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Human extracellular superoxide dismutase (EC-SOD) expression in transgenic chicken

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Extracellular superoxide dismutase (EC-SOD) is a metalloprotein and functions as an antioxidant enzyme. In this study, we used lentiviral vectors to generate transgenic chickens that express the human EC-SOD gene. The recombinant lentiviruses were injected into the subgerminal cavity of freshly laid eggs. Subsequently, the embryos were incubated to hatch using phases II and III of the surrogate shell *ex vivo* culture system. Of 158 injected embryos, 16 chicks (G0) hatched and were screened for the hEC-SOD by PCR. Only 1 chick was identified as a transgenic bird containing the transgene in its germline. This founder (G0) bird was mated with wild-type hens to produce transgenic progeny, and 2 transgenic chicks (G1) were produced. In the generated transgenic hens (G2), the hEC-SOD protein was expressed in the egg white and showed antioxidant activity. These results highlight the potential of the chicken for production of biologically active proteins in egg white. [BMB Reports 2013; 46(8): 404-409]

INTRODUCTION

The importance of, and demand for, biopharmaceutical proteins are rapidly growing (1). Until recently, recombinant proteins used for the treatment of human diseases have been produced mainly by living microbial, insect, or animal cells using industrial bioreactors (2-4). Since using a cell-based manufacturing system to produce new recombinant proteins is an expensive and time-consuming process, alternative platforms for production of pharmaceutical proteins are needed; the use of transgenic livestock as bioreactors offers one such possibility.

The need in the pharming industry for new production systems has provided a strong impetus for the development and improvement of transgenic techniques. The mammary glands of livestock have attracted a great deal of attention for producing human recombinant proteins (5). Despite being successfully commercialized (6), these mammalian transgenic systems have several drawbacks, in that they require a relatively large area for breeding of the animals and require a few years for the animals to become sexually mature. Chickens have several merits over mammalian systems as bioreactors, such as a short generation time, high protein productivity in eggs, the small space required for breeding, the ease of scale-up of transgenic flocks, and being free of prion diseases. In addition, the N-glycosylated carbohydrate of chicken has N-acetylneuramic acid at its end, as does that of humans (7-9). The most successful method for generating transgenic birds is through use of viral vectors as a means of delivering genes to embryos in newly laid eggs (10-12). Previously, several studies have succeeded in producing transgenic chickens expressing human recombinant proteins in egg white (8, 12).

One protein that is of interest in the biopharmaceutical field is the Cu-Zn-containing extracellular superoxide dismutase (EC-SOD or SOD3), which is one of the 3 isoforms of superoxide dismutases (SOD) present in mammals, the other 2 being cytoplasmic and nuclear Cu-Zn-containing SOD (SOD1) and mitochondrial Mn-containing SOD (SOD2) (13). The enzymes of the SOD family are partly responsible for maintaining low levels of reactive oxygen species (ROS), by metabolizing superoxide anion into oxygen and hydrogen peroxide (14). The greatest amount of the superoxide radical is produced in the metabolism of molecular oxygen. These reactive oxygen species are generally formed in the body by several different mechanisms, including cellular respiration and phagocytosis.

EC-SOD is a homotetrameric glycoprotein showing high affinity for heparin and other sulfated-glycosaminoglycans, and is highly expressed in the lungs, kidneys, uterus, and blood vessels (15, 16). It is located in the ECM and is known to be the only antioxidant that enzymatically scavenges superoxide in the extracellular compartment. Recently, it has been proposed that EC-SOD protects the brain, lungs, and other tissues from oxidative stress (17).

