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Production of human monoclonal antibody in eggs of chimeric chickens

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The tubular gland of the chicken oviduct is an attractive system for protein expression as large quantities of proteins are deposited in the egg, the production of eggs is easily scalable and good manufacturing practices for therapeutics from eggs have been established. Here we examined the ability of upstream and downstream DNA sequences of ovalbumin, a protein produced exclusively in very high quantities in chicken egg white, to drive tissue-specific expression of human mAb in chicken eggs. To accommodate these large regulatory regions, we established and transfected lines of chicken embryonic stem (cES) cells and formed chimeras that express mAb from cES cell–derived tubular gland cells. Eggs from high-grade chimeras contained up to 3 mg of mAb that possesses enhanced antibody-dependent cellular cytotoxicity (ADCC), nonantigenic glycosylation, acceptable half-life, excellent antigen recognition and good rates of internalization.

During the past two decades, 17 monoclonal antibody (mAb) therapeutic products have been approved by the US Food and Drug Administration, and it is likely that multiple mAbs will be approved annually in the next few years. Hence, the demand for the production of mAbs will increase at a rate that will tax the existing capacity of traditional production systems such as Chinese hamster ovary (CHO) or murine myeloma cells. In addition, the long timeline for and high cost of building large fermentation facilities make mammalian cell culture an expensive and time-consuming option. There have been numerous attempts to produce mAbs in plants[1], filamentous fungi[2] and mammals[3–5], but none of these systems can quickly produce a mAb that is appropriately glycosylated using an easily scalable procedure. Here we show that a mAb produced in the tubular gland cells of the chicken oviduct is secreted into egg white to yield eggs containing milligram quantities of the antibody. Using transgenes with large regions of regulatory sequence from the ovalbumin (Ov) locus, tissue-specific and developmentally regulated expression of the mAb is demonstrated. When compared with the same mAb produced from CHO cells, the mAb purified from egg white binds antigen with equal affinity, is internalized into cells expressing the antigen at the same rate, has enhanced ADCC and has an acceptable half-life in mice.

RESULTS

Construction of transgenes

We constructed two expression vectors, Ov7.5mAbdns and Ov15mAbF1, with 7.5 kb and 15 kb of the 5′ flanking region, respectively, and 15.5 kb of the 3′ flanking region from the ovalbumin gene controlling expression of the mAb (**Fig. 1a**). Both vectors contained all the known regulatory elements that are believed to be responsible for oviduct specificity and steroid induction of ovalbumin gene expression in laying hens[6–8]. In addition, the expression vectors were designed and constructed in such a way that unique V genes from a mAb can be easily inserted into the vectors. In the experiments described below, a chimeric human-mouse mAb specific for the dansyl hapten, which was designated mAbdns, and a fully human mAb specific for prostate-specific membrane antigen, which was designated mAbF1, were used to demonstrate the production of mAb in eggs from chimeras.

Establishment of cES cell lines carrying OV expression vectors

The Ov7.5mAbdns construct was stably transfected into a line of male cES cells. One clone (OVA) out of six was identified by PCR to contain the mAb cassette (data not shown). Analysis by Southern blot indicated integration of more than one copy of the transgene into the cES cell genome (**Supplementary Fig. 1** online).

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The Ov15mAbF1 construct was stably transfected into a female cES cell line. One clone (OVH) was shown by Southern blot analysis to contain one integrated copy of at least 10 kb of the 5′ ovalbumin promoter sequences (**Supplementary Fig. 1** online).

Production of chimeras

A total of 398 Stage X (EG&K) embryos were injected with the OVA cell line and 97 survived to day 14 of incubation, when chimerism was assessed by feather pigmentation. Sixty-seven of the 97 embryos (69%) were chimeric, and 70 embryos (18% of the Stage X (EG&K) that were injected with cES cells) hatched.

A total of 593 Stage X (EG&K) embryos were injected with the OVH cell line and 218 survived to day 14 of incubation. At this time, 167 of the 218 embryos were chimeric (77%) and embryos that were judged to have in excess of 40% black plumage were hatched. Eighty-three chicks were hatched, yielding a rate of 14% high-grade chimeras from the 593 Stage X (EG&K) embryos that were injected.

The contribution of cES cells to chimeras was also estimated by histological examination of enhanced green fluorescent protein (EGFP) expression in the magnum of the chimeras. In some chimeras, extensive contributions to both the tubular gland cells and the adjacent epithelial lining of the magnum were revealed (**Fig. 1**). Although high-grade chimeras could be made with male cES cells (see **Supplementary Fig. 2** online) they masculinized female chimeras, and egg production was restricted to birds carrying small contributions from the cES cells. By contrast, female cell lines (e.g., OVH) produced high-grade, egg-laying chimeras (**Table 1**); two examples of these birds are shown in **Supplementary Figure 2** online.

The contribution of cES cells to the germ line was assessed by mating male and female chimeras to Barred Plymouth Rocks. From this cross, contributions to the germ line would be evident by the production of black chicks. Black chicks were not observed among 8,862 offspring from 20 chimeras, indicating that the cells did not contribute to the germ line under the conditions of these experiments (**Supplementary Table 1** online).

Production of mAb using the Ov7.5mAbdns construct

Tissue-restricted and hormonally induced expression in chimeras carrying the Ov7.5mAbdns were revealed in six female chimeras (OV10-17, OV10-41, OV10-55, OV10-66, OV10-79 and OV10-102) that were injected for 10 d with 1 mg/day of 17-β-estradiol beginning at 12 d of age. After 10 d the magnum portions of the oviducts (where the tubular gland cells are located) were collected and observed macroscopically for EGFP expression (the gene encoding EGFP driven by a ubiquitous promoter was included in the Ov7.5mAbdns vector to allow identification of the cES cell–derived cells in chimeras). RT-PCR was conducted with primers located in the signal peptide and 3′ of the constant region of the heavy (H)- and light (L)-chain transcripts (**Supplementary Table 2** online). Out of the five magnum samples in which EGFP expression was observed macroscopically, L- and H-chain transcripts were identified in three: OV10-17, OV10-66 and OV10-79 (**Fig. 1b**). However, as we had observed in DT-40 cells (see Methods),

