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**—Technical Note—**

# Production of a Transgenic Pig Expressing Human Albumin and Enhanced Green Fluorescent Protein

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**Abstract.** We introduced a fusion gene of human albumin and enhanced green fluorescent protein (EGFP) into porcine oocytes using the sperm vector method, and produced a piglet that showed clear expression of GFP in the hooves and skin. PCR and Southern blotting analysis of genomic DNA extracted from the piglet's tissues, including the liver, showed that the tissues carried the transgene. RT-PCR analysis demonstrated that both the human albumin and EGFP genes were expressed in the tissues. The fact that human albumin gene was integrated and expressed in the liver of the transgenic pig opened a way for us to achieve our goal, which was the use of transgenic pigs for the bioartificial liver support system.

**Key words:** Bioartificial liver support, Enhanced green fluorescent protein (EGFP), Human serum albumin, Sperm vector method, Transgenic pig

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Production of a recombinant protein in the milk of transgenic dairy animals is currently being tested as an alternative way to produce a number of blood factors. Complex proteins like factor IX, factor VIII, and fibrinogen, in addition to simpler proteins like antithrombin III and albumin, have been produced using transgenic livestock [1–3]. Another promising option for the practical application of transgenic animals is production of tissues and organs that can be used for human medicine. For example, transgenic pigs can be organ donors for xenotransplantation. To control xenogeneic immunorejection, genetically modified pigs expressing human complement regulatory proteins have been developed [4–6].

We have been developing bioartificial liver support systems for treatment of patients with severe liver failure. For extracorporeal perfusion through bioartificial liver support using porcine whole liver or hepatocytes, xenogeneic immunorejection is not an issue because it can be avoided by employing semipermeable membranes

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or adsorbent columns. Donor pigs expressing major human hepatic proteins, but less porcine proteins, are ideal to minimize xenogeneic protein influx.

In the present study, we focused on producing transgenic pigs that express human albumin in the liver because albumin accounts for two-thirds of total serum protein and one-fourth of daily protein production in the liver. We introduced a recombinant human albumin (hAlb) gene coupled with an enhanced green fluorescent protein (EGFP) gene as a marker for transgenic animals. GFP co-expression would also be useful to trace transgenic hepatocytes when porcine-derived artificial livers are used for clinical purposes in future. We first produced transgenic mice carrying the fusion gene to examine its biological consequences. Subsequently, we produced transgenic pigs using the sperm vector method [7] and *in vitro* matured (IVM) oocytes.

## Materials and Methods

*Chemicals*

Chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.) unless otherwise indicated.

*DNA construct*

The 6.5 kb Sal 1-Bam H1 fragment of plasmid pCX-h-Alb/EGFP [9] (Genome Information Research Center, Osaka University, Osaka, Japan) was used to produce a transgenic mouse and pig. As shown in Fig. 1, this fusion gene was constituted with the 3.0 kb CAG-EGFP cDNA [8,9] and 3.5 kb CAG-human albumin cDNA.

*Production of a transgenic mouse*

Animals and collection of embryos: Seven-week-old female B6D2F1 and ICR mice were purchased from CLEA Japan (Tokyo, Japan) and used as embryo donors and recipients, respectively. The mice were housed in a room for over a week after purchase under conditions of constant temperature (20–23 C) and humidity (60–70%) and a 12-h light/dark cycle.