In the present study, we aimed to generate transgenic birds

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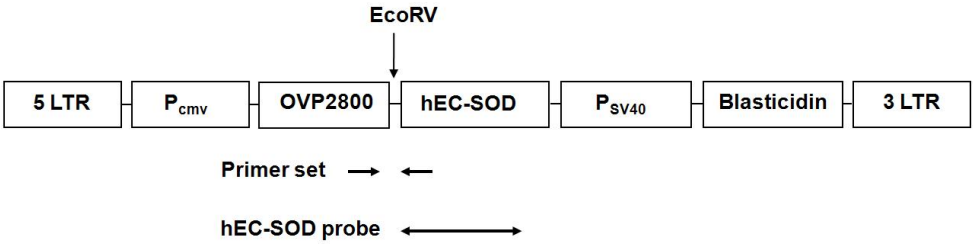

Fig. 1. Structure of the OVP-hEC-SOD lentiviral vector. LTR, long terminal repeat; P_{CMV}, CMV promoter; OVP2800, 2.8-kb chicken ovalbumin promoter; hEC-SOD, human extracellular superoxide dismutase; P_{SV40}, SV40 early promoter; Blasticidin, blasticidin-resistance gene. The approximate positions of the probe for Southern blot analysis and the primer set for detection of target sequences are indicated below the vector. *Eco*RV cuts once inside the lentiviral vector. The figure is not drawn to scale.

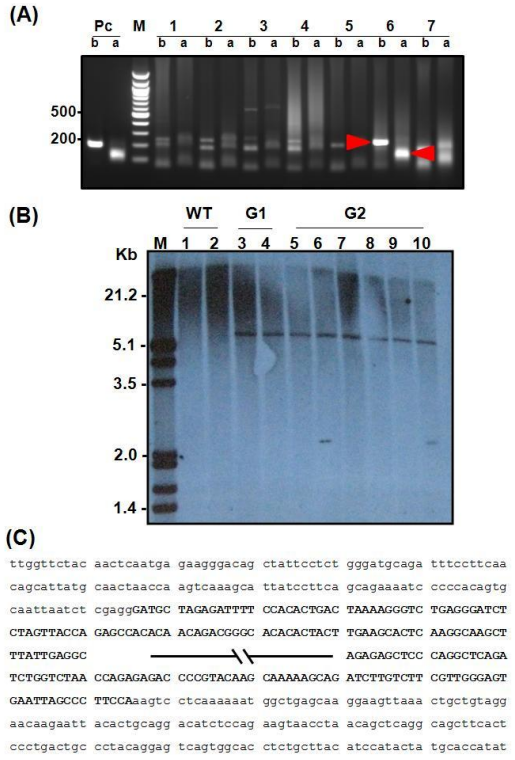

Fig. 2. Identification of the hEC-SOD gene for transgenic chicken. (A) PCR transgene analysis. M, 100-bp DNA ladder; Pc, positive control using the OVP-hEC-SOD/pLenti6/TR plasmid; lanes 1-7, genomic DNA samples isolated from the semen of the hatched roosters (G0); b, before *Eco*RI digestion; a, after *Eco*RI digestion. One rooster (lane 6) was positive for the transgene in the germline. The red arrowheads indicate the *Eco*RI-digested amplified DNA fragments (a) or the undigested amplified DNA fragment (b). (B) Southern blot analysis of transgenic chickens. Genomic DNA samples extracted from the whole blood of wild-type and transgenic chickens (G1 and G2) were digested with *Eco*RV, electrophoresed, blotted, and hybridized with the probe for the hEC-SOD gene. M, DIG-labeled DNA marker; lanes 1 and 2, wild-type chickens; lane 3, the G435; lane 4, the H148; lanes 5-7, the G435's offspring; lanes 8-10, the H148's offspring. (C) Nucleotide sequences flanking the hEC-SOD provirus. Small letters, the chicken cAMP-dependent protein kinase type I-beta regulatory subunit gene; bold capital letters, the provirus.

expressing the hEC-SOD gene using the lentiviral vector and to investigate whether transgenic chickens could be used as bioreactors for the production of the hEC-SOD protein. Using this approach, we successfully generated transgenic birds secreting the recombinant hEC-SOD protein in egg white. Significantly, this is the first report of the production of the hEC-SOD protein in transgenic chicken.