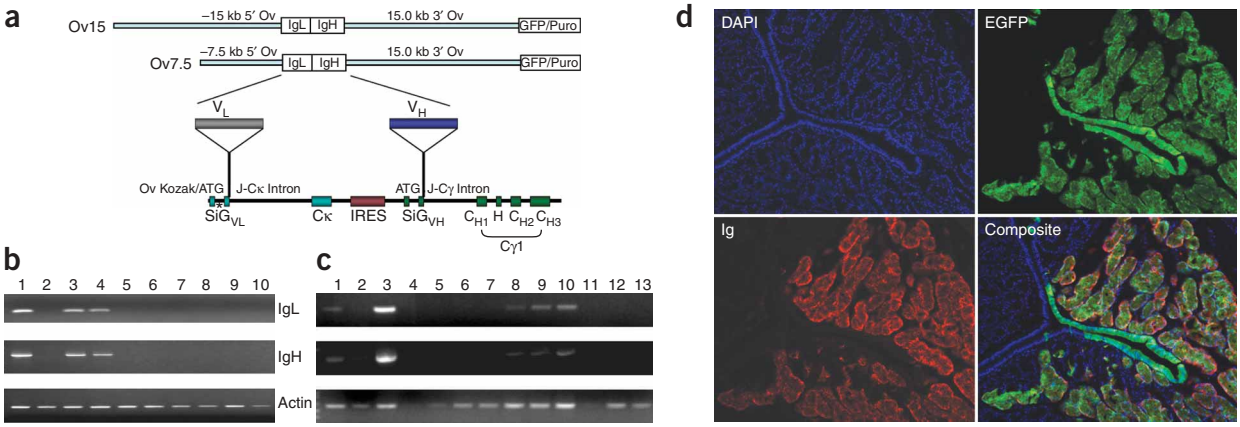

Figure 1 Tissue-restricted expression of mAb from Ov7.5mAbdns and Ov15mAbF1 vectors. (**a**) Diagram of the Ov7.5mAbdns and Ov15mAbF1 expression vectors. Positions of the 5′ and 3′ regulatory sequences of ovalbumin are indicated. Included in the 3′ end of the vector is a cassette with both *EGFP* and *puromycin* (*Puro*) driven by a promoter (*Cx*) that functions in all cell types to facilitate the isolation of stably transfected lines of cES cells and the identification of the cES cell contribution in the chimeras. Details of the mAb cassette are illustrated in the lower part of the diagram. The thin black line represents intron sequences or nontranslated sequences derived from the human L and H chains. SiG_{VL}, sequence of the signal peptide of the L chain; VL, sequence of the V gene of the L chain; Cκ, sequence of the constant region of the Kappa L chain; IRES, internal ribosomal entry sequence; SiG_{VH}, sequence of the signal peptide of the H chain; VH, sequence of the V gene of the H chain; C_{H1}, H, C_{H2} and C_{H3}, coding sequences of the C_H1, Hinge, C_H2 and C_H3 domains of the gamma1 H chain. *intron sequence present in mAbdns but not in mAbF1. (**b**) RT-PCR of EGFP-positive tissue samples from estrogen-induced Ov7.5mAbdns chimeric chicks for IgH, IgL and β-actin. Lanes: 1, OV10-17 magnum; 2, OV10-55 magnum; 3, OV10-66 magnum; 4, OV10-79 magnum; 5, OV10-102 magnum; 6, OV10-55 gut; 7, OV10-79 pancreas; 8, OV10-79 brain; 9, OV10-102 brain; 10, OV10-102 leg muscle. (**c**) RT-PCR of EGFP-positive tissue samples from estrogen induced Ov15mAbF1 chimeric chicks for IgH, IgL and β-actin. Lanes: 1, OV15-34 magnum; 2, OV15-76 magnum; 3, OV15-83 magnum; 4, OV15-121 magnum; 5, OV15-76 stomach; 6, OV15-76 duodenum; 7, OV15-76 jejunum; 8, OV15-76 ileum; 9, OV15-76 cecum; 10, OV15-76 colon; 11, OV15-111 brain; 12, OV15-111 liver; 13, OV15-34 muscle. Note the detection of both full-length L and H transcripts. Although the β-actin transcript was weak in OV15-111, the conclusion that Ig was not expressed in brain was supported by the absence of Ig expression with a strong β-actin transcript in a brain sample from OV14-53 (data not shown). (**d**) Frozen sections of the magnum portion of the oviduct from an estrogen-induced chimeric female chick produced from cES cells transfected with Ov7.5mAbdns. Images of a section under DAPI staining showing the location of the cells, EGFP fluorescence showing the contribution of cES cells in chimera, human Ig Ab staining showing the transgene expression and composite of all three images. Note the restricted transgene expression in donor cES cell–derived tubular glands (arrow) but not in epithelial cells (arrowhead).

Table 1 Human IgG in eggs from Ov15mAbF1 chimeras

Mean of mAb/egg (mg)	Number of hens	Mean extent of chimerism (%)	Range of [mAb] in egg (μg/ml)	Range of mAb/egg (mg)
>1.0	4	93	34.6–147.9	0.7–3.4
>0.5 to 1.0	6	97	9.0–61.6	0.2–1.4
>0.1 to 0.5	7	79	3.3–23.2	0.1–0.6
>0.01 to 0.1	13	75	0.2–5.4	0.01–0.1

The mean value of mAb/egg from each hen was an average of values from 2 to 10 eggs. The extent of chimerism was scored by comparing the extent of black down relative to that of the Barred Plymouth Rock breed from which chicken ES cells were derived.

the L-chain transcript was aberrant, with the leader sequence directly spliced to Cκ. In two samples (OV10-55 and OV10-102), EGFP expression was not observed in magnum tissue but only in associated connective tissue and muscle, and RT-PCR failed to amplify either L- or H-chain transcripts.

To determine whether the transgene expression was tissue specific, we collected tissues derived from cES cells (as determined by the presence of EGFP expression macroscopically), including brain, pancreas, gut and muscle, from the estrogen-induced female chimeras and conducted RT-PCR as described above. None of these tissues showed detectable expression of L or H chains (**Fig. 1b**). Taken together, these results indicated that the ovalbumin regulatory sequences present in the Ov7.5mAbdns expression vector were sufficient to direct tissue-restricted expression of the mAb transgene.

The magnum samples from two of the chimeras (OV10-17 and OV10-66) were analyzed to determine if the mAb transgene was correctly expressed in EGFP-positive cES cell–derived tubular gland cells by immunohistochemical staining with rabbit anti-human IgG. EGFP-positive tubular gland cells derived from donor cES cells were positive for mAb, indicating that the transgene was being both transcribed and translated (**Fig. 1d**). As additional evidence for tissue specificity, mAb protein production was detected only in the cES cell–derived (that is, EGFP-expressing) tubular gland cells (**Fig. 1d**, arrow) but not in the adjacent cES cell–derived epithelial cells (**Fig. 1d**, arrowhead). Detection of the human mAb protein in EGFP-positive tubular gland cells indicates that the oviduct-specific regulatory sequence from the ovalbumin gene was functional and drove tissue-restricted and hormonally induced production of the human mAb in chimeras.

Deposition of human mAb in eggs from Ov7.5mAbdns chimeras

Egg white samples from the nine chimeras carrying the Ov7.5mAbdns transgene and White Leghorn hens (negative controls) were prepared by ammonium sulfate precipitation and analyzed by enzyme-linked immunosorbent assay (ELISA). No mAb deposition was detectable in eggs from White Leghorn hens or from six chimeric hens (OV11-17, OV11-53, OV11-73, OV11-88, OV12-97 and OV13-13). Low levels of mAb were deposited in eggs from three different chimeric hens (~1.4–6.3 ng/ml for eggs from hen OV11-13, ~2.0–2.9 ng/ml for eggs from hen OV11-37 and ~2.9–10.8 ng/ml for eggs from hen OV11-43 as determined by ELISA for human IgG).

Taken together, the data from estrogen-induced chimeras and from the mature chimeric hens show that the Ov7.5mAbdns vector delivered tissue-restricted, hormonally induced and developmentally regulated gene expression in chimeric hens, and the transgene product was exported from the tubular gland cells and deposited in egg white. However, the concentration of mAb in eggs from these hens was very low (1.4–10.8 ng/ml). Evaluation of donor contribution in these chimeras revealed extremely low cES cell contribution (0–0.1%) to the tubular gland cells where the transgene expression was confined, which was probably the main reason for the low levels of expression in eggs. Because the transgene was transfected into a male cES cell line, chimeras with high donor cell contributions were masculinized, yielding hens that did not produce eggs. Therefore, production of mAb could only be evaluated in low-grade chimeras. In addition, the L-chain mRNA was inappropriately spliced into a molecule that did not contain the variable region.

Production of mAb using the Ov15mAbF1 construct

Tissue-restricted and hormonally induced expression of the Ov15mAbF1 transgene was demonstrated by injecting 12-d-old female chimeras made with the OvH clone with 1 mg/day of 17-β-estradiol for 10 d. Donor cES-derived areas that contain EGFP-positive (that is, cES cell–derived) cells and wild-type (that is, recipient-derived) cells

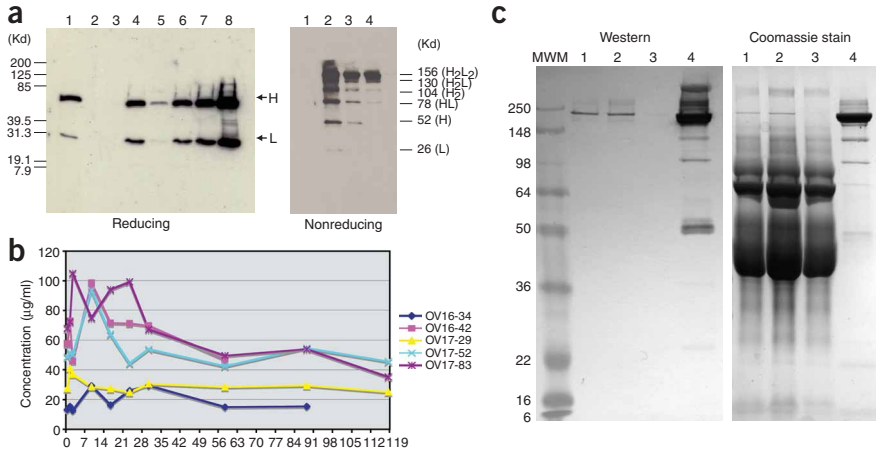

Figure 2 Deposition of mAbF1 into eggs from Ov15mAbF1 chimeras. (**a**) Western blot analysis of egg white samples from Ov15mAbF1 chimeric hens under reducing (left) and nonreducing conditions (right). Left gel lanes: 1, 4 ng of purified human IgG1, κ (Sigma) as a positive control; 2, MW marker; 3, egg white from a wild-type White Leghorn hen as negative control; 4, egg white from OV15-17; 5, egg white from OV15-29; 6, egg white from OV15-71; 7, egg white from OV16-34; 8, egg white from OV16-42. Right gel lanes: 1, egg white sample from a wild-type White Leghorn hen; 2, egg white sample from OV16-42; 3, egg white sample from OV16-34; 4, human IgG1, κ control (8 ng). Note the presence of L and H chains of the correct size in all samples. (**b**) Concentration of mAb in eggs from representative chimeric hens during the first 120 d after the first oviposition. At each time point, the mean values were calculated from at least two independent preparations of egg white that were assayed in two independent ELISA assays. (**c**) Purification of mAbF1 from egg white. Lanes: 1, starting material; 2, protein A load; 3, protein A flow through; 4, protein A elute. Both Coomassie stain and western blot analysis are under nonreducing conditions. HRP-labeled goat anti-human IgG was used for detection in the western blot analysis.

