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Original article

In vivo gene transfer into the blastoderm of early developmental stage of chicken

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Summary — An attempt was made to improve gene transfer into chick embryos in order to produce transgenic chickens. The β-actin-*lacZ*/ MiwZ, a marker gene in transfection reagent, was injected into the blastodisc of either unincubated fertilized eggs (stage X) or eggs induced from the shell gland by treating the hens intravenously with oxytocin or arginine vasotocin (stages IV–VI). All the manipulated embryos were incubated to reach stage XIV, the period at which primordial germ cells (PGCs) migrate from the germinal crescent to the gonadal anlage via the blood stream. MiwZ was detected in the embryos, extraembryonic tissues and blood by the histochemical staining method of β-galactosidase. The MiwZ DNA was detected in 57% (127/221) of the survival embryos and in 9% (12/127) of the embryonic tissues. The expression was observed mosaically in the epidermis, heart and neural tube. The PGCs in the blood collected from the vitelline artery or dorsal aorta also showed a positive histochemical staining. However, the expression of MiwZ using the soft shelled eggs was more intense in the extraembryonic tissues, although it did not emerge in the embryos. Thus, it is possible to introduce an exogenous gene into the embryonic tissues using incubated fertilized eggs without sacrificing the hens. This technique for successive genetic operations should facilitate the production of transgenic chickens.

transgenic chicken / *LacZ* / primordial germ cells / chick embryo / PCR

Résumé — Transgenèse dans le blastoderme de jeunes embryons de poulet. Ce travail a été réalisé pour tenter d'améliorer l'efficacité du transfert de gènes d'intérêt dans l'embryon de poulet afin d'obtenir des poussins transgéniques. Un gène marqueur, le β-actin-*lacZ*/MiwZ, a été injecté dans le blastodisque d'œufs fécondés soit immédiatement après la ponte (stade X), soit

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juste après leur expulsion de l'utérus (stades IV-VI ; expulsion provoquée par injection intraveineuse d'occytocine ou d'arginine-vasopressine). Tous les embryons traités ont ensuite été incubés jusqu'au stade XIV qui correspond à la période pendant laquelle les cellules germinales primordiales (CGP) migrent (transport par voie sanguine) du croissant germinal vers le site d'implantation des gonades. L'expression du gène MiwZ dans l'embryon, les tissus extra-embryonnaires et le sang a été détectée par coloration histochimique (β-galactosidase). Cette expression a pu être retrouvée dans l'ADN de 57 % des embryons survivants (127/221) et dans 9 % des tissus extra-embryonnaires. Dans cette partie, le gène MiwZ s'est exprimé (distribution en mosaïque) dans l'épiderme, le cœur et le tube neural. Les CGP retrouvées dans le sang artériel (artère vitelline ou aorte dorsale) ont, elles aussi, montré une réaction colorée positive. Par ailleurs, l'expression du MiwZ dans les œufs prélevés dans l'utérus est apparue nettement plus intense que celle observée dans les tissus extra-embryonnaires mais ne s'est pas manifestée dans les embryons de ces œufs. Pour conclure, nos observations montrent qu'il est possible d'introduire des gènes exogènes dans les tissus embryonnaires de poulet à partir d'œufs juste pondus, ce qui évite le sacrifice de la femelle. Cette technique devrait ainsi faciliter dans l'avenir la production de poussins transgéniques.

transgénèse / poulet / cellules germinales primordiales / amplification par PCR

INTRODUCTION

A complete culture system has been established for the chick embryo from the single cell stage of hatching (Perry, 1988). This technique was a breakthrough for the investigation of developmental events at early embryonic periods in vitro, and it will be a useful method for analyzing embryogenesis, particularly at the molecular level. In fact, various methods have since been developed to produce transgenic chicken. Sang and Perry (1989) reported that foreign DNA was injected into the germinal disc of fertilized ova obtained from the magnum of the oviduct. Naito et al (1994) observed that the introduction of foreign DNA into the somatic and germ cells was successfully achieved by injection into the germinal disc of fertilized ova at the single cell stage. In these methods, however, the hens were sacrificied to obtain fertilized ova and most of the chick embryos expressed the DNA mosaically (Naito et al, 1991). In addition, Petitte et al (1990) developed somatic and germline chimeras by injecting dispersed donor blastdermal cells into the compromised recipient embryos, thus providing a practical technique for the production of transgenic chimera chickens. Expression of the introduced DNA, with MiwZ as a marker gene, was observed in extraembryonic tissues (Otsuka et al, 1990); however, the rates of DNA transfection into the donor cells and their incorporation into the germlines were very low. Furthermore, γ-irradiation of the recipient embryo, prior to injection of the donor cells, consistently yielded somatic and germline chimeric chickens (Carsience et al, 1993). More recently, a technique using primordial germ cells (PGCs) as donor cells has been developed (Tajima et al, 1993). With this technique, the PGCs are noted for manipulations of germlines, sexing and conservation of the genetic materials using the methods identical to embryonic stem cells in the mice.