RESULTS

Generation of transgenic chickens expressing the hEC-SOD

The OVP-hEC-SOD lentiviral vector (Fig. 1), pseudotyped with vesicular stomatitis virus glycoprotein (VSV-G), was used to produce transgenic birds. A total of 158 eggs were each injected with about 5,000 virus particles, and 16 G0 chicks (10.1%) were finally hatched. To screen transgenic chickens, all chicks were raised to sexual maturation. Semen samples from 7 surviving roosters were screened by PCR. Only 1 G0 cockerel (A354) was identified as having the foreign gene present in the germ line (Fig. 2A). For further confirmation, *Eco*RI-digestion of the 233-bp PCR-amplified product was assessed; since the fragments contain the *Eco*RI enzyme site approximately in the middle of the fragment, the DNA fragments were digested into 2 smaller fragments (Fig. 2A).

The transgenic rooster (A354) was crossed to wild-type counterparts, and 1195 chicks were produced and screened, among which 2 transgenic G1 offspring were identified by PCR (data not shown). The hEC-SOD insertion of these G1 birds (G435, H148) was further confirmed by Southern blot analysis. The G1 transgenic birds were males and were crossbred to wild-type hens to generate G2 transgenic chickens. The 122 resulting progeny (G2) were screened by PCR to determine the presence of the transgene (data not shown). The ratio of transgenic to non-transgenic offspring, 56/122 (G435, 30/60; H148, 26/62), did not significantly differ from the expected Mendelian ratio. The G1 chickens and their six offspring were randomly selected to determine the copy number. It has been found that they carry only single copy of the transgene and have the same proviral insertion site, indicating that they were derived from the same germ cell precursor (Fig. 2B).

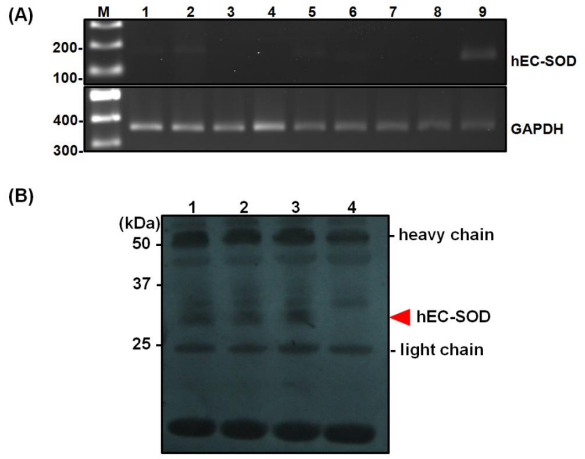

Fig. 3. Expression of the hEC-SOD in transgenic chickens. (A) RT-PCR of the hEC-SOD transgene. Total RNA was isolated from the liver (1), pancreas (2), heart (3), muscle (4), brain (5), intestine (6), kidney (7), ovary (8), and oviduct (9) tissues of an adult G2 hemizygous transgenic hen. The hEC-SOD gene was mainly expressed in oviduct tissue; the GAPDH gene was used as an internal control. M, 100 bp DNA ladder. (B) Western blot analysis of the hEC-SOD protein. The egg white samples of wild-type and transgenic hens were immunoprecipitated and then immunoblotted using a mouse anti-hEC-SOD antibody. The arrowhead indicates the expressed hEC-SOD protein. Lanes 1-3, transgenic chickens; lane 4, wild-type chicken.

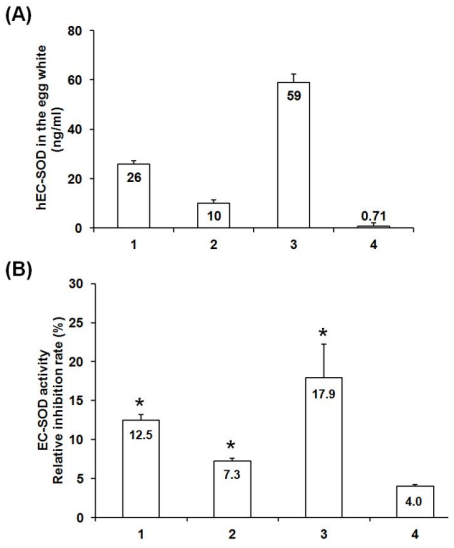

Fig. 4. The hEC-SOD protein assays of egg white samples. Egg white samples isolated from wild-type (lane 4) and G2 transgenic chickens (lane 1-3) were diluted with 4 volumes of PBS (pH 7.4). The expression level (A) and the antioxidant activity (B) of the hEC-SOD in egg white samples were measured by ELISA and EC-SOD Assay Kit, respectively. Error bars represent the mean ± standard deviations of three independent experiments. The asterisks indicate a significant difference of the hEC-SOD activity between wild-type and transgenic egg white ($P > 0.05$).