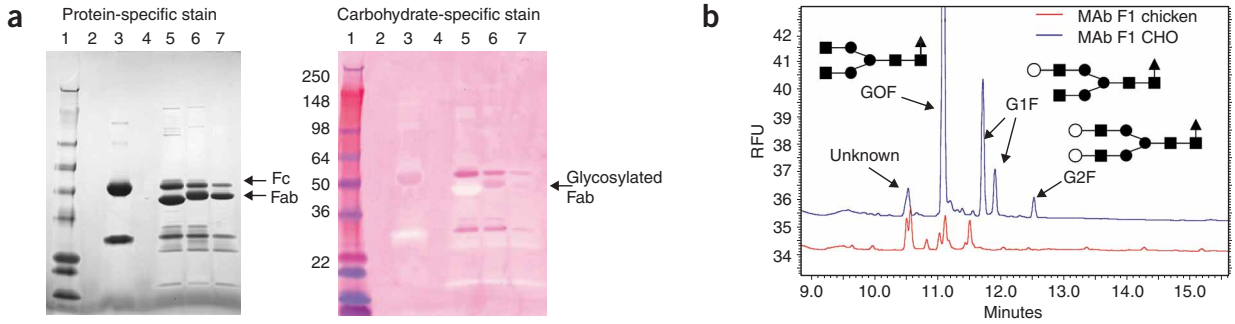

Figure 3 Glycosylation of mAbF1 protein produced in chicken tubular gland cells. (**a**) Analysis of Fab glycosylation. SDS-PAGE with protein-specific staining showing both Fc and Fab fragments (left). Western blot with a carbohydrate-specific stain showing that both Fc and Fab are glycosylated (right). Lanes: 1, MW standard; 2, empty; 3, anti-CTLA4 mAb control, reduced; 4, empty; 5, anti-CTLA4 mAb control papain digest, nonreduced; 6, mAbF1 CHO papain digest, nonreduced; 7, mAbF1 chicken papain digest, nonreduced. The anti-CTLA4 mAb is only glycosylated in the Fc region. (**b**) Oligosaccharide profiles of mAbF1 produced in chicken tubular gland cells and in CHO cells. APTS-labeled oligosaccharides were separated by capillary electrophoresis with LIF detection. Known glycan structures of the CHO-produced mAb are indicated. Glycan structures were identified based on the retention time of oligosaccharide standards; glycan structures were also confirmed by MS analysis. Mannose is shown as a black circle; galactose is shown as an open circle; N-acetyl glucosamine is shown as a black square; fucose is shown as a black triangle.

from various tissues, including the magnum portion of the oviduct, muscle, brain, liver and gut, were collected. RT-PCR with primers specific for L and H chains were used to monitor expression of the transgene. L- and H-chain transcripts were detected in the magnums from two out of four estrogen-induced female chicks (OV15-34, OV15-76 and OV15-83) but not in muscle or liver (**Fig. 1c**). No conclusion can be drawn from lane 11 (**Fig. 1c**, brain) as no β-actin amplification was observed, although a brain sample from another chick (OV14-53) with strong expression of β-actin did not express L and H chains (data not shown). Low-level expression was detected in ileum, cecum and colon (**Fig. 1c**). Immunohistochemistry of sections from estrogen-induced magnums confirmed the expression of mAb in EGFP-positive tubular gland cells but not in EGFP-positive epithelial cells (data not shown). Taken together, these data demonstrate that the Ov15mAbF1 vector directed transgene expression in the tubular gland cells of the oviduct. The low-level ectopic expression detected in the gut suggests that 10–15 kb of the 5′ regulatory sequences of the ovalbumin gene may not be sufficient to confer fully tissue-specific expression of the transgene. This may be due to a lack of regulatory sequences or a positional effect of the transgene integration site.

Deposition of human mAb in eggs from Ov15mAbF1 chimeras

Sixty-nine chimeras were generated from OVH cES cells carrying the Ov15mAbF1 to evaluate the deposition of the fully human mAb into eggs. Thirty chimeric hens were raised to sexual maturity and commenced laying. Eggs from all of the hens contained mAb in their egg white, as indicated by ELISA analysis. The concentration and amount of mAb in eggs varied among the chimeric hens, presumably owing to the varying degree of chimerism in the magnum (**Table 1**).

Table 2 Monosaccharides in chicken and CHO-produced mAbF1

Monosaccharide	CHO HumAb pmol (% total)	Chicken HumAb pmol (% total)
Fucose	692 (18)	0
Glucosamine	1,536 (40)	1,571 (52)
Galactose	671 (17)	43 (1)
Mannose	940 (25)	1,513 (47)
Total	3,839 (100)	3,127 (100)

Eight chimeric hens laid eggs that contained >1 mg of mAb per egg, and the maximal level among the 30 chimeras was 3.4 mg of mAb per egg. Four chimeric hens laid eggs whose mean level of mAb was between 1.2 and 1.6 mg per egg. Six chimeric hens laid eggs whose mean level of mAb was between 0.5 and 1.0 mg per egg.

The presence of mAb in egg white was further analyzed by western blot with a rabbit anti-human IgG (H+L) that recognizes both the H and L chains. Full-length L and H chains were detected in egg white

Table 3 Carbohydrate composition of mAbF1 in eggs

Observed mass + Na^+	Theoretical mass + Na^+	Composition	Possible structure
1136.4	1136.5	$(Man)_3(GlcNAc)_3$	
1257.4	1257.6	$(Hex)_2(Man)_3(GlcNAc)_2$	
1298.4	1298.44	$(Hex)(Man)_3(GlcNAc)_3$	
1339.46	1339.5	$(Man)_3(GlcNAc)_4$	
1460.49	1460.5	$(Hex)_2(Man)_3(GlcNAc)_3$	
1501.53	1501.5	$(Hex)(Man)_3(GlcNAc)_4$	
1542.56	1542.6	$(Man)_3(GlcNAc)_5$	
1663.6	1663.58	$(Hex)_2(Man)_3(GlcNAc)_4$	or
1704.6	1704.6	$(Hex)(Man)_3(GlcNAc)_5$	
1745.66	1745.6	$(Man)_3(GlcNAc)_6$	
1866.7	1866.7	$(Hex)_2(Man)_3(GlcNAc)_5$	
1948.7	1948.68	$(Man)_3(GlcNAc)_7$	Not determined

Man, mannose, shown as black circle; Hex, hexose (mannose or galactose), shown as open circle; GlcNAc, N-acetyl glucosamine, shown as black square. The vertical line indicates that the last hexose could be connected to any one of Man or GlcNAc residues along the line.

samples from five chimeric hens (OV15-17, OV15-29, OV15-71, OV16-34 and OV16-42) (**Fig. 2a**). Fully assembled H_2L_2 was also seen, although small amounts of assembly intermediates were also present (**Fig. 2a**).

To determine the variation of the mAb concentration deposited in egg white, we monitored eggs from five different chimeric hens (OV16-34, OV16-42, OV17-29, OV17-52 and OV17-83) over a period of 3–4 months. In three hens, the amount of mAb peaked during the first few weeks, then dropped approximately twofold and remained stable over the next few months (**Fig. 2b**). In two hens, the expression level remained relatively stable over the time course (**Fig. 2b**). If not all of the tubular glands secrete and deposit ovalbumin protein at the same time as the egg is passing through the magnum portion of the oviduct, the amount of mAb deposited in the egg white could vary owing to the chimeric nature of the tubular glands in these hens.