The 8 to 12-week-old female B6D2F1 mice were intraperitoneally given 7.5 IU of equine chorionic gonadotropin (eCG; Teikoku Zouki, Tokyo, Japan) and then 8.25 IU of human chorionic gonadotropin

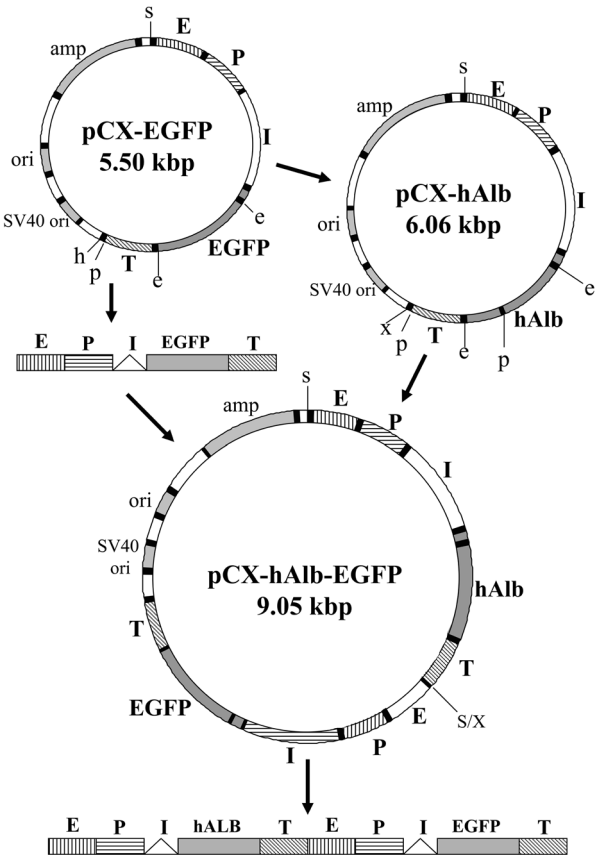


**Fig. 1.** Construction of pCX-hAlb-EGFP. E: CMV-IE enhancer, P: chicken $\beta$-actin promoter, I: intron, T: rabbit $\beta$-globin poly A signal, EGFP: EGFP cDNA, hAlb: human albumin cDNA. s: Sal I, e: EcoR I, p: Pst I, h: Hind III, x: Xho I, s/x: Sal I/Xho I.

(hCG; Teikoku Zouki) 47–48 h later to induce superovulation. They were mated with males of the same strain, and the females presenting a vaginal plug on the following morning were used as embryo donors. Pronuclear embryos were collected at 20–21 h after the hCG injection for the experiment.

Embryonic culture: Embryos were collected in M2 medium (CLEA Japan) and cultured in CZB medium [10] at 37.5 C in a humidified atmosphere of 5% $CO_2$ in air.

Production of transgenic mice by pronuclear injection and progeny tests: Pronuclear injection was performed as described by Hogan *et al.* [11]. The hAlb-EGFP construct was diluted to 5 or 10 ng/$\mu$l with TE buffer (pH 7.4) [11, 12] for injection. The DNA suspension, at either concentration, was injected into embryos and incubated for 5 days

until blastocyst stage. To determine which concentration was more efficient, the number of the blastocysts expressing EGFP was examined under a fluorescent microscope (TE300, Nikon, Tokyo, Japan). Based on the analysis, the 10-ng/$\mu$l DNA suspension was used for actual production of transgenic mice. The DNA suspension was injected into another set of pronuclear embryos and incubated for 4 days to the morula and early blastocyst stages. The embryos expressing GFP were transferred to the uterine horn of a pseudopregnant recipient. An 8 to 12-week-old female ICR mouse was used as a recipient; the mouse was mated with a vasectomized male and received transgenic embryos at 3 dpc (the time that vaginal plugs were found was taken as 0.5 dpc, days post coitus). The recipient was dissected on 19.5 dpc, immediately before parturition, to remove fetuses. The fetuses were nurtured for 3 weeks by an other female mouse from the same strain that had given birth on the same day. Expression of the GFP gene was examined under a fluorescent stereo microscope (MZ FLIII, Leica, Germany) using 2 to 3-mm sections of the tails cut from these transgenic mice. DNA was also isolated from the tail fragments for PCR analysis.

To examine germline transmission of the introduced genes, the 8-week-old founder mouse was mated with B6D2F1 to produce progeny (G1 mice). At 3 weeks, 2 to 3-mm pieces of the tails were cut from the G1 mice and analyzed for fluorescence as described above.

