-fold, regardless of the target gene and gene size for viral packaging (Fig. 2). CHO-K1 cells transiently transfected with the WPRE-containing plasmids also exhibited enhanced productions of

hEpo and hEpo/Fc by approximately 6-fold and 2-fold, respectively (data not shown). Although the WPRE does not always enhance the viral titer and transgene expression (Klein et al. 2006), the sequence was effective in our constructs. Consequently, we used the WPRE-incorporated retroviral vectors to generate GM chickens expressing hEpo and hEpo/Fc.

Generation of GM chickens expressing hEpo/Fc

Based on the observations that human IgG2 and a human IgG2-derived Fc-fusion protein exhibited more efficient transport into the egg yolk than other human IgG subclasses (Kawabe et al. 2006b), hEpo was genetically fused to the Fc region of human IgG2. In this case, a papain digestion site was introduced into the joint region between hEpo and Fc (Wenig et al. 2004) so that hEpo could be separated by digestion with the enzyme. A retroviral vector encoding an expression cassette for hEpo/Fc under the control of the chicken β-actin promoter (Fig. 1) was injected into the heart of developing chicken embryos at stage 15 according to Hamburger and Hamilton (1951). In this study, a total of 74 chicken embryos in 4 experiments were injected with the retroviral solution (1.5-4.0 μl) with titers of 0.11-2.2×10^8 infectious units (IU)/ml. After the retroviral vector injection, the embryos were cultured until they hatched. The hatchability of the embryos was in the range of 13-50%, and 28% on average (Table 1). The birds that hatched exhibited no apparent abnormalities during breeding and female chickens produced eggs after sexual maturation. The long-term productions of hEpo/Fc in the serum and eggs of the GM chickens were determined by ELISA (Fig. 3). hEpo/Fc was stably produced throughout the measurement period. The production levels of hEpo/Fc varied among the GM chickens, and might be related to the retroviral titer, injection volume and

embryonic stage at the transduction because these chickens were highly mosaic and chimeric for integration of the vectors. The concentrations of hEpo/Fc in the serum, egg white and yolk were in the ranges of 27-144, 9-66 and 12-41 μg/ml, respectively (Table 1). These findings demonstrate that the GM chickens produced hEpo/Fc in their whole body, including eggs, without any toxic effects on the birds. Since hEpo production has been reported to exert toxic effects on animal health when mammalian transgenic bioreactors are used (Massoud et al. 1996; Kind and Schnieke 2008), chickens may be advantageous over mammals as transgenic bioreactors for the production of human cytokines and hormones. Furthermore, since hEpo is not produced in the yolk in hEpo-expressing chickens (Kodama et al. 2008), the Fc region of hEpo/Fc facilitated the transport to and accumulation in yolk.

Analyses of hEpo/Fc produced by GM chickens

Biochemical characterization of the hEpo/Fc produced in the serum of GM chickens was performed by Western blotting under reducing and non-reducing conditions using two different antibodies (anti-hEpo and anti-human IgG) (Fig. 4). Under reducing conditions, the bands corresponding to hEpo/Fc resolved as broad bands in the molecular weight range of 62-68 kDa, suggesting that the protein was modified with oligosaccharide chains (Fig. 4a, b). Since the Fc region used for the engineered hEpo/Fc included a hinge region, it was expected that hEpo/Fc would be produced as S-S-linked dimers in the cells. The structure was confirmed by non-reducing SDS-PAGE followed by Western blotting (Fig. 4c, d). The molecular weights of the bands corresponded to 120-140 kDa, suggesting that the protein formed dimers. These results indicate that hEpo/Fc produced in the serum of GM chickens was correctly synthesized

and assembled.