Blood samples from 26 chimeric hens and a Barred Plymouth Rock hen (negative control) were analyzed by ELISA for the presence of human IgG. Eighteen hens had detectable levels of mAb in blood with the highest concentration of 39 ng/ml observed in hen OV17-83. The correlation coefficient (r) between the level of mAb in blood and the concentration of mAb in egg white was 0.67.

Characterization of mAb produced in tubular gland cells

MAb was purified from egg white as described in Methods. Most of the purified material was a fully assembled H_2L_2 with a purity >90% (determined by ELISA and A280) (**Fig. 2c**). The mAbF1 produced in chicken was compared in several assays with mAbF1 produced in conventional CHO cell culture. Size-exclusion high-performance liquid chromatography (SEC-HPLC) analysis showed that both were >90% IgG monomer. Sequence analysis by Nano liquid chromatograph-tandem mass spectrometry (LC-MS/MS) showed no sequence difference (**Supplementary Table 3** online). Liquid chromatograph/mass spectrometric (LC/MS) analysis of the L chain showed that both had identical mass (±3 Da.). In addition, capillary isoelectric focusing analysis indicated that both had similar isoelectric points, although CHO-derived mAbF1 had greater charge heterogeneity than chicken-derived mAbF1 (**Supplementary Fig. 3** online). The thermal stabilities of mAbF1 produced in the two systems were obtained using differential scanning calorimetry (DSC) and compared with corresponding forms that were deglycosylated in their Fc domain[9]. The unfolding profiles of the CHO- and chicken-derived antibodies were quite different, whereas the unfolding profiles of the Fc-deglycosylated antibodies were almost identical (**Supplementary Fig. 4** online). These data suggest that glycosylation of mAbF1 produced in chicken tubular gland cells confers greater thermal stability than the carbohydrate residues attached to mAbF1 produced in CHO cells.

Glycosylation of mAb produced in chicken

We compared glycosylation of mAbF1 produced in our chicken expression system and in CHO cells. MAbF1 produced in both systems was glycosylated in the Fab and Fc regions, whereas a control anti-CTLA4 expressed in CHO cells was glycosylated only in the Fc region (**Fig. 3a**). However, the oligosaccharide profiles were quite different, as shown by capillary zone electrophoresis (**Fig. 3b**) and matrix-assisted laser desorption ionization/mass spectrometry (MALDI-MS) (data not shown). Monosaccharide analysis of chicken- and CHO-produced mAbF1 revealed a difference in carbohydrate composition (**Table 2**). Exoglycosidase analysis of glycans by CE and mass spectrometry to identify the terminal sugar residues confirmed the absence of terminal sialic acid and fucose residues in mAbF1 produced in chicken tubular gland cells. Monosaccharide analysis also showed the presence of N-acetyl glusosamine residues, mannose

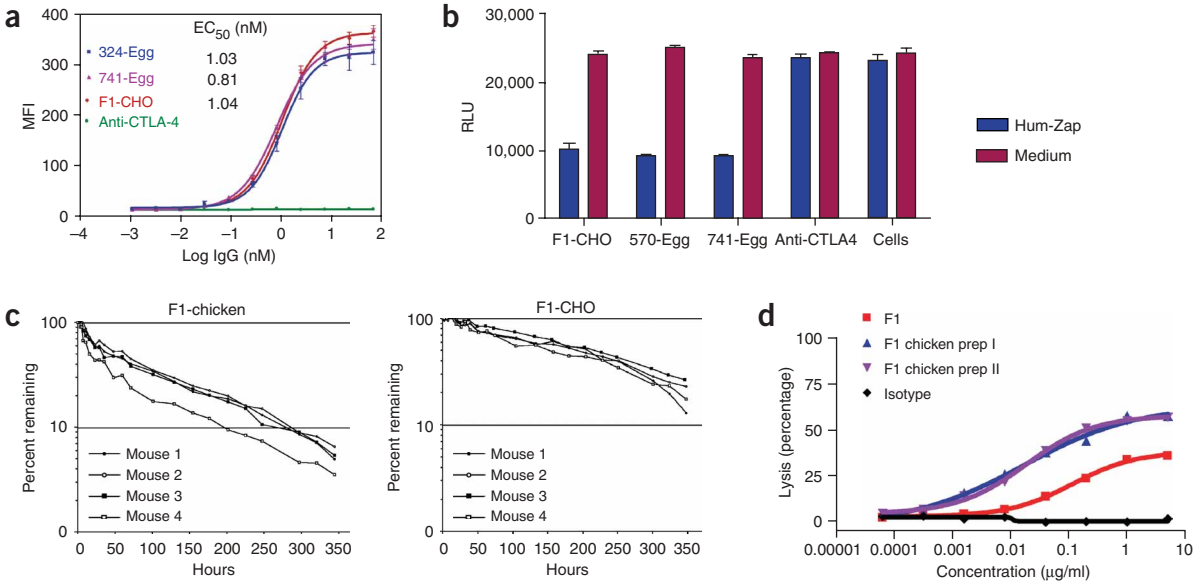

Figure 4 Biological activities of mAbF1 produced by chicken tubular gland cells in comparison to that produced by CHO cells. (**a**) Binding of chicken or CHO cell produced mAbF1 to PSMA expressed on LNCaP cells. 324-Egg and 741-Egg are two different preparations of mAbF1 isolated from eggs. F1-CHO is mAbF1 produced by CHO cells. Anti-CTLA4 is a human IgG1 control mAb not recognizing antigen on LNCaP cells. (**b**) Internalization assay of mAbF1 produced in chicken tubular gland cells and CHO cells. 570-Egg and 741-Egg were two preparations of mAbF1 isolated from eggs. 300 ng/well each of mAb was added either with Hum-Zap (anti-human IgG, Saporin conjugate) or cell culture medium to LNCaP Cells. Cell viability was determined 48 h after addition of mAb and Hum-Zap. The *y*-axis is relative luminescence units (RLU). (**c**) *In vivo* clearance of mAbF1 produced in chicken tubular gland cells (F1-chicken) and CHO cells (F1-CHO). Four BALB/c mice were injected with each radiolabeled antibody preparation. The residual radioactivity was measured over a period of 350 h using a whole-body counter. Data are expressed as the percentage of the injected dose remaining. (**d**) ADCC assay of mAbF1 produced in chicken tubular gland cells (F1 chicken prep I and II) and CHO cells (F1). A human IgG1 not recognizing antigen on LNCaP cells was used as isotype control.

residues and very low content of galactose residues in mAbF1 produced in chicken. The carbohydrate composition and possible glycosidic linkages of 11 major glycans in chicken-derived mAbF1 were analyzed by MALDI time-of-flight analysis (TOF)-MS (**Table 3**). The oligosaccharide structures contained high-mannose type, complex type and hybrid type N-glycans.

MAb produced in chicken tubular gland cells is biologically active

We compared the antigen binding property of mAbF1 produced in chicken tubular gland cells and in CHO cells. Both antibody preparations produced nearly identical binding curves to prostate-specific membrane antigen (PSMA) expressed on LNCaP cells LNCaP (ATTC ref. no. CRL 1740) with similar effector concentration for half-maximum response (EC_{50}) values (**Fig. 4a**). The data demonstrate that even though the chicken- and CHO-derived antibodies are glycosylated differently, they recognize and bind antigen equivalently.

Binding of mAbF1 to PSMA leads to internalization of the antibody[10]. In one potential application, mAb could be conjugated with cytotoxins to target and destroy PSMA-expressing tumor cells. Therefore, the internalization of chicken-derived and CHO-derived mAbF1 was evaluated with the Hum-Zap assay. Cells are killed when the mAbF1/Hum-Zap complex binds to PSMA on the cell surface and is internalized, whereas antibody or Hum-Zap alone is not toxic to cells. Both antibody preparations internalized with a similar efficiency (**Fig. 4b**). When tested over a range of antibody concentrations, the EC_{50} values for internalization of both the chicken-derived and CHO-derived mAbF1 were 0.49 nM.

The *in vivo* serum half-life of chicken-produced and CHO-produced mAbF1 was analyzed in BALB/c mice by intravenous injection of radiolabeled antibodies. Residual radioactivity was determined over time by whole-body counting. MAbF1 produced by chicken tubular gland cells cleared with a half-life ($t_{1/2}$) of 102.4 ± 0.9 h, whereas mAbF1 produced by CHO cells cleared more slowly, with a half-life of 207.5 ± 18.3 h (**Fig. 4c**).