To identify the insertion site of the hEC-SOD in the transgenic chicken genome, we analyzed the genomic DNA of the G435 chicken. The transgene was integrated at the ninth intron of the cAMP-dependent protein kinase type I-beta regulatory subunit gene which is derived by automated computational analysis using gene prediction method (NCBI's GNOMON), and located on the chromosome 14 (Fig. 2C). In hemizygous transgenic birds, we did not find any problems in producing the next generation of transgenic chickens.

Oviduct specific hEC-SOD expression in transgenic chicken

We aimed to drive transgene expression to the oviduct of laying hens by using chicken ovalbumin 5′-regulatory sequences to control expression of the human EC-SOD. RT-PCR analysis showed that the hEC-SOD mRNA was successfully expressed in the oviduct tissue of transgenic hens (data not shown). To identify whether transgene expression was restricted to the oviduct tissue of laying hens, total RNA samples were collected from the liver, pancreas, heart, muscle, brain, intestine, kidney, ovary, and oviduct tissues of adult G2 hemizygous transgenic hens. The mRNA transcripts of the hEC-SOD were mainly limited to oviduct tissue, but ectopic transgene expression was also detected in pancreas, intestine, and brain tissues (Fig. 3A).

Recombinant hEC-SOD proteins in egg white are functional

Eggs were collected from a wild-type and 3 hemizygous transgenic hens (G2) randomly selected. Expression of recombinant hEC-SOD protein was identified in the egg white of the transgenic hens. Immunoprecipitation and western blot analysis showed that the recombinant protein size was approximately 30 kDa (Fig. 3B). The mean values for the recombinant hEC-SOD protein in eggs from the three transgenic hens were between 10 and 59 ng/ml (Fig. 4A). To investigate whether the recombinant protein was functional, the egg white samples from a wild-type and the three transgenic hens were assayed; the latter showed higher antioxidant activities compared to that of the wild-type (Fig. 4B).

DISCUSSION

We have previously showed that lentiviral vector systems can be utilized for the production of transgenic chickens, and stable transmission of the integrated vector through the germ line was demonstrated with conserved transgene expression in the G1 and G2 generations (18). In the current study, we generated transgenic chickens expressing the recombinant hEC-SOD (rhEC-SOD) protein in egg white using a lentiviral vector system for transgene delivery.

The rhEC-SOD has previously been expressed in CHO cells (19), insect cells (20), *Pichia pastoris* (21), and *Escherichia coli* (22), but only limited amounts of EC-SOD protein could be produced in these systems. Especially, the rhEC-SOD in *E. coli* has a problem that it is expressed in the form of an inclusion

body and must be refolded to exhibit hEC-SOD activity. In this study, we, for the first time, introduced transgenic birds as an alternative platform for production of recombinant hEC-SOD proteins and attempted to generate the transgenic hEC-SOD chickens. Immunoprecipitation and western blot analysis showed that this approach was successful, as evidenced by the expression of the hEC-SOD proteins in egg white of the G2 transgenic chicken. Moreover, hEC-SOD overexpression in rabbit caused a lactation problem (23); however, we did not find any difficulties in producing the next generation of transgenic chickens. Therefore, the chicken is an excellent system for production of pharmaceutical proteins without inducing health problems in the host.