Further analyses of the hEpo/Fc proteins produced in the serum, egg white and yolk of GM chickens were carried out by Western blotting using hEpo/Fc samples purified with staphylococcal protein A-Sepharose beads. The egg white-derived hEpo/Fc exhibited a single major band with a lower molecular weight compared with those of the serum- and CHO-derived hEpo/Fc proteins (Fig. 5a, b). On the other hand, two (Fig. 5a) or three (Fig. 5b) major bands were detected for the yolk-derived hEpo/Fc, suggesting that the hEpo/Fc in yolk was separated into two fractions during the transport from serum. The lowest molecular weight band in Fig. 5b corresponded to Fc protein, indicating that hEpo/Fc was partially digested to hEpo and Fc during the transport. A similar phenomenon was previously observed for scFv-Fc production by GM chickens and quails (Kamihira et al. 2005; Kawabe et al. 2006a), although the joint region sequence between scFv and Fc differed from that in the present study. Since such digestion was not observed in whole antibodies produced in the yolk of GM chickens (Kamihira et al. 2009), redesign of the joint region sequence is still necessary for efficient collection of intact Fc-fused proteins from yolk.

In our previous study, the structures of the oligosaccharide chains of hEpo produced in GM chickens differed between the hEpo proteins isolated from serum and egg white (Kodama et al. 2008). To identify the glycoforms of hEpo/Fc produced in the serum and eggs of GM chickens, lectin blotting was performed using sugar-specific lectins (Fig. 5c-e). All the hEpo/Fc samples derived from GM chickens reacted with ECA lectin (Fig. 5c), indicating that a terminal galactose was incorporated into the *N*-linked carbohydrates of hEpo/Fc. The MAM and SSA lectin blot analyses revealed that the serum- and CHO-derived hEpo/Fc proteins contained α2-3-linked sialic acid residues (Fig.

5d), while the CHO-derived hEpo/Fc did not contain α2-6-linked sialic acid residues (Fig. 5e). These results are consistent with a previous report that CHO cells are capable of α2-3-linked sialic acid incorporation into recombinant glycoproteins but incapable of α2-6-linked sialic acid incorporation (Grabenhorst et al. 1999). α2-6-linked sialic acid residues were detected in the oligosaccharide chains of the serum- and yolk-derived hEpo/Fc, whereas the sialylation of the egg white-derived hEpo/Fc was negligible (Fig. 5d, e). These results indicate that significant amounts of the oligosaccharide chains of the egg white-derived hEpo/Fc terminated with galactose, resulting in a lower molecular weight of the protein. Although it is assumed that the yolk-derived hEpo/Fc was transported from the serum, the sialylation level of the yolk-derived hEpo/Fc was not as high as that of the serum-derived hEpo/Fc, indicating that the yolk transport mediated by Fc receptors on the ovum may be selective and influenced by the structures of the oligosaccharide chains and/or the fusion partners of Fc-fused proteins. Zhu et al. (2005) reported recombinant antibody production in the egg white of chimeric chickens using an oviduct-specific ovalbumin promoter for antibody expression. The oligosaccharides of the egg white-derived recombinant antibody were mostly asialo-oligosaccharides terminated with *N*-acetyl glucosamine, and only a small portion of the oligosaccharides were terminated with galactose. We also reported the oligosaccharide structures of recombinant antibodies produced in the serum and eggs of GM chickens (Kamihira et al. 2009), and the oligosaccharides of recombinant antibodies purified from the serum and yolk contained sialyl-oligosaccharides. These results were consistent with the lectin blot analysis for hEpo/Fc produced by the GM chicken.

Next, the *in vitro* biological activity of hEpo/Fc was measured (Fig. 6).