The ADCC activities of the mAbs were compared in a modified ^{51}Cr ADCC assay. CHO-derived mAbF1 induced a dose-dependent cell lysis, which reached a plateau at 38% lysis with an EC_{50} of 0.11 μg/ml (**Fig. 4d**). In contrast, the maximum ability of mAbF1 derived from chicken eggs to lyse target cells was increased to 60% and the EC_{50} was 0.018 μg/ml.

DISCUSSION

We have shown that cES cells transfected with a transgene designed to express a human mAb can be used to derive chimeric chickens whose eggs contain fully functional antibody. This work demonstrates the potential for producing proteins in chicken eggs as a viable alternative to mammalian cell culture systems. The introduction of genetic modifications via cES cells overcomes the limitation of viral expression systems on the size of the foreign DNA that can be introduced (~8 kb)[11–17]. We show here that 41- to 49-kb transgenes can be readily introduced into cES cells; recent studies at Origen suggest that the amount of 5′ and 3′ sequence can be increased to 125 kb and 25 kb, respectively. Thus, large portions of regulatory sequence can be included to further increase transgene expression levels in a tissue-restricted, hormonally induced and developmentally regulated manner.

To accommodate large regulatory regions, we have extended cES cell technology by maintaining the cells indefinitely, developing a system for transfecting the cells with large transgenes and demonstrating that the genetically modified cells retain the ability to make somatic chimeras. Previously, somatic chicken chimeras had not been made from cells cultured for more than 3 weeks[18,19], and insertion of transgenes required a promoter trap strategy[20]. In addition, tissue-restricted expression from a tissue-specific promoter had not been demontsrated in any *in vivo* chicken expression system. Although we observed low-level expression of the ovalbumin transgene in the gut of Ov15mAbF1 chimeras, expression was largely tissue specific and restricted to the tubular gland cells in the oviduct. The ectopic expression suggests that more than 10–15 kb of 5′ sequence and 15 kb of 3′ sequence are required to ensure tissue specificity regardless of the site of integration. However, integration into several distinct locations within the genome has yielded tissue-restricted expression with as little as 7.5 kb of 5′ sequence (Ov7.5mAbdns; L.Z. *et al.*, unpublished data). Detection of human mAb in EGFP-positive tubular gland cells using both the Ov7.5mAbdns and Ov15mAbF1 expression vectors indicates that the regulatory sequences from the ovalbumin gene are functional and can drive tissue-specific production of the human mAb in chimeras. The transgene products are deposited into egg white at a concentration of 1–3 mg per egg, which is 20- to 30-fold higher than previously reported with non-tissue-specific, virus-derived promoters and enhancers[16]. In addition, the expression system described here has been designed to allow rapid insertion of unique V sequences for production of different mAbs.

MAb produced in the chicken oviduct and in CHO cells had very similar physical characteristics. Mass spectrometric sequence analysis showed no sequence difference. Both chicken tubular gland cells and CHO cells can glycosylate the antibodies at the conserved Asn297 site in the C_H2 domain and at a site in V_H. However, the N-linked oligosaccharide profiles were different. The most striking differences were the presence of high-mannose type N-glycans in the Fc region (Asn297), the absence of fucose and the very low content of galactose residues in the chicken-produced antibody. Despite the difference in glycosylation, the chicken- and CHO-produced mAbs had nearly identical binding curves and similar affinities, and were internalized equally by antigen on LNCaP cells. MAb produced in the chicken oviduct showed a shorter serum half-life in mice compared with that produced in CHO cells, presumably because of the increased content of high-mannose type N-glycans. However, the pharmaceutical potential of the mAb produced in chicken tubular gland cells remains to be evaluated in primates and ultimately in humans. The DSC data indicate that the differences in thermal stabilities of the CHO- and chicken-derived mAbs are primarily due to the different glycosylation patterns present in their C_H2 domains. Of the several melting transition points, the first one (T_{m1}) is critical as it determines the overall stability of the antibodies at lower temperatures. The difference in T_{m1} of chicken-derived antibody (63.8 °C) compared with CHO-expressed antibody (62.7 °C) predicts that the former should be more stable under the typical storage conditions of therapeutic mAbs.

The absence of α1-6 fucose residues on the Asn297 linked N-acetyl glucosamine residue of human immunoglobulins substantially increases ADCC[21–24]. Although the immunoglobulin molecules produced in B cells of birds possess α1-6 fucose residues[25,26], the α1-6 fucose linkage is absent from the major egg white proteins[27–33]. Similarly, the Asn297-linked N-acetyl glucosamine of mAbF1 is not fucosylated when produced in the chicken tubular gland cells and this property is associated with an increase in ADCC. In some therapeutic applications (e.g., rituximab (Rituxan) and trastuzumab (Herceptin)[24,34,35]), this attribute is beneficial.

Transgenic plants and transgenic dairy animals including cows and goats have been used to produce antibodies. Yet, all mAbs in clinical use are produced in cell culture systems. Production of mAbs in plants such as tobacco has the advantages of low cost,

relatively short generation time and easy scale-up[1]. However, the presence of immunogenic plant-specific glycans and biosafety issues regarding containment need to be addressed[1]. Transgenic goats can be used to produce recombinant proteins in milk at concentrations between 1 and 5 g/l, and the proteins are glycosylated with patterns typical of mammalian proteins, although oligomannose structures were more abundant on human antithrombin produced in transgenic goat milk[3–5]. However, a long time period is required for generating and rearing lactating transgenic animals (18 months for goat and 3 years for cow).

In comparison with plant and mammalian transgenic systems, the chicken system for producing human mAb is attractive; the mAbs possess enhanced ADCC, acceptable half-lives, adequate glycosylation, excellent antibody binding and good rates of internalization. From a production point of view, the chicken is advantageous because (i) the interval from transfection of the cES cells to production of eggs by chimeras is only 8 months, (ii) the egg is a sterile and compact compartment providing a good starting material for isolation and purification and (iii) conditions for good manufacturing practices are well known in poultry production for the rearing of elite breeding stock and for vaccine production in eggs. These attributes have provided the impetus to develop technologies for protein production in eggs, although the traditional emphasis has been on the production of fully transgenic chickens[36,37]. The ability to incorporate the transgenes described here into fully transgenic chickens would, however, simplify production and scalability of therapeutic antibodies in eggs and therefore, we are continuing to develop technology that will facilitate introduction of large transgenes into the chicken genome.

METHODS

Animal care. Animal experiments were done according to protocols reviewed and approved by the Institutional Animal Care and Use Committee at Origen Therapeutics.

Isolation and culture of cES cells. To obtain chicken cES cell lines, we isolated the area pellucida from Stage X (EG&K)[38] Barred Plymouth Rock embryos as described previously[39], washed twice with culture medium and dispersed using a 200-µl pipette into a single cell suspension. The cES cells became visible approximately 1 week after seeding the blastodermal cells. The cells were small with a large nucleus and a pronounced nucleolus (**Supplementary Fig. 2** online) and grew in single layers with clearly visible individual cells. Typically, the cells were grown to 80–100% confluency before they were passaged at daily intervals in a 1:2 ratio. To maintain optimum growth and to prevent differentiation, we passaged the cells by transferring 30–50% of the medium covering them into the new well. STO cells at a concentration of 10^4 cells/cm^2 were required to support the growth of cES cells. The cells were dispersed using either a short trypsin (0.25%) wash or a 1- to 2-min incubation in Ca/Mg-free PBS and passaged in small clumps.

The parental cell line for OVA was derived from a pool of embryos on mitotically inactivated STO cells seeded on gelatinized tissue culture plates at a concentration of 10^4 cells/cm^2. The parental cell line for OVH was isolated from a single Barred Plymouth Rock embryo under the same conditions as OVA. To obtain mitotically inactivated STO cells, we grew the cells to confluency, irradiated and cryopreserved at 10^7 cells/vial until further use. The cES cell culture medium consisted of conditioned DMEM or Knockout DMEM (Invitrogen) supplemented with 10% fetal calf serum (FCS), 1% of a solution containing 10,000 units of penicillin and 10,000 units of streptomycin C; 2 mM glutamine, 1 mM pyruvate, 1× nucleosides, 1× nonessential amino acids and 0.1 mM β-mercaptoethanol. Before use, the DMEM or knockout DMEM medium was conditioned on Buffalo Rat Liver (BRL) cells as described previously[39]. Briefly, after BRL cells were grown to confluency, medium containing 5% serum was added and conditioned for 3 d. The medium was removed and a new batch of medium was conditioned for 3 d. This was repeated 1× and the three batches were combined, frozen at −20 °C and thawed to make the cES cell culture medium. Chicken ES cells were cryopreserved using standard procedures in 10% DMSO in medium containing DMEM + 25 mM HEPES or CO_2-independent medium (Invitrogen) supplemented with 10% serum.