Sperm vector method: Introduction of DNA into the mouse oocytes by the sperm vector method was carried out as described previously [7, 14]. Briefly, mouse epididymal sperm frozen-thawed in CZB were co-incubated at a concentration of 2–7 $\times$ $10^5$ with the 6.5 kb fragment of pCX-hAlb-EGFP for 5 min at 0 C. After co-incubation, sperm heads, isolated by inflicting a Piezo-impact to the neck portion of spermatozoa, were individually microinjected into oocytes using a Piezo micromanipulator. Microinjected oocytes were cultured in CZB for 5 days to examine fertilization, development to blastocysts, and *in vitro* expression of the EGFP gene.

*Production of a transgenic pig*

*In vitro* maturation of porcine oocytes: Ovaries were collected at a local abattoir and transported to the laboratory in Dulbecco's phosphate buffered saline (PBS) containing 75 $\mu$g/ml potassium penicillin G, 50 $\mu$g/ml streptomycin sulfate, and 0.1% (w/v) polyvinyl alcohol (PVA). Cumulus-oocyte complexes (COCs) were collected from the antral follicles that were 3.0–6.0 mm in diameter by aspiration. COCs having at least three layers of compacted cumulus cells were selected and cultured in NCSU23 medium [13] supplemented with 0.6 mM cysteine, 10 ng/ml epidermal growth factor (EGF), 10% (v/v) porcine follicular fluid, 75 $\mu$g/ml potassium penicillin G, 50 $\mu$g/ml streptomycin sulfate, and 10 IU/ml eCG (Teikoku Zouki) and hCG (Teikoku Zouki). The COCs were cultured for 22 h twice, with or without hormones, in a humidified atmosphere of 5% $CO_2$ and 95% air at 38.5 C.

IVM oocytes with expanded cumulus cells were treated with 1 mg/ml hyaluronidase dissolved in Tyrode's lactose medium supplemented with 10 mM Hepes, and 0.3% (w/v) polyvinylpyrrolidone (PVP) (Hepes-TL-PVP), and denuded of cumulus cells by gentle pipetting. Oocytes having evenly granulated ooplasm and an extruded first polar body were selected for the experiments.

ICSI procedure and embryo transfer: As the sperm vector method was shown to be effective for introducing a transgene into mouse embyos, we applied it to the production of transgenic pigs.

Introduction of DNA into the IVM oocytes was carried out by the sperm vector method [7, 14]. Briefly, porcine sperm frozen-thawed in BTS [15] were co-incubated with DNA under the same conditions used for mouse sperm. After co-incubation, isolated sperm heads were microinjected individually into preactivated oocytes. Oocyte activation was induced by electrical stimulation (DC 150V/mm, 100 $\mu$sec) in activation solution consisting of 0.3 M mannitol, 50 $\mu$M $CaCl_2$, 100 $\mu$M $MgSO_4$, and 0.01% PVA (300 mOsm). Sperm-injected oocytes were cultured in NCSU23 supplemented with 4 mg/ml BSA [13] for 1 or 2 days.

Crossbred (Large White/Landrace $\times$ Duroc) prepubertal gilts weighing between 100 and 105 kg were used as recipients of sperm-injected embryos. To induce estrus, gilts were treated with a single intramuscular injection of 1500 IU eCG, followed by an intramuscular injection of 1500 IU hCG given 71 h later. Embryos were transferred to the oviduct of the recipients 3 days after hCG injection.