Partially purified hEpo/Fc proteins produced in the serum, egg white and yolk of GM chickens were applied to a proliferation bioassay system involving Ba/F3 cells expressing the murine Epo receptor, in which the cell proliferation is directly dependent on the hEpo concentration (Ueda et al. 2000; Kodama et al. 2008). The yolk-derived hEpo/Fc exhibited an almost equivalent performance to the serum- and CHO-derived hEpo/Fc proteins, in contrast to the egg white-derived hEpo/Fc (Fig. 6). The EC_{50} values for the serum-, egg white-, yolk- and CHO-derived hEpo/Fc proteins were calculated to be 28.4, 0.89, 33.5 and 42.9 mIU/ml, respectively. It was reported that the carbohydrate content of hEpo is inversely related to its *in vitro* bioactivity based on receptor binding, and that this effect is entirely caused by inhibitory effects of sialic acid residues on the Epo-Epo receptor interactions (Elliott et al. 2003; Elliott et al. 2004). The biological characteristics of the yolk-derived hEpo/Fc were more similar to the characteristics of the serum-derived hEpo/Fc than to those of the egg white-derived hEpo/Fc, indicating that the yolk-derived hEpo/Fc was transported from serum.

In conclusion, hEpo/Fc produced in the serum of GM chickens was recovered from the yolk and retained some of the biological characteristics. If necessary, hEpo can be prepared from hEpo/Fc by digestion with papain. The data presented here suggest that transport of Fc fusion proteins to the yolk in transgenic chicken bioreactors may represent an attractive approach for the production of pharmaceutical glycoproteins containing more potent carbohydrate structures.

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Figure Legends

Fig. 1 Retroviral vectors. The provirus structures of MSCV/GΔAhEpo (**a**), MSCV/GΔAhEpoW (**b**), MSCV/GΔAhEpo/Fc (**c**) and MSCV/GΔAhEpo/FcW (**d**) are shown. LTR, long terminal repeat derived from mouse stem cell virus (MSCV); Ψ^+, virus packaging signal sequence derived from MSCV; GFP, green fluorescent protein gene; Pact, chicken β-actin promoter; hEpo, human erythropoietin gene; hEpo/Fc, hEpo gene joined with the Fc region gene derived from human IgG2; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element.

Fig. 2 Effects of the WPRE on the retroviral titer. Retroviral vectors encoding the hEpo and hEpo/Fc genes with or without the WPRE were transduced into NIH-3T3 cells. The retroviral titer was determined by the number of fluorescent cells. Statistical significance was evaluated by an unpaired Student's *t*-test. *$p<0.05$, vs. the absence of the WPRE.

Fig. 3 Long-term production of hEpo/Fc by GM chickens. The concentrations of hEpo/Fc in the serum (**a**) and eggs (**b**) of representative chickens were measured by ELISA.

Fig. 4 Western blotting of hEpo/Fc produced in the serum of GM chickens. Western blot analyses of serum samples were performed under reducing (**a**, **b**) and non-reducing (**c**, **d**) conditions using anti-hEpo (**a**, **c**) and anti-human IgG (Fc) (**b**, **d**) antibodies.

Fig. 5 Analysis of hEpo produced by GM chickens. The hEpo/Fc fusion proteins produced in the serum, egg white and yolk of GM chickens and recombinant CHO clones were purified using staphylococcal protein A-Sepharose beads. (**a**, **b**) Western blotting was performed using anti-hEpo (**a**) and anti-human IgG (Fc) (**b**) antibodies. (**c**-**e**) The oligosaccharide chains modified with terminal galactose, α2-3-linked sialic acid and α2-6-linked sialic acid residues were detected using ECA (**c**), MAM (**d**) and SSA (**e**) lectins, respectively. Lane 1, serum-derived hEpo/Fc; lane 2, egg white-derived hEpo/Fc; lane 3, yolk-derived hEpo/Fc; lane 4, Fc fragment protein from human IgG; lane 5, CHO-derived hEpo/Fc.

Fig. 6 *In vitro* biological activity of hEpo/Fc produced by GM chickens. Partially purified hEpo/Fc proteins derived from the serum, egg white and yolk of GM chickens were subjected to a proliferation bioassay involving Ba/F3 cells expressing the murine Epo receptor.