Genetic modification of cES cells. Chicken ES cells were transfected as adherent cells using a Petri-Pulser (Genetronics). Adherent cells in a 9.5-cm^2 well were washed with PBS and 1 ml of electroporation buffer (Specialty Media) containing 5 µg of linearized DNA was added. The Petri Pulser was set into the well and a 15-ms square wave pulse varying from 675–750 V/cm was given. After 10 min, 2 ml of medium were added and the culture was returned to the incubator. Antibiotic selection was initiated by the addition of 1 µg/ml of puromycin approximately 2 d after transfection. Resistant clones became visible 8–14 d after transfection and were transferred to new wells for expansion.

Production of chimeras using Stage X (EG&K) recipients. The transfected Barred Plymouth Rock cES cells were injected into White Leghorn embryos, which are homozygous at the dominant white locus. The resulting chimeric chickens have black feathers that are derived from the cES cells and white feathers that are derived from the recipient embryo. Between 1 and 5 µl of cell suspension containing between 5,000 and 20,000 cells was injected into the subgerminal cavity of recipient embryos. To allow easy access to the embryo during injection of the cES cells, Stage X (EG&K) recipient embryos were first transferred to a surrogate shell[40]. The cES cells were injected into the subgerminal cavity and the egg was closed with Saran Wrap. After incubation at 37.5–38 °C for 3 d the embryo was transferred to a second surrogate shell, and incubated until hatch at 37.5–38 °C and 50% humidity. To increase the contribution of cES cells, we compromised the recipient embryos by irradiating the eggs with ~660 rads from a cesium source[41] within 6 h of injection of the donor cells. After injection of cES cells, the embryos were held at 15 °C for 3–7 d to maintain embryonic diapause before development was initiated by incubating at 37.5 °C. While the embryos were maintained in diapause, they were rocked through 90° at hourly intervals.

Construction of ovalbumin-based expression vector. A 918-bp fragment located in the 5′ flanking region of the ovalbumin gene (GenBank J00895) was amplified from chicken genomic DNA. Primers OV-1 and OV-2 (**Supplementary Table 2** online) were designed based on the above-referenced sequence. The PCR product was cloned and used to screen a chicken genomic BAC library (020-CHK-H3- Texas A & M BAC center)[42]. A 150-kb BAC clone containing the ovalbumin locus was identified. The BAC was digested individually with *Kpn*I, *Bam*HI or *Xho*I to obtain a subclone library of the BAC. Subclones were screened by a combination of restriction mapping, PCR and end sequencing. Subclones K4, K8, K3 and B9 cover a region of 37 kb with 15 kb of 5′ flanking sequence and 15.5 kb of 3′ flanking sequence in the ovalbumin locus. Based on the above identified sequences, 37 kb and 45 kb expression vector backbones (Ov7.5 and Ov15, respectively) were constructed to contain 9.2 kb and 16.8 kb of the 5′ sequence from the ovalbumin gene, respectively and 15.5 kb of the 3′ flanking sequence including the 3′ UTR (**Fig. 1a**). Assembly of the ovalbumin expression vectors involved construction of intermediate vectors for the assembly of the 5′ ovalbumin sequences, the 3′ ovalbumin sequences and final assembly of all the sequences. In a first step, the 5′ ovalbumin sequence was assembled into 5′ Ov7.5 by joining a 9.2-kb *Kpn*I-*Hin*cII fragment from the K8 subclone and a 125-bp *Hin*cII-*Xba*I fragment obtained by PCR to restore the 3′ end including the Kozak consensus sequence and adding an engineered *Not*I restriction site for subsequent cloning of the gene to be expressed. 5′ Ov15 was assembled by adding an additional 7.5-kb fragment onto 5′ Ov7.5. In a second step, the 3′ ovalbumin sequence was assembled by joining a 120-bp PCR fragment including the 3′ UTR sequence and an engineered *Not*I restriction site, a 3.5-kb *Xba*I-*Bam*HI fragment from the K3 subclone and a 12-kb *Bam*HI fragment from the B9 subclone. An *Asc*I site was introduced right after the 3′ ovalbumin sequences to facilitate the cloning of a selectable marker for stable transfection (here a *CxEGFP/CxPuro* cassette). An *EGFP* gene driven by a ubiquitous promoter *Cx* (*CxEGFP*) was included in the puromycin-selection cassette (*CxPuro*) at the 3′ end of the ovalbumin vectors to allow identification of the cES cell–derived cells in chimeras. The *Cx* promoter contains a CMV enhancer and a chicken β-actin promoter[43]. The resulting 3′ Ov15CxEGFP/CxPuro contains 15.5 kb of the

3′ flanking sequence of the ovalbumin gene. In a third step, a low copy number plasmid vector was designed for the assembly of 5′ ovalbumin sequence and 3′ ovalbumin sequence. The pBeloBAC11 vector (New England Biolab) was modified to have multiple cloning sites including *Rsr*II, *Kpn*I, *Not*I and *Srf*I in a 5′-to-3′ direction. The 5′ ovalbumin sequence (5′ Ov7.5 or 5′ Ov15) was cloned into *Kpn*I and *Not*I sites, and the 3′ ovalbumin sequence was cloned into *Not*I and *Srf*I sites. The resulting vectors Ov7.5 and Ov15 contain a *Not*I site for cloning any transgene to be expressed.

Monoclonal antibody cassettes. The mAb cassette was designed and assembled with unique restriction sites allowing easy insertion of V genes. The mAb cassette expressed by Ov7.5mAbdns is a mouse-human hybrid anti-dansyl monoclonal antibody (mAbdns). The V-gene fragments were amplified from the V_L and V_H that were used to make chimeric anti-dansyl proteins[44]. Appropriate restriction sites were added at both ends for subsequent cloning. Both the L and H chains of the mAb are expressed from a single vector by using an internal ribosomal entry site (IRES) element (Clontech). The mAb cassette was assembled with genomic DNA sequences that include intron sequences (**Fig. 1a**). The sequences for signal peptides and constant regions with appropriate restriction sites added at both ends were obtained by PCR from human genomic DNA (Clontech) based on published sequences (GenBank X17262 for SiG_{VL}, GenBank X67858 for Cκ, GenBank Z14241 for SiG_{VH}, GenBank Z17370 for Cγ1) and confirmed by DNA sequencing. The mAbdns cassette was released from the vector and cloned into the *Not*I site of Ov7.5 to obtain Ov7.5mAbdns for transfection into cES cells.

The mAbdns cassette was tested in chicken DT40 cells (ATCC) under a ubiquitous *Cx* promoter. The V_L exon was alternatively spliced out in the L-chain transcript to produce predominantly a L chain without the V region. However, a small portion of full-length mAb molecules capable of antigen binding were produced as indicated by RT-PCR and western blot analysis (data not shown).

The second mAb cassette (mAbF1) is a fully human antibody produced at Medarex from human immunoglobulin transgenic mice immunized with LNCaP membrane[45,46]. mAbF1 binds to a conformational epitope on the extracellular domain of human PSMA and exhibits high-affinity binding to viable LNCaP cells by flow cytometry, and shows efficient internalization into PSMA-expressing cells. V genes corresponding to mAbF1 were obtained by PCR and used to replace the V genes in mAbdns. In addition, aberrant splicing in mAbdns was corrected by fusing the SiG_{VL} directly to the V_L sequence (confirmed by transfecting a *Cx* promoter driven mAbF1 cassette into chicken DT40 cells, data not shown), and lysine and glycine at positions 97 and 314 of the C_H region were changed to arginine and alanine, respectively. The mAbF1 cassette was cloned into the *Not*I site of Ov15 to obtain Ov15mAbF1 for transfection.

Induction of ovalbumin expression in female chicks. At 10–14 d of age female chimeras were injected daily for 10 d with 1 mg of 17-β-estradiol in sesame oil (Sigma) that stimulates growth of the oviduct and induces activation of the ovalbumin promoter. After 10 d the birds were euthanized and EGFP-expressing portions of the magnum were collected. Magnum samples were used for RT-PCR of L and H chains from mRNA, histology for the presence of EGFP and human IgG by immunohistochemistry.