*Gene analysis of the transgenic mouse and pig*

Genomic DNA of the transgenic mouse and pig was extracted for PCR analysis by Qiagen DNeasy Tissue Kit (Qiagen, Valencia, CA, U.S.A.). The PCR reaction mix consisted of 200 $\mu$M dNTP, 0.5 $\mu$M of two primers, 2.5 mM of $MgCl_2$, 0.1 volume of 10 $\times$ reaction buffer, 1.25 U of Taq DNA polymerase and 50–100 ng DNA template. The hAlb cDNA primers, 5′-GCCGCTGCAGATCCTCATGAAT-3′ (forward primer) and 5′-TTATAAGCCTAAGGCAGCT-3′ (reverse primer), were used to obtain a targeted PCR product of 700 bp using a Robocycler (Stratagene, La Jolla, CA, U.S.A.). The PCR condition was 94 C for 5 min; 35 cycles of 94 C for 1 min, an annealing temperature of 60 C for 1 min, and 72 C for 1 min; and 72 C for 7 min.

For RT-PCR analysis, mRNA was extracted from the tissues by repeated freeze-thawing and use of a Qiagen RNeasy Mini Kit (Qiagen) according to the manufacturer's instruction. The frozen-thawed tissues were treated in lysis buffer containing 1% mercaptoethanol followed by homogenization in a QIAshredder column (Qiagen). The lysate was added to glycogen (250 $\mu$g/ml, molecular biology grade, Ambion, TX, U.S.A.) as an RNA carrier, and applied onto an RNeasy Mini Spin Column (Qiagen) for adsorption of RNA molecules. RNA was eluted with 50 $\mu$l RNase-free water containing 5 U RNase inhibitor, treated with DNase I (Takara, Kyoto, Japan), and stored at –80 C. The RT-PCR reaction mix consisted of 400 $\mu$M dNTP, 0.6 $\mu$M of each primer, 2.5 mM of $MgCl_2$, 0.2 volume of 5 $\times$ reaction buffer, 5 U of RNase inhibitor, 1 U of Qiagen OneStep RT-PCR Enzyme Mix (Qiagen), and approximately 10 ng RNA template. For human albumin, annealing temperature was 60 C, and the forward (5′-AAGCAAAAAGAATGCCCT-3′) and reverse (5′-CTTGGGCTTGTGTTTCAC-3′) primers were designed to obtain a targeted PCR product of 289 bp. For EGFP, the annealing temperature was 56 C, and the forward and reverse primers were designed to obtain a targeted PCR product of 306 bp as follows: 5′-TGAACCGCATCGAGCTGAAGGG-3′, and 5′-TCCAGCAGGACCATGTGATCGC-3′. The RT-PCR conditions were 50 C for 30 min and 95 C for 15 min for RT; and 94 C for 5 min; 40 cycles of 94 C for 1 min, annealing temperature for 1 min, and 72 C for 1 min; and 72 C for 7 min for PCR.

For Southern blot hybridization analysis, DNA was extracted by a conventional method from tissues of the founder transgenic pig, and was expected to have hAlb-EGFP insertions. For determination of the transgene copy number, pCX-hAlb-EGFP was first diluted to 1, 3, 10, 30, and 100 copies/genome, added to 5 $\mu$g wildtype DNA, and used as a positive control. In addition, 5 $\mu$g wildtype DNA was used as a negative control.

The genomic DNA of each sample, as well as the positive and negative controls, were completely digested with EcoRI, and the products of the DNA fragments were separated by electrophoresis on 1.2% agarose gel and transferred onto a nylon membrane (Hybond-XY, Amersham Biosciences, Piscataway, NJ, U.S.A.). The EGFP constructs were labeled with [$^{32}$P] by random priming (Megaprime DNA Labelling System, Amersham Biosciences) and hybridized to the nylon membrane. After washing the membrane to remove nonspecific radioactivity, the [$^{32}$P]-labelled EGFP probe, complementarily bound to the target DNA fragments, was detected by autoradiography.

The pig, whose sample had a radioactive band at 0.7 kb, was determined to have the albumin-EGFP transgene. The signal intensity of each sample was compared to that of the positive controls to determine the approximate copy number of the transgene.