The most attractive feature of the hen as a bioreactor is its capacity for egg production. Egg white contains about 4 g of dry weight protein, over half of which is ovalbumin that is derived from the single ovalbumin gene. Hence, the regulatory region of the ovalbumin gene has been used to express recombinant foreign proteins in the oviduct of laying hens (12, 24). Since lentiviral vector systems can deliver approximately 5 kb of foreign DNA, viral titers generally decrease as the size of the insert increases. Considering virus packaging efficiency, and as highlighted in our previous report (18), the maximum size of the 5'-regulatory region of the ovalbumin gene that can be packaged into virus particles was selected. The 4.3-kb OVP-hEC-SOD composed of the 2.8-kb chicken ovalbumin promoter containing the transcription initiation site and 1.4-kb hEC-SOD gene fragment was constructed and used to produce the recombinant virus particles. The length of the 5'-regulatory region of the ovalbumin gene used in the present study was approximately 0.8 kb longer than that employed previously (18).

The chicken ovalbumin promoter showed mainly oviduct expression of the foreign gene in the transgenic chickens, but ectopic expression of transgene mRNA was also detected in some other tissues (Fig. 3A). The above results again suggest that the 1.3-kb region upstream of the transcription initiation site of the ovalbumin gene is the minimum fragment that possesses the full ability to drive oviduct-specific expression. It also emphasizes that the 5'-UTR of the ovalbumin gene is essential for high-level expression of foreign proteins in egg white, perhaps due to its positive effect on transcribed mRNA stability (8, 18).

Light and heavy chain positions of immunoglobulin as well as protein marker sizes indicated that the molecular weight of the expressed hEC-SOD protein was approximately 30 kDa (Fig. 3B). Native hEC-SOD is composed of 222 amino acids and has a calculated molecular weight of 26.7 kDa. These differences in the expected and actual molecular weights suggested that the hEC-SOD protein expressed in egg white was a glycosylated form of the protein. Thus far, the role of glycosylation in hEC-SOD activity is not clearly understood (22). The highest concentration of the hEC-SOD measured in the egg white of G2 transgenic chickens was approximately 60 ng/ml (Fig. 4A). Although the quantity of the recombinant hEC-SOD protein in egg white was not as high as the yield reported previously (12), the proteins showed biologically antioxidant activity, which varied among the G2 transgenic hens (Fig. 4B). Although expression levels of the recombinant proteins were related to the chromosomal location of the transgene insertion; positional effects of chromosomal insertion on transgene expression have previously been reported in transgenic (25, 26). Variations of the hEC-SOD protein expression levels among the G2 transgenic hens might depend on the individual difference rather than positional effects because the transgenic birds showed the same insertion site. Therefore, selection of high-producer chickens in terms of production of the foreign protein in egg white will be necessary for commercial production using transgenic progeny.

In summary, we have developed a new system for recombinant hEC-SOD production using transgenic chickens expressing a foreign gene in the egg white. This system holds promise for commercial production of recombinant proteins using transgenic chickens as bioreactors.

MATERIALS AND METHODS

Animals

The study protocol and standard operating procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the National Institute of Animal Science in Korea (2010-008). The experimental procedures used in animal management, reproduction, and embryo manipulation followed the standard operating procedures of our laboratory.

Lentiviral vector construction

The hEC-SOD gene and the 2.8-kb fragment (-2791 to +9) of the chicken ovalbumin promoter (OVP) were amplified from the hEC-SOD cDNA and chicken genomic DNA respectively, using the following primers: hEC-SOD sense, 5'-gaattcggg ctgggtgcagctctcttttc-3'; antisense, 5'-gaattcgggttttttgaattgctgaatt t-3'; OVP sense, 5'-acgcgtcaaatagagaagctttaaa-3'; antisense, 5'-ctcgagatccgatgctgtatgtacagac-3'. These amplified PCR fragments were cloned into the pCR®II-TOPO® vectors (Invitrogen, Carlsbad, CA) and digested with *Mlu*I/*Xho*I or *Eco*RI respectively. The digested fragments were then sub-cloned into *Mlu*I/*Xho*I and *Eco*RI sites of pcDNA3.1 (Invitrogen), respectively. The OVP-hEC-SOD/pcDNA3.1 construct was digested with *Hind*III, end-filled with Klenow fragment (Promega, Madison, WI), and ligated to create the OVP-hEC-SOD/pLenti6/TR. The DNA sequences of the OVP-hEC-SOD/pLenti6/TR were confirmed by DNA sequencing. A schematic of the construct is presented in Fig. 1.