Table 1 Retroviral vector injection into chicken embryos

(a) Hatchability of virus-injected embryos

Exp. No.	Viral titer (IU/ml)	Hatchability	
		Injected	Hatched (%)
1	5.3×10^7	18	9 (50%)
2	1.1×10^7	18	4 (22%)
3	1.3×10^8	23	6 (26%)
4	2.2×10^8	15	2 (13%)
Total	–	74	21 (28%)

(b) List of representative chickens

Exp. No.	Chicken ID (Sex [M, male; F, female])	Injection volume (μl)	Injection stage[a]	Average of hEpo/Fc conc. (μg/ml)		
				Serum	Egg white	Yolk
1	#106 (F)	1.5	15	118±28	66±25	41±9
2	#210 (F)	1.5	14	53±20	16±8	21±4
	#212 (M)	3.5	15	37±11	–[b]	–[b]
	#215 (M)	3.5	15	35±9	–[b]	–[b]
3	#305 (M)	2.0	14	94±29	–[b]	–[b]
	#312 (F)	4.0	14	61±17	16±9	25±2
	#317 (F)	2.0	14	27±12	9±3	12±6
	#319 (M)	2.0	14	144±17	–[b]	–[b]
4	#402 (M)	0.5	14	139±15	–[b]	–[b]

[a] Developmental stage according to Hamburger and Hamilton (1951).

[b] Not available.