In the present report, we have improved an exogenous gene transfer into the chick embryo by using fertilized eggs with hard shells. This method is advantageous in the production of transgenic chickens, because the gene transfer is easily achieved without sacrificing the hens, leading to higher hatchability.

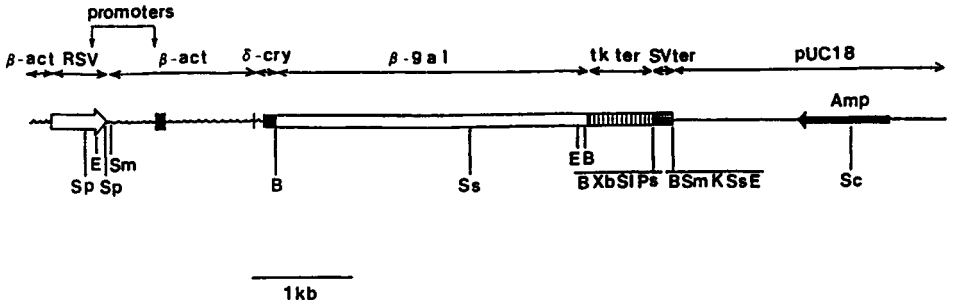

Fig 1. Structure of the recombinant plasmid, MiwZ. β-actin (β-act) and δ-crystallin (δ-cry) sequences are shown by the wavy line and their exon sequences by the solid boxes. Bacterial sequences (β-gal) are indicated by the open box, HSV-tk sequences by the vertically striped box, SV40 sequences by the horizontally striped box, the RSV LTR sequences by the open arrow and plasmid vector sequences by the solid horizontal line. Promoters and termination signals (tk ter and SV ter) are also indicated. The restriction sites are indicated by B, BamHI; E, EcoRI, K, KpnI; Ps, PstI; Sc, ScaI; Sl, SalI; Sm, SmaI, Sp, SphI; Ss, SstI; and Xb, XbaI.

MATERIALS AND METHODS

Preparation of the DNA solution

Figure 1 shows the structure of the constructed plasmid MiwZ marker gene, which contains the *E coli*-β-galactosidase gene *(lacZ)* under the control of RSV enhancer and the chicken β-actin gene promoter/enhancer (Suemori et al, 1990). The circular form MiwZ (6.25 μg) was mixed with 22.5 μg of transfection reagent (Boehringer, Germany), and then diluted up to 50 μL with Hepes-buffered saline (HBS; 20 mM Hepes containing 150 mM NaCl, pH 7.4), in a polystyrene tube on ice according to the instructions but with slight modification. A micropipette (G-1, Narishige, Tokyo, Japan), whose tip was developed down to the outside diameter of about 40 μm, was filled with the DNA solution prior to microinjection.

Microinjection of the DNA solution

Fertilized eggs laid by a strain of Rhode Island Red hens were used as host embryos. The eggs with soft shells were obtained by treating the hens intravenously with oxytocin or arginine vasotocin at 7 h after oviposition of the preceding egg. Three trials and two control experiments were carried out as follows:

When both unincubated eggs (stage X; trial 1; Eyal-Giladi and Kochav, 1976) and temporarily preserved eggs at 4 °C for 3 h as soon as laid (stage X; trial 2) were used as host embryos (about 6 x 10^4 cells), the window of 10–15 mm in diameter was opened at the sharp end of the egg shell and the thick albumen around the germinal disc was removed at a volume of about 1.5 mL. The micropipette filled with the DNA solution was inserted into the central area of the germinal disc through the vitelline membrane; the injection volume of the DNA solution was about 0.5 mL. The window of the shell was then closed carefully with a tape. All the procedures for the injection and embryo culture were carried out under sterile conditions.