Southern blot analysis. We used 10 μg of genomic DNA isolated from transfected cES cells in each digestion. The digested DNA was run on a 1% agarose gel (Invitrogen) with a PPI-200 Programmable Power Inverter (MJ Research). The probe DNA was a 4.7-kb *Not*I fragment of the relevant mAb cassette labeled with [P^{32}] dCTP by the Megaprime Labeling System (Amersham). Hybridization was conducted with Rapid-hyb Buffer (Amersham).

RT-PCR to detect transcription of mAb cassette. mRNA was prepared from tissue samples with Oligotex Direct mRNA Kit (Qiagen). cDNA was then synthesized from 9 μl of mRNA using ThermoScript RT-PCR System for First-Strand cDNA Synthesis (Invitrogen). We used 2 μl of cDNA in the subsequent PCR reaction. The primers used can be found in **Supplementary Table 2** online. Primers HuVLSig-F and Hu-Cκ-R were used to amplify a 559-bp (for mAbdns) or a 552-bp (for mAbF1) fragment from the L-chain transcript. Primers HuVHSig10-F and HuIgG1-C-R were used to amplify a 1,382-bp (for mAbdns) or 1,376-bp (for mAbF1) fragment from the H-chain transcript. Primers EGFP-1F and EGFP-1R were used to amplify a 545-bp fragment from the EGFP transcript. Primers Act-RT-1 and Act-RT-2 were used to amplify a 597-bp fragment from the endogenous chicken β-actin transcript. PCR reactions were performed with AmpliTaq Gold (Applied Biosystems) following the manufacturer's instruction.

Immunohistochemical localization of mAb. Magnums were collected and fixed overnight in 4% paraformaldehyde. After several washes in PBS, they were incubated overnight in 30% sucrose at 4 °C. Blocks were prepared and frozen sections were cut at 5 μm. The section was stained with a rabbit anti-human IgG (H+L) (Jackson ImmunoResearch Lab) at 1:2,500 dilution and visualized with a goat anti-rabbit IgG (H+L) conjugated with Alexa Fluor 594 (Molecular Probes) at 1:1,000 dilution. The sections were stained with 300 nM DAPI for 2 min before mounting with the Prolong Antifade Kit (Molecular Probes).

ELISA analysis of mAb in egg white. Egg white samples were diluted in PBS and ELISA was carried out essentially according to Mohammed *et al.*[48] The microtiter plates were coated with goat anti-human IgG (Zymed) at a concentration of 5 μg/ml and the presence of human mAb in the egg white samples was revealed by horseradish peroxidase (HRP)-labeled goat anti-human IgG (γ-chain specific) (Southern Biotechnology) diluted at 1:5,000. The standard curves were established with purified human IgG1,κ (Sigma). The sensitivity of the ELISA was 0.8 ng/ml. All samples were run as duplicates. The coefficient of variations for intra- and interassays were 1.9% and 11.0%, respectively.

Western blot analysis of mAb. Egg white samples were prepared by ammonium sulfate precipitation[47]. Samples were run on 12% or 4–15% Tris-HCl ready gel (Bio-Rad). After transfer onto a membrane, Western blot was performed with Super Signal West Pico Chemiluminescent Substrate Kits (Pierce) as instructed. A rabbit anti-human IgG (H+L) was used as a primary antibody (1:4,800 dilution) and a ImmunoPure Peroxidase conjugated goat anti-rabbit IgG (H+L) (Pierce, 1:100,000) was used as a secondary antibody.

W-chromosome PCR. PCR with primers W-chr1 and W-chr2 (see **Supplementary Table 1** online) to amplify a 276-bp fragment in the sequence of the *Xho*I repeat in pUGD0600 (ref. 48) was used to identify the presence of W-chromosome in the genome of cES cells and chimeras. In chickens, females are ZW and males are ZZ. Primers Act-1 and Act-2 (see **Supplementary Table 1** online) were used to amplify a 410-bp fragment in the endogenous chicken β-actin DNA.

Extraction and purification of human antibody. Egg white was first mixed at a low shear rate for 30 min at 20–22 °C and then ovomucin precipitated by a modified method described previously[49]. One volume of homogenized egg white suspension was added to three volumes of reverse osmosis water and stirred for 30 min. The diluted suspension was adjusted to pH 6.0 using 0.5 M phosphoric acid and then centrifuged for 20 min at 12,100*g*. Approximately 3% of the egg white protein, containing mostly ovomucin, was removed by this method. The supernatant was adjusted to pH 7.4 using 0.5 M dibasic sodium phosphate and 150 mM sodium chloride concentration with crystalline salt. The human IgG was purified on a Protein A-Sepharose FF column (Amersham Biosciences) at a 120 cm/h linear flow rate. The adsorbed human IgG was washed with five column volumes of the loading buffer (PBS, pH 7.4) and then eluted with 3 mM phosphoric acid. The eluted human IgG fraction was adjusted to pH 7.5 using 60 mM sodium phosphate (pH 7.5) containing 230 mM NaCl to achieve a final concentration of 40 mM sodium phosphate and 150 mM NaCl. The sample was then filtered through a 0.2 μm syringe filter (Pall).

Protein characterization of mAbF1. SEC-HPLC analysis. About 10 μg of IgG sample was analyzed on a Waters 2795 HPLC using a 4.6 x 300 mm BioSep SEC S3000 column (Phenomenex). Chromatography was carried out in 0.1 M sodium phosphate, 0.15 M NaCl and 0.1 M sodium sulfate, pH 7.2, at 0.4 ml/min flow rate for 20 min. Separations were monitored at A_{280}. Molecular weight standards (Bio-Rad) were used to determine approximate molecular weight.

Nano LC electrospray ionization MS/MS analysis. Both mAb preparations were reduced and alkylated, dialyzed overnight and digested with trypsin for 4 h at 37 °C in 25 mM ammonium bicarbonate (pH 8). MS/MS of both tryptic

digests was performed on a Nano HPLC system (Dionex) interfaced to a QSTAR pulsar mass spectrometer (MDS Sciex) operation in positive ion mode and using collision-induced dissociation (CID) to obtain tandem spectra. A 1-µl aliquot of each sample was injected and separated using a 75-µm diameter Pepmap C18 column at a flow rate of 250 nl/min. The mobile phase was solvent A (95% water, 5% acetonitrile, 0.5% formic acid) and solvent B (20% water, 80% acetonitrile, 0.5% formic acid). The instrument was operated in information-dependent acquisition mode with 7 s operating cycles, recording a full spectrum for 2 s then selecting the most intense ion to record a CID spectrum during the next 5 s. Spectra were analyzed using Mascot (Matrix Science).

LC-MS analysis. IgG samples (50 µg) in 4-M guanidine HCl were reduced in 25 mM DTT by incubating the samples at 60 °C for 90 min. Samples were then alkylated in 45 mM iodoacetic acid for 15 min at 20–22 °C in the dark and the reaction was stopped with 22 mM DTT. Prior to LC-MS, samples were dialyzed against 1 L of 25 mM ammonium bicarbonate and 50 µl of each sample was injected to a Poros R1/10 2.1 × 100 mm column (Applied Biosystems) using a Waters 2795 HPLC equipped with a Micromass ZQ mass spectrometer and analyzed in positive ion mode. The mobile phase was (A) 0.1% formic acid and 0.01% trifluoroacetic acid (TFA) and (B) 100% CH_3CN with 0.1% formic acid and 0.01% TFA. Elution (0.25 ml/min) was conducted by a linear gradient of 10–60% of (B) in (A) developed over 100 min.

CE-IEF analysis. IgG samples (50 µg each at 1.0 mg/ml) were dialyzed against water to remove salts before CE analysis. Capillary electrophoresis isoelectric focusing was performed on a P/ACE MDQ CE system (Beckman) with normal polarity, using a 50 µm internal diameter eCAP neutral capillary (Beckman) with 30-cm effective length. Sample was injected for 60 s by pressure injection at 20 psi. Separation was carried out using 15 kV for 45 min. The temperature of the capillary was 20 °C. The anolyte was 20 mM phosphoric acid and the catholyte was 40 mM sodium hydroxide. The rinse solution was 10 mM phosphoric acid and the cathodic mobilizer solution was used as provided (Beckman). The separations were monitored at A_{280}.

Thermal stability by differential scanning calorimetry (DSC). Calorimetric measurements of melting temperatures (T_m) were carried out on a VP-Capillary DSC differential scanning microcalorimeter platform that was combined with an autosampler (MicroCal LLC). Sample cell volume was 0.144 ml. Denaturation data on the glycosylated and deglycosylated forms of the antibodies were obtained by heating the samples, at a concentration of 2.3 µM, from 30 to 95 °C at a rate of 1 °C/min. The protein samples were present in PBS at pH 7.4. The same buffer was used in the reference cell to obtain the molar heat capacity by comparison. The observed thermograms were baseline corrected and normalized data were analyzed using Origin v7.0.