## Results

*Production of a transgenic mouse*

When the 5- and 10-ng/$\mu$l DNA suspension was injected into pronuclear-stage embryos (Table 1), 73.4% (47/64) and 65.4% (34/52) of the injected embryos developed to blastocysts, respectively. Of these, fluorescent signals were detected in 4.3% (2/47) for the 5-ng/$\mu$l group and in 20.6% (7/34) for the 10-ng/$\mu$l group, indicating significantly higher expression ($p<0.05$) of the transgene in the latter. There was no significant difference in the rate of blastocyst formation between the two DNA concentrations.

When the sperm vector method was used to introduce DNA into mouse oocytes, rates of cleavage (97.5%, 39/40) and blastocyst formation (62.5%, 25/40) were equal to those obtained by pronuclear injection. The proportion of blastocyts expressing GFP was significantly ($p<0.05$) higher in the sperm vector group.

**Table 1.** *In vitro* development of mouse embryos following gene transfer by the pronuclear injection or sperm vector method

| | No. of embryos cultured* | No. of normally cleaved embryos (%) | No. of embryos developed to blastocysts | GFP expressing embryos/ blastocysts (%) |
|---|---|---|---|---|
| Pronuclear injection (5 ng/$\mu$l) | 64 | 59 (92.2) | 47 (73.4) | 2/47 (4.3)[a] |
| Pronuclear injection (10 ng/$\mu$l) | 52 | 43 (82.7) | 34 (65.4) | 7/34 (20.6)[b] |
| Sperm vector | 40 | 39 (97.5) | 25 (62.5) | 15/25 (60.0)[c] |

A 6.5 kb fragment of pCX-human albumin-EGFP was introduced.
* Embryos with two pronuclei were cultured.
[a, b, c] Values with different superscript differ significantly.

Seventeen GFP expressing morula- to early blastocyst-stage embryos obtained by pronuclear injection (10-ng/$\mu$l) were transferred to the uterine horn of a recipient, which, as a result, produced 3 morphologically normal fetuses. Of these fetuses, a male (33.3%) was GFP positive in its tail fragment and showed a 0.7-kb PCR product that corresponds to the hAlb gene. This founder transgenic mouse was then mated with a female B6D2F1 to produce 12 G1 progeny. Six (50%) of these G1 showed GFP fluorescence in their tail fragments. The sex ratio (male/female) of the G1 was 4/8, and those expressing GFP were all female.

*Production of a transgenic pig*

Transfer of 614 microinjected oocytes into 3 recipient gilts resulted in 2 pregnancies, one of which ended in miscarriage on day 24 of gestation. An apparently normal, female transgenic piglet was born 115 days after transfer. The piglet was born at midnight and found dead the following morning; therefore, it was unclear whether it was born alive or stillborn. The piglet showed clear expression of GFP in the hooves, and the skin of the piglet also showed fluorescence. Faint fluorescence in the blood and other organs, including the liver, was observed during autopsy. Autopsy did not show any sign of anatomical abnormalities.

*Gene analysis of the piglet*

Figure 2 shows analysis of integration and expression of the introduced gene. PCR analysis of genomic DNA extracted from several tissues, including the skin, hoof, muscle, aorta, lung, liver, uterus, and ovary, showed that all the tissues examined carried the transgene (Fig 2-a). Expression of EGFP and hAlb genes was confirmed by RT-PCR, as shown in Fig 2-b and c.

The genomic southern blot revealed a 0.7 kb band in the DNA sample of the obtained piglet. We determined that this founder piglet had integrated about 100 copies of the transgene by comparing it with the band intensity of the positive control.

## Discussion

When the development of embryos injected with the DNA suspension prepared at 5 or 10 ng/$\mu$l was examined *in vitro*, there was no difference in the rate of blastocyst formation between these concentrations. The embryos injected with the 10-ng/$\mu$l DNA suspension, however, showed a significantly higher rate of EGFP expression ($p<0.05$) at the blastocyst stage. Based on this result, we used the 10-ng/$\mu$l DNA suspension for production of transgenic mice.