The eggs with soft shells used as host embryos (about 100 cells) (stage IV–VI; trial 3) were washed twice in Ringer's solution supplemented with dihydrostreptomycin (1 mg/mL) and penicillin (10 IU/mL). The shell membrane was removed, and the DNA solution was injected into the germinal disc vertically through the thick albumen at a volume of about 0.3 mL. MiwZ DNA (control 1) or Boehringer reagent (control

2) diluted with HBS was injected into the germinal disc of the laid eggs with hard shells as described earlier.

Culture of the manipulated embryos

Manipulated eggs (trials 1, 2 and controls) were incubated for 50–72 h while rocking intermittently at an angle of 90° in hourly cycles at 37.5 °C with a relative humidity of 60%. They were cultured to reach stage XIV (Hamburger and Hamilton, 1951), the period at which PGCs begin to migrate from the germinal crescent to the gonadal anlage via the blood stream (Kuwana, 1993). The ova (trial 3), however, were placed in a glass jar (100 mL), without adding a culture medium, at 41.5 °C for 18 h. They were then transferred to recipient eggshells without the thick albumen and cultured to reach the same stage according to Perry's culture system.

Detection of MiwZ DNA expression

The expression of MiwZ DNA was detected by a histochemical staining method of β-galactosidase. Blood samples were collected from the vitelline artery or dorsal aorta by a fine glass pipette. PGCs enriched by cytologic screening were smeared on a glass slide and fixed with 1% glutaraldehyde in phosphate-buffered saline (PBS) for 5 min at 4 °C. After washing with PBS, the smears were stained for 1 h at room temperature with 0.05% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, Sigma, USA), 1 mM $MgCl_2$, 0.1% Triton X-100, 3 mM potassium ferrocyanide and 3 mM potassium ferrocyanide in PBS. The embryos and extraembryonic tissues were removed from the yolk immersed in PBS and fixed with 1% glutaraldehyde in PBS for 20 min at 4 °C. After washing three times with PBS, the samples were stained for 2 h at 37 °C with X-gal. The intensity of the expression was estimated by counting the positive cell colonies in the extraembryonic tissues.

DNA extraction and PCR analysis

Whole embryos (stage XIV) were removed from the yolk and washed with TNM buffer (20 mM Tris-HCl buffer, pH 7.5, containing 100 mM NaCl and 1.5 mM $MgCl_2$). The total DNA was extracted from the homogenized embryos by the phenol-chloroform method (Sambrook et al, 1989). The polymerase chain reaction (PCR) analysis was carried out on 500 ng DNA samples in order to detect the presence of the *lacZ* gene, as appropriate. To estimate the copy number, PCR reactions of the endogenous gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were also performed on each DNA sample under standard procedures. Five μL of the products of each reaction were then combined and subjected to 1% agarose gel electrophoresis (Love et al, 1994).

RESULTS

The survival rates for the manipulated embryos of eggs with no incubation (trial 1) and temporal preservation at 4 °C for 3 h (trial 2) were 73.8 and 79.7% at stage XIV, respectively. However, in the case of the manipulated embryos obtained by premature oviposition (trial 3), the survival rate was 42.9%. The expression of the MiwZ DNA, as revealed by β-galactosidase activity, was detected in 57.8 (trial 1), 56.6 (trial 2) and 66.7% (trial 3) of the survival embryos. Most of them were detected only in the extraembryonic tissues around the embryo (fig 2A), but the embryonic expression of the MiwZ was detected in six cases (11.5%) of the 52 embryos at trial 1 and six cases (8.7%) of the 69 embryos at trial 2 (fig 2B). There was little difference in intensity of the expression between trial 1 and trial 2. However, in trial 3, the expression in the extraembryonic tissues was much more intense than the other two trials, although no expression was detected in the embryo (fig 2C) (table I). When the DNA (control 1) or transfection reagent (control 2) was injected into the embryos individ-