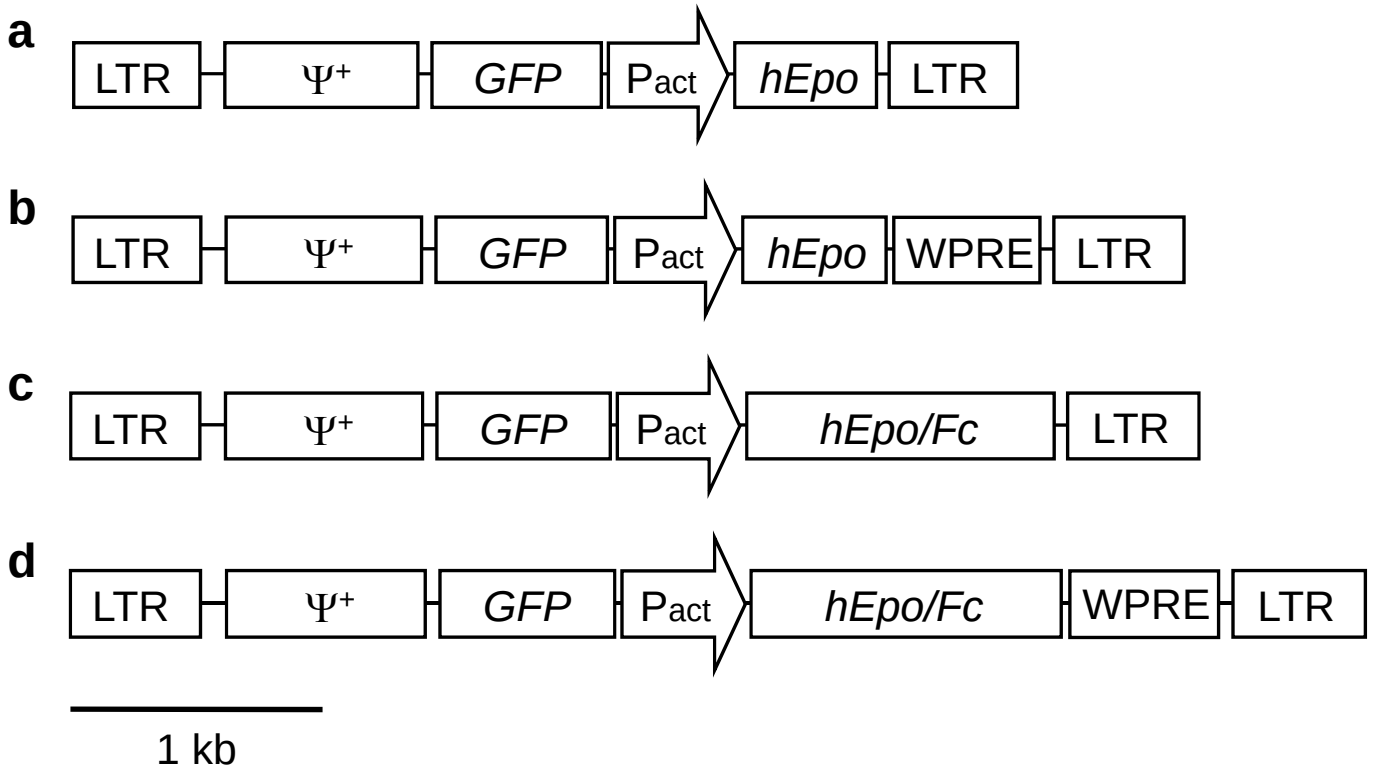

FIG. 1
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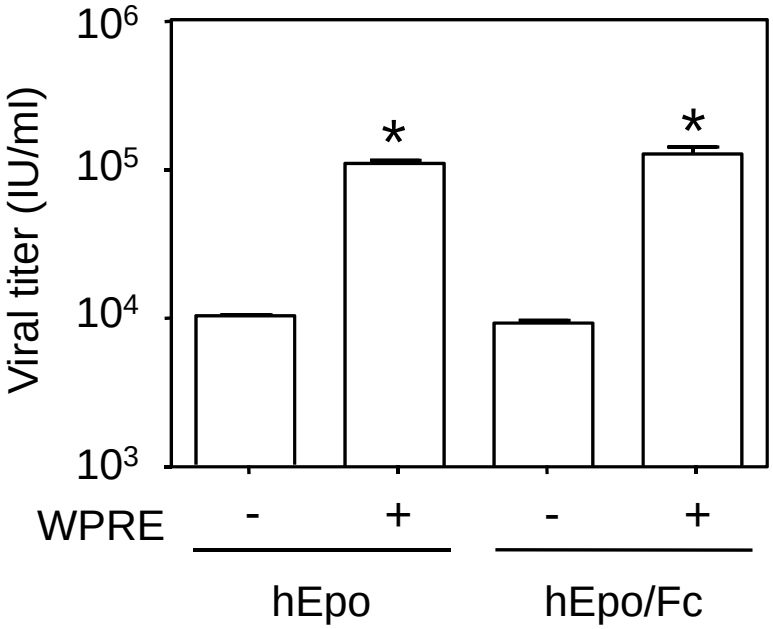