In this study, we selected transgenic embryos using EGFP expression as a marker and then transferred them to a recipient. As a result, a single transfer successfully produced transgenic progeny. This result indicated that pre-transfer selection using EGFP is an efficient method to screen transgenic embryos [16, 17], though embryos with transient expression could not be eliminated. Nevertheless, the number of embryos to be transferred to produce transgenic offspring could be significantly reduced by pre-transfer selection.

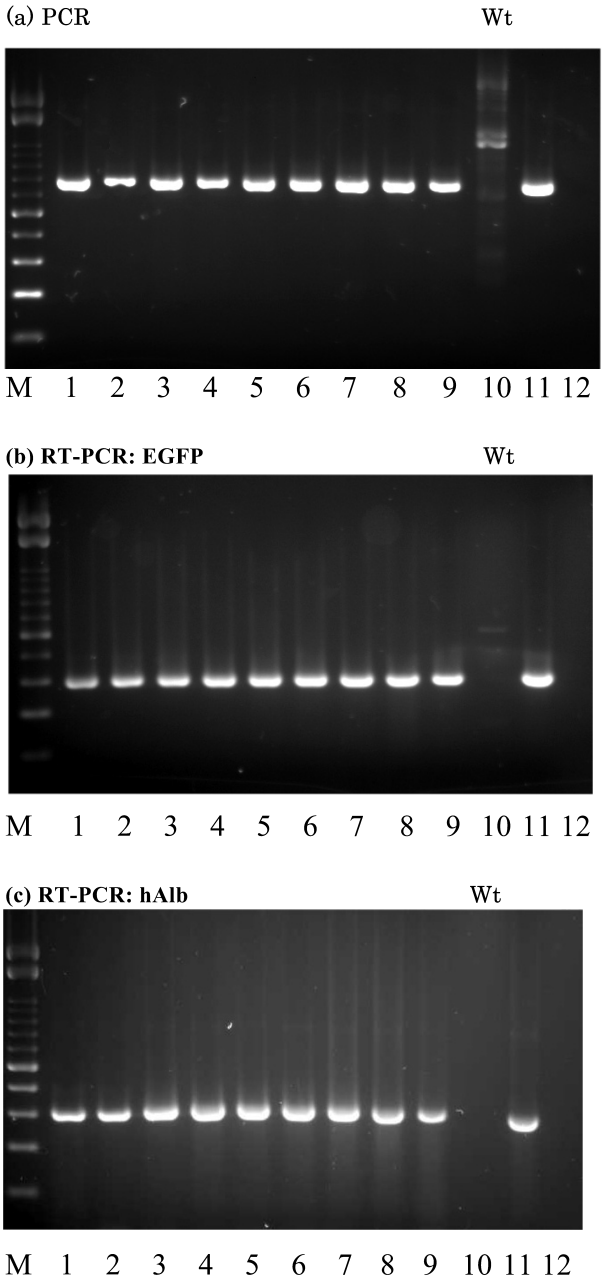


**Fig. 2.** Analysis of the transgenic pig carrying pCX-hAlb-EGFP. Lane M: DNA ladder, 1: skin, 2: hoof, 3: muscle of the left anterior limb, 4: muscle of the right anterior limb, 5: aorta, 6: liver, 7: lung, 8: uterus, 9: ovary, 10: ovary of a wild-type pig, 11: pCX-hAlb-EGFP, 12: negative control. (a) PCR analysis of genomic DNA extracted from the above tissues. (b) RT-PCR analysis of EGFP messenger RNA extracted from the above tissues. (c) RT-PCR analysis of human albumin messenger RNA extracted from the above tissues.

In addition, germline transmission of the transgene was confirmed by progeny test of the transgenic mice.