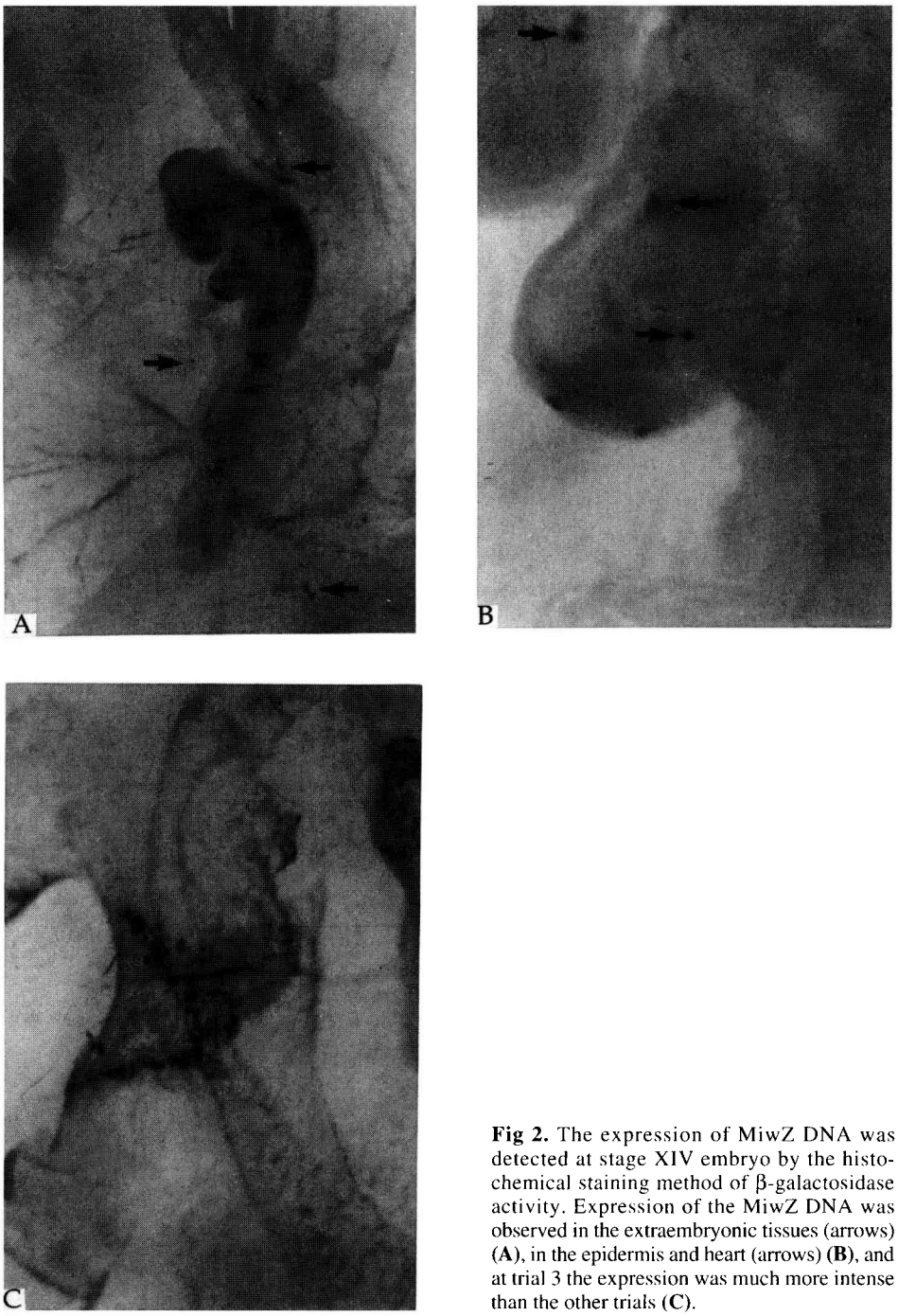

Fig 2. The expression of MiwZ DNA was detected at stage XIV embryo by the histochemical staining method of β-galactosidase activity. Expression of the MiwZ DNA was observed in the extraembryonic tissues (arrows) **(A)**, in the epidermis and heart (arrows) **(B)**, and at trial 3 the expression was much more intense than the other trials **(C)**.

Table I. Expression of MiwZ DNA in chick embryos at stage XIV and survival rate.