FIG. 2
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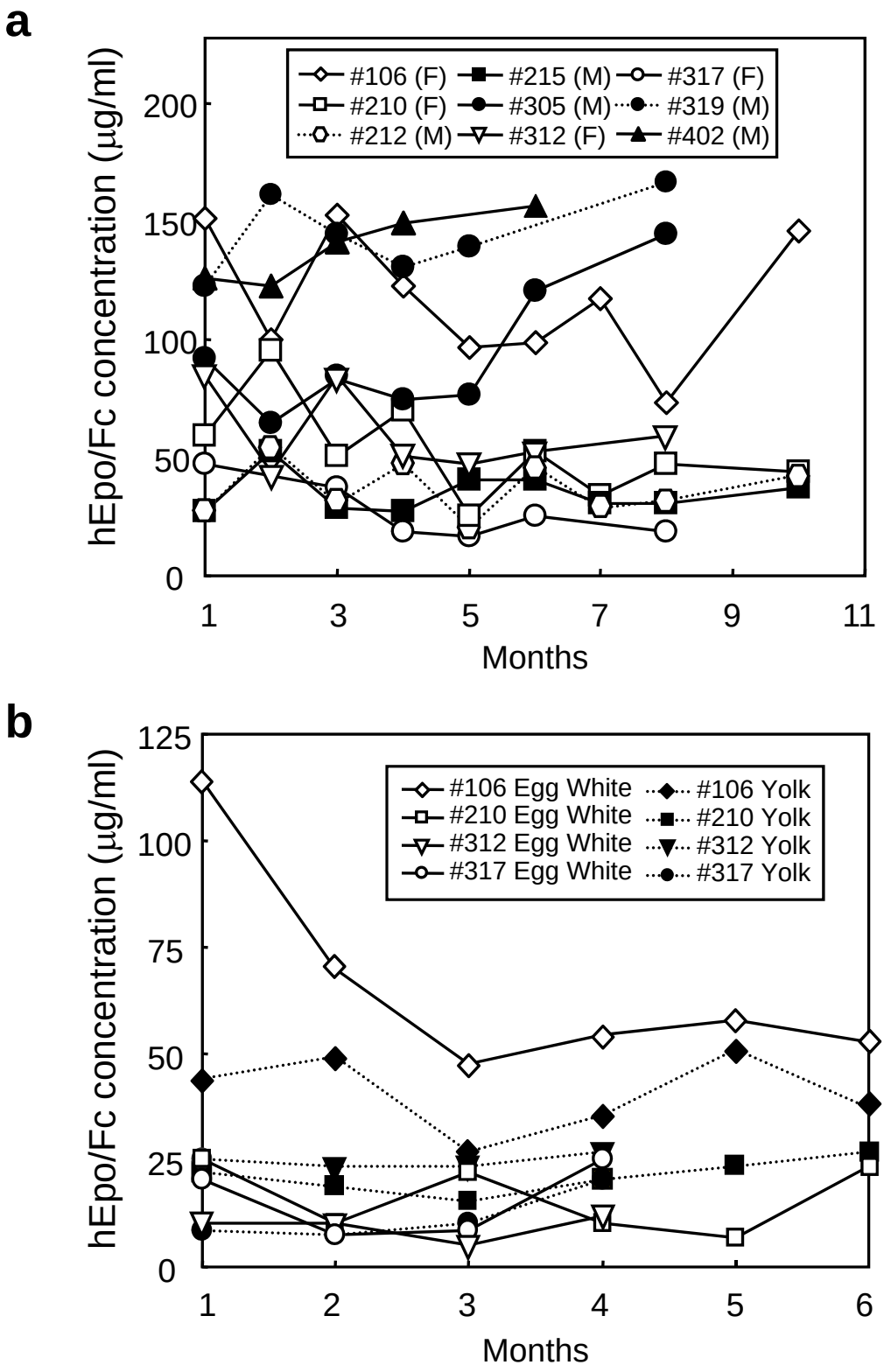