It has been reported that transgenic mice expressing pCX-EGFP are easily identified by detecting fluorescence in the skin under a portable UV light [9]. The hAlb-EGFP expressing mouse in this study, however, did not have sufficient fluorescence detectable using a portable UV light. Nonetheless, GFP fluorescence in tissue sections was detectable under a fluorescent microscope, and the result was consistent with that of PCR analysis. The level of GFP expression was low in this study, probably because the copy number of the integrated hAlb-EGFP was small, or the construct was inserted to a chromosome region where gene transcription was not very active. Southern blot analysis was not performed for the transgenic mouse, but EGFP was expressed not only in the founder mouse but also in its progeny, suggesting that the hAlb-EGFP construct had been integrated into the chromosomal DNA. Further, the transgenic mouse showed normal development and fertility, indicating that human albumin and EGFP expression in the mouse did not have biologically harmful influences. Since the results in mice indicated that introduction of the hAlb-EGFP gene is less likely to perturb early embryonic development and produces transgenic progeny capable of germline transmission, we concluded that the gene construct could be also used for production of transgenic pigs.

Following the experiment in mice, we successfully produced a transgenic pig by the sperm vector method. We used modified sperm vector method [7], which was developed from the original method described by Perry *et al.* [14]. It is noteworthy that our study showed that the sperm vector method can be also used in pigs. Further, we used *in vitro* matured (IVM) oocytes, not pronuclear-stage embryos, as traditionally described [for review see 18], as sperm recipients. This will greatly reduce the cost and workload required for transgenic pig production, making the application of transgenic pigs more practical. The method still requires improvment, as the efficiency of transgenic progeny production is still low. In addition, the transgenic pig produced in this study did not survive, which might be attributed to the large copy number for transgene integrated in the transgenic pig. Nevertheless, the sperm vector

method using IVM oocytes is promising as a practical technology.

For development of transgenic animals as bioreactors that secrete human pharmaceuticals, significant technological advances for introduction of foreign genes into the endocrine system of livestock have been made. Traditionally, human serum albumin was isolated from human blood as a plasma protein fraction by the hypothermic ethanol fractioning method. More recently, recombinant human serum albumin was developed and has been used since for large scale synthesis in yeast [19]. However, these methods are disadvantageous as they require a supply of human blood and have high production costs. On the other hand, the use of transgenic livestock has enabled efficient yield of transgene products in their tissues, milk, and blood [20, 21]. For instance, large-scale, safe, and low-cost production of the human antihemophilic factor by transgenic livestock may greatly contribute to the treatment of hemophilia [20, 21].

The results of our study demonstrated that the transgene was integrated and expressed in the porcine liver, which is crucial for our goal of using the transgenic liver for bioartificial liver support. Preferably, promoters for liver-specific proteins, such as albumin or metallothionein, should be used for recombinant gene expression, as we are targeting production of human albumin in the porcine liver. In the present study, we demonstrated that the CAG promoter consisting of the chicken $\beta$-actin promoter and CMV-IE enhancer [22] induced systemic expression in various organs and tissues, including the liver of the transgenic pig. Moreover, the fused gene used in this study showed that human albumin cDNA and EGFP cDNA, both of which have their own CAG promoters, could be expressed stably in various tissues in the transgenic pig.

The aim of the present study was to collect fundamental data for production of transgenic pigs by the sperm-vector method using IVM oocytes. As a first step, we investigated whether pre-transfer selection of transgenic embryos can be efficiently performed using a DNA construct that consisted of CAG promoter and EGFP. In fact, a transgenic mouse was obtained from the pre-selected embryos. This result encourages pre-transfer selection of transgenic porcine embryos produced by the sperm-vector method, provided that developmental ability of porcine embryos can be maintained during the prolonged culture period.

In conclusion, our study showed that it is possible to produce transgenic pigs, which express human albumin not only in the liver but also in all over the body; these genetically modified pigs will greatly contribute to research into regenerative medicine, transplantation, and bioartificial organ production.

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