	No of manipulated embryos	*Survival rate (%)*	*Rate of MiwZ DNA expression*		*Intensity of expression **
			Extraembryonic (%)	*Embryonic (%)*	
Trial 1	122	90/122 (73.8)	52/90 (57.8)	6/52 (11.5)	41.8
Trial 2	153	122/153 (79.7)	69/122 (56.6)	6/69 (8.7)	30.7
Trial 3	21	9/21 (42.9)	6/9 (66.7)	0/6 (0.0)	2608.0
Control 1	71	63/71 (88.7)	0/63 (0.0)		
Control 2	72	67/72 (93.1)	0/67 (0.0)		

* The intensity of expression was estimated by counting the positive cell colonies.

ually, they showed no expression of the MiwZ DNA. Table II shows the expression sites of MiwZ in the 12 embryos at trials 1 and 2. These expression sites were detected in the epidermis (50%), heart (33.3%) and neural tube (16.7%) in a mosaic manner. As for the other expression sites, the caput site was noted in particular. In trial 2, the expression was also detected in the PGCs in blood collected from the vitelline artery or dorsal aorta of the embryos (16.7%) (fig 3).

On the one hand, the manipulated embryos (trials 1 and 2) were classified as single copy, mosaic and negative at stage XIV, demonstrating that the rates of MiwZ DNA detection as a single copy were 1.7% for trial 1 and 4.2% for trial 2, respectively (table III). More than 60% of the embryos were classified as mosaic in trials 1 and 2 (table III).

Table II. Expression sites of MiwZ DNA in embryonic tissues.

Expression site	*No of embryos **
Epidermis	6
Heart	4
Neural tube	2
Primordial germ cells	2
Others	6

* Data show the number of 12 embryos that expressed MiwZ in the embryonic tissues.

Table III. Copy number of the plasmid DNA, MiwZ.

Class	*Trial 1 (%)*	*Trial 2 (%)*
(i) Negative	19 (31.7)	19 (26.4)
(ii) Mosaic	40 (66.6)	50 (69.4)
(iii) Single-copy	1 (1.7)	3 (4.2)
Total	60	72

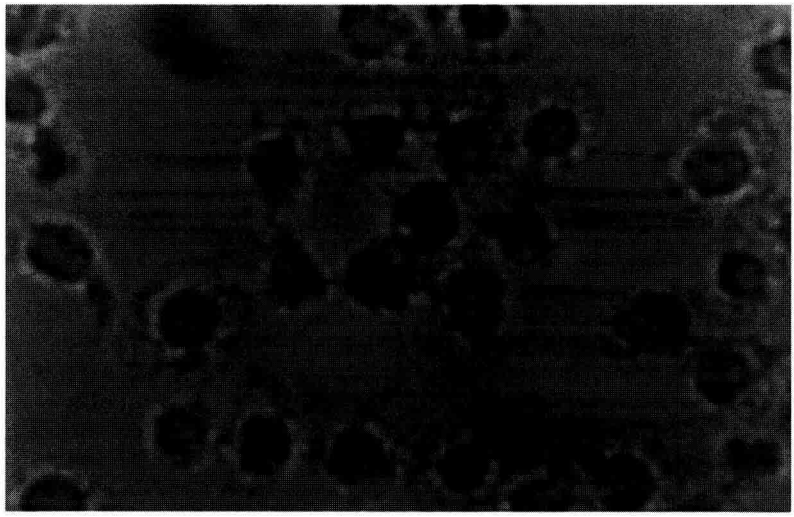

Fig 3. Expression of the MiwZ DNA in the primordial germ cells (PGCs). At trial 2, the expression was detected in the PGCs in blood collected from the vitelline artery or dorsal aorta of the embryos (arrows).

DISCUSSION

In this report we described the expression of the MiwZ DNA in the chick embryos, extraembryonic tissues and PGCs by in vivo transfection. At first, we expected that a temporal inhibition of the embryonic metabolism by preserving at 4 °C for 3 h would keep the host embryos in a stable condition and thus increase the survival rates for the manipulated embryos and improve MiwZ DNA expression at restricted regions. Consequently, temporarily preserved eggs were used as host embryos (trial 2). Unfortunately, there were little differences in intensity, survival rates and DNA expression between trial 1 and trial 2, at which fertilized eggs with no incubation were used as host embryos. However, it is interesting to note that 4.2% (trial 2) and 1.7% (trial 1), at a level equivalent to one copy per cell from a whole embryo, were analyzed using the PCR method. Furthermore, the MiwZ expression was detected in the PGCs in blood collected from the vitelline artery or dorsal aorta only in trial 2. In the same way, exposure to g irradiation was used to compromise the development of recipient embryos (stage X) prior to injection with blastodermal cells. The irradiation caused an increase in the rate of somatic and germline chimeras (Thoraval et al, 1994). Thus, it may be possible to obtain a transgenic chimera with chimerism limited to a particular germline and a restricted region by controlling the metabolic condition of the host embryos as well as the site of injection. The embryonic expression was detected in particular at the epidermis of the caput, suggesting that some of the transferred DNA cells are incorporated with the epiblast while others are caught by the hypoblast sheet that is formed. In this experiment, DNA solution was injected just under the blastoderm. At this stage of embryonic development, it has been reported that a single cell layer is formed and PGCs are observed in the central area of the germinal disc (Ginsburg and Eyal-Giladi, 1987). However, the central area of blastodermic layers is a neural presumptive region and many cells in this area are destined to form the brain and the epidermis of the caput (Rudink, 1948). In the experiment using the embryos obtained by premature oviposition, the expression was as intense as using the fertilized ovum at the single-cell stage (Naito et al, 1991). Taking