Recombinant virus production

The OVP-hEC-SOD transducing particles were produced using the ViraPower™ Packaging Mix (Invitrogen) according to the manufacturer's instructions. Titers of the produced virus particles were approximately 5×10^6 colony forming units (cfu)/ml. Recombinant virus stocks were resuspended in

DMEM (Invitrogen), aliquoted, and stored at −80°C.

Microinjection of virus and embryo culture

The embryo culture and microinjection procedure of virus particles were performed as previously described (5, 18). About 5,000 virus particles were microinjected into the central part of the subgerminal cavity below the developing embryo of each newly laid egg (stage-X) from the Hy-Line brown laying hens. The embryos were incubated to hatching through phases II and III of the surrogate shell ex vivo culture system.

Analysis of transgenic chickens

To screen the transgene in transgenic roosters, genomic DNA samples were extracted from the semen of the hatched roosters (G0). PCR was performed using FastStart PCR master (Roche Applied Science, Indianapolis, IN) with each primer (sense, 5'-ggtcaaacttctgaagggaacctgtg-3'; antisense, 5'-agcaggcaggaacacagtagcg-3'). The transgene in individual G1 and G2 chickens was analyzed by PCR. To estimate copy number of the transgene, Southern blot analysis was performed with DIG-labeled hEC-SOD probes using DIG High Prime DNA Labeling and Detection Starter Kit II (Roche). The integrated provirus was cut by *Eco*RV between the OVP and the hEC-SOD gene (Fig. 1). The integration site of the transgene in the transgenic chicken was analyzed using the GenomeWalker Universal Kit (TaKaRa) according to manufacturer's protocol. The transgene-flanking region was identified by comparing the sequence of the cloned insert DNA with chicken genome sequences using BLAST program (http://www.ncbi.nlm.nih.gov).

RT-PCR

Total RNA was isolated from the liver, pancreas, heart, muscle, brain, intestine, kidney, ovary, and oviduct tissues of an adult G2 hemizygous transgenic hen. The cDNA was prepared from 2 μg of total RNA using the ImProm-II™ reverse transcription system (Promega) according to the manufacturer's instructions. The following primers were used for PCR amplification: hEC-SOD sense, 5'-agacaccttccactctgagg-3'; antisense, 5'-gtttc ggtacaaatggaggc-3'; chicken GAPDH sense, 5'-gattctacacacgga cacttcaagg-3'; antisense, 5'-acaatgccaaagttgtcatggatgac-3' (27). Thirty-five cycles of amplification were performed for hEC-SOD and 25 cycles for GAPDH. Amplification of GAPDH was used to normalize cDNA loading into each lane of gels.

Western blot analysis

To assay the hEC-SOD protein expression in the egg white of the transgenic hens, eggs were harvested from wild-type and transgenic hens (G2); they were carefully cracked, and the egg white was collected free of yolk. The egg white was diluted with 4 volumes of ice-cold phosphate-buffered saline (pH 7.4). The hEC-SOD protein was immunoprecipitated from this egg white using 5 μg of mouse anti-hEC-SOD antibody (Abcam, Cambridge, MA) at 4°C overnight. The precipitated proteins were resolved on SDS-PAGE and transferred to nitrocellulose membrane (Roche). The membrane was incubated with a mouse anti-hEC-SOD antibody (Abcam) at 4°C overnight. Then, the specific antibody-antigen complexes were detected using an ECL detection system (GE Healthcare, Fairfield, CT).

Human EC-SOD protein analysis

The concentration and activity of the human EC-SOD proteins in the egg white samples used for Western blot analysis were assayed using the hEC-SOD ELISA Kit (Abfrontier, Korea) and the Superoxide Dismutase Assay Kit (Trevigen, Gaithersburg, MD) according to manufacturer's protocols, respectively. All assays were repeated three times. Results are represented as mean ± standard deviation.

Statistical analysis

All numerical data were analyzed by *t*-test. Differences were considered significant at a value of $P < 0.05$.

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