Assay for glycosylation. Screening of mAb Fab fragment for glycosylation. Fab and Fc fragments were produced by adding 1.25 µg of activated papain to 25 µg of IgG samples containing 1 mM cysteine. Samples were incubated at 40 °C for 4 h and the reactions stopped with 30-mM iodoacetamide. Samples were analyzed by 4–20% Tris-Glycine SDS-PAGE followed by electro-blotting onto polyvinylidene fluoride membrane. The carbohydrate-specific staining of the blot was carried out using the Gel Code Glycoprotein Staining Kit (Pierce) following the protocol suggested by the manufacturer.

Oligosaccharide characterization of mAbs by CE-LIF. Oligosaccharides were released from IgG samples (100 µg) by overnight incubation of the samples with 12.5 mU PNGaseF (Prozyme) at 40 °C. Under the conditions used, the N-linked glycans from the Fc portion of human mAbF1 expressed in CHO cells and chicken tubular gland cells were released. After ethanol precipitation to remove mAb protein, the supernatant containing the glycans was dried by vacuum centrifugation and resuspended in 19 mM 8-aminopyrine-1,3,6-trisulphonate (APTS) (Beckman) in 15% acetic acid and 1 M sodium cyanoborohydride in THF (Sigma). The glycan labeling reaction was allowed to continue overnight at 40 °C followed by 25-fold dilution of sample in water. APTS-labeled glycans were applied to capillary electrophoresis with laser-induced fluorescence on a P/ACE MDQ CE system with reverse polarity, using a 50-µm internal diameter N-CHO-coated capillary (Beckman) with 50-cm effective length. Samples were pressure (8 s) injected and separation was carried out at 20 °C using carbohydrate separation gel buffer (Beckman) at 25 kV for 20 min. The separations were monitored using a laser-induced fluorescence detection system (Beckman) with a 3-mW argon ion laser and excitation wavelength of 488 nm and emission of 520 nm.

Oligosaccharide characterization of mAbF1. Purified antibodies (100 µg each at 1.0 mg/ml) were treated with 4 µl of PNGaseF, an endoglycosidase that cleaves N-linked carbohydrates (Prozyme). The samples were incubated at 37 °C overnight followed by dialysis overnight against 50-mM ammonium bicarbonate (pH 8). The protein was removed by ethanol precipitation. The released carbohydrates were desalted on a micro-carbon column from Glygen essentially following the vendor's protocol, but the elution volume was reduced to 5 µl. A 1-µl aliquot of each glycan sample was mixed 1:1 (vol/vol) with matrix solution (α-cyano-4-hydroxycinnamic acid or 2,5-dihydroxybenzoic acid (Applied Biosystems), spotted on a MALDI target plate and allowed to air-dry. MALDI-Q-TOF tandem analysis was used to perform the analysis of intact glycoconjugates. All mass spectra were recorded on a QSTAR pulsar i mass spectrometer equipped with an o-MALDI source 2 (MDS Sciex), which provides the mass (composition) of classes of carbohydrates. After the glycan profiling of both the mAbs, the possible glycosidic linkage in each glycan was investigated. High CID mass spectra were recorded on a 4700 Proteomics analyzer with a TOF/TOf optics (Applied Biosystems). For high-CID MS/MS experiments, the collision energy was set at 1 kV. Inside the collision cell, the selected oligosaccharide ions were collided with argon at a pressure of 2×10^{-6} Torr.

Monosaccharide analysis by HPLC with HPAE-PAD. IgG samples (200 µg) were subjected to acid hydrolysis using either 2-N TFA (for estimating neutral sugars) or 6-N HCl (for estimating amino sugars) at 100 °C for 4 h. Samples were dried by vacuum centrifugation at 20–22 °C and were reconstituted in 200 µl water before analysis by high-performance anion exchange chromatography with pulsed amperometric detection (HPAE-PAD) (Dionex). Monosaccharides were separated using a CarboPac PA10 4 × 250-mm column with pre-column Amino Trap and Borate Trap (Dionex). Procedures were followed according to Dionex Technical Note 53. Monosaccharide peak identity and relative abundance were determined using monosaccharide standards (Dionex).

Assay for binding affinity. PSMA on LNCaP cells (ATCC) was used as antigen to assay for binding. Duplicate aliquots of 200,000 cells/well were incubated for 30 min with 50 µl of antibody at the indicated concentrations. Cells were washed twice before addition of goat anti-human IgG PE labeled antibody (Jackson ImmunoResearch) at 1:200 dilution in 50 µl/well for 30 min at 4 °C. Cells were washed twice in PBS with 1% BSA and assayed by fluorescence-activated cell sorting. EC_{50} values of mAb binding to PSMA on LNCaP cells were determined from binding curves utilizing GraphPad Prism 3.0 (GraphPad Software). Cells were grown in RPMI 1640 medium supplemented with 10% FBS, 10 mM HEPES, 2 mM L-glutamine, and 1 mM sodium pyruvate.

Antibody internalization assay. Internalization of antibody binding to PSMA on LNCaP cells was determined by incubating cells with mAb and Hum-Zap (Advanced Targeting Systems). Hum-Zap is a goat anti-human IgG antibody conjugated to the ribosome-inactivating protein, saporin. LNCaP cells (10,000/well) were incubated in triplicate for 48 h at 37 °C in 150 µl of culture medium containing 300 ng Hum-Zap and 300 ng of mAbF1 or control mAb. Cell proliferation and survival were determined with the CellTiter-Glo Luminescent Cell Viability Assay (Promega). Internalization assays were also done by incubating dilutions of antibody in cell culture medium with 10,000 adherent LNCaP cells/well for 2 h at 4 °C. Antibody solutions were gently removed and replaced with 150 µl of medium containing 200 ng of Hum-Zap. Cell viability was determined following 48 h of incubation at 37 °C. EC_{50} values for antibody internalization were determined graphically with Prism 3.0 (GraphPad Software).

Clearance of mAb in BALB/c mice. Ten micrograms of mAb protein were lightly iodinated (less than one I per antibody) with ^{125}I using the Iodobead method (Pierce). Six week-old female BALB/c mice (Taconic Farms) were fed 0.1 mg/ml potassium iodide in their drinking water for 1 week before the experiment. Four mice per protein were injected intravenously into the tail vein with approximately 600,000 c.p.m. of labeled mAb and whole-body

radioactivity was measured at selected times using a whole-body gamma counter (Wm. B. Johnson NaI crystal detector with a Ludlum scaler). Half-life was calculated by exponential regression analysis of the residual radioactivity.

ADCC assay. LNCaP-C42b cells were tested in a modified ^{51}Cr ADCC assay. Human peripheral blood mononuclear cells were purified from heparinized whole blood by standard Ficoll-Paque separation. The cells were resuspended (at 1×10^6 cells/ml) in RPMI 1640 medium containing 10% FBS and 10 U/ml of human IL-2 and incubated overnight at 37 °C. The following day, the cells were collected and washed once in culture medium and resuspended at 2×10^7 cells/ml. Two million target LNCaP-C42b cells were incubated with 200 µCi ^{51}Cr in 1 ml for 1 h at 37 °C. The target cells were washed once, resuspended in 1 ml of medium and incubated at 37 °C for an additional 30 min. After the final incubation, the target cells were washed once and brought to a final volume of 1×10^5 cells/ml. For the ADCC assay, 100 µl of labeled LNCaP cells were incubated with 50 µl of effector cells and 50 µl of antibody. The final target to effector ratio was 1:100. In all studies, human IgG1 isotype control was run and compared to CHO-derived mAbF1. Other controls included were (i) target and effector cells but no antibody, (ii) target cells with no effector cells and (iii) target and effector cells in the presence of 3% Triton X-100. After 4 h incubation at 37 °C, the supernatants were collected and counted on a gamma counter (Cobra II auto-gamma, Packard Instruments) with a reading window of 240–400 keV. The c.p.m. were plotted as a function of antibody concentration and the data were analyzed by nonlinear regression, sigmoidal dose response (variable slope) using Prism software. The percent lysis was determined by the following equation:

$$\%\,\text{Lysis} = (\text{Sample c.p.m.-No antibody c.p.m.})/(\text{TritonX c.p.m.-No antibody c.p.m.}) \times 100.$$

Note: Supplementary information is available on the Nature Biotechnology website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Biotechnology* website for details).

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