FIG. 3
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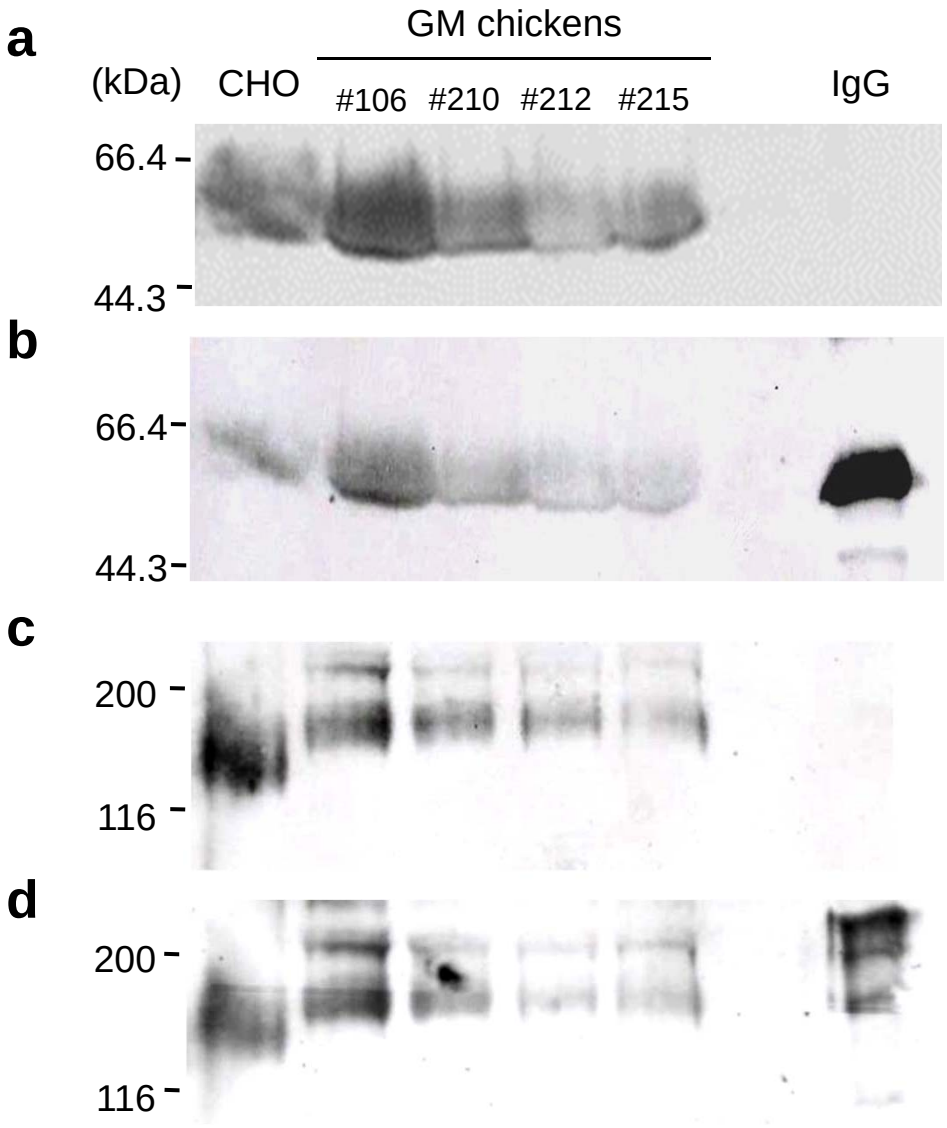

FIG. 4
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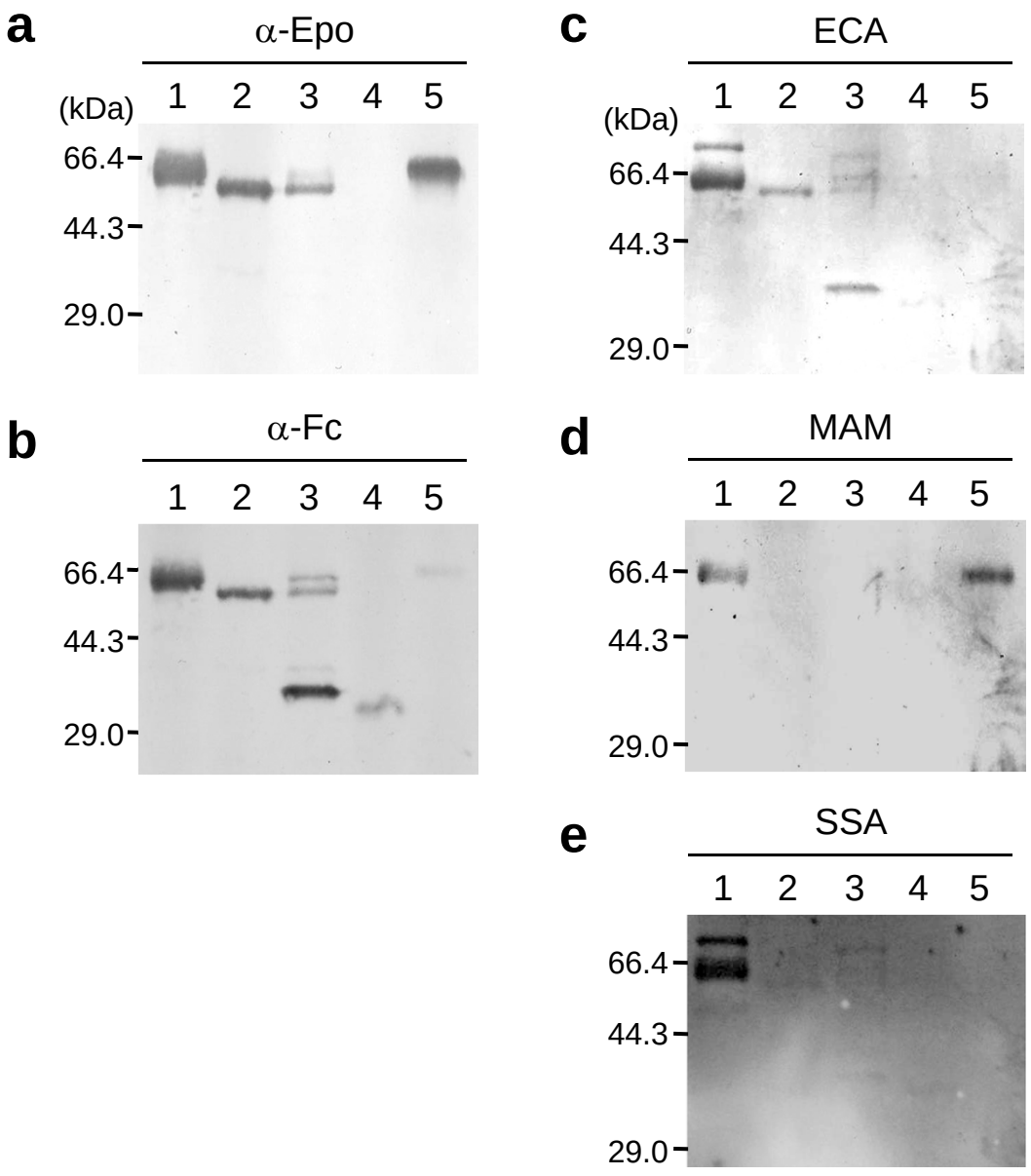

FIG. 5
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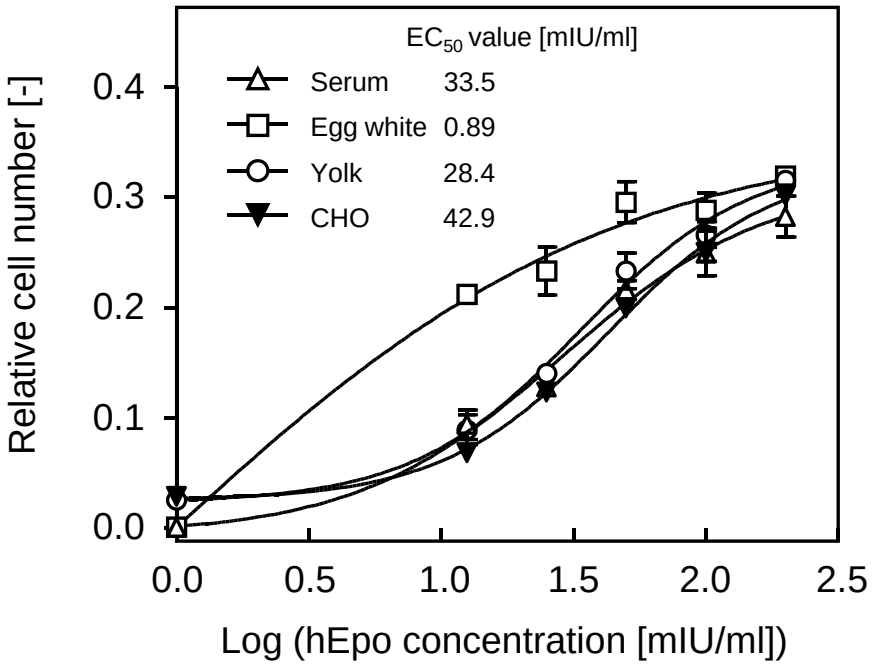

FIG. 6
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