these data together, it is suggested that the mosaicism is inhibited by injecting into the chick embryo at the earlier stage of development. However, the burdens of in vitro culture and microinjection at the early stage resulted in the low survival rate in this experiment (42.9%).

An advantage of our method is that an exogenous gene is able to be easily transferred into the embryonic tissues without sacrificing the hens, thus resulting in a higher hatchability (39.0%, 25/64) than with a conventional method of in vitro culture (Naito et al, 1994). This technique for successive genetic operations should facilitate the investigation of embryogenesis in early chick embryos at the molecular level. However, two questions remain unanswered: the reason that many expressions of DNA were detected in extraembryonic tissues and the frequence that the DNA was integrated with the host chromosomes. In order to assure its chromosome integration, devises for introducing foreign DNA may be essential and an increased efficiency in the production of a germline chimera may be necessary for the production of transgenic chickens. Consequently, further investigation into the establishment of avian PGC lines in the same manner as embryonic stem cells of mice is anticipated.

REFERENCES

Carsience RS, Clark ME, Gibbins AMV, Etches RJ (1993) Germline chimeric chickens from dispersed donor blastocermal cells and compromised recipient embryos. *Development* 117, 669-675

Eyal-Giladi H, Kochav S (1976) A complementary normal table and a new look at the first stages of the development of the chick. *Dev Biol* 49, 321-337

Ginsburg M, Eyal-Giladi HE (1987) Primordial germ cells of the young chick blastoderm originate from the central zone of the areapellucida irrespective of the embryo-forming process. *Development* 101, 209-219

Hamburger V, Hamilton H (1951) A series of normal stages in the development of the chick embryo. *J Morphol* 88, 49-92

Kuwana T (1993) Migration of avian primordial germ cells toward the gonadal anlage. *Dev Growth Differ* 35, 237-243

Love J, Gribbin C, Mather C, Sang H (1994) Transgenic birds by DNA microinjection. *Bio/Technol* 12, 60-63

Naito M, Agata K, Otuka K, Kino K, Ohta M, Hirose K, Perry MM, Eguchi G (1991) Embryonic expression of β-actin-*lacZ* hybrid gene injected into the fertilized ovum of the domestic fowl. *Int J Dev Biol* 35, 69-75

Naito M, Sasaki E, Ohtaki M, Sakurai M (1994) Introduction of exogenous DNA into somatic and germ cells of chickens by microinjection into the germinal disc of fertilized ova. *Mol Reprod Dev* 37, 167-171

Otsuka K, Kino K, Ohta M, Hirose H, Mochii M, Agata K (1990) Transplantation of blastderm cells transfected with foreign DNA into chicken blastderm. *Jpn Poult Sci* 27, 221

Perry MM (1988) A complete culture system for the chick embryo. *Nature* 331, 70-72

Petitte JN, Clark ME, Liu G, Verrinder Gibbins AM, Etches RJ (1990) Production of somatic and germline chimeras in the chicken by transfer of early blastodermal cells. *Development* 108, 185-189

Rudink D (1948) Prospective areas and differentiation potencies in the chick blastoderm. *Ann New York Acad Sci* 49, 761-772

Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn (C Nolan, ed), Cold Spring Harbor Lab Press, NY, USA

Sang H, Perry MM (1989) Episormal replication of cloned DNA injected into the fertilized ovum of the hen. *Mol Reprod Dev* 1, 98-106

Suemori H, Kadodawa Y, Goto K, Araki I, Kondoh H, Nakatsuji N (1990) A mouse embryonic stem cell line showing pluripotency of differentiation in early embryos and ubiquitous β-galactosidase expression. *Cell Differ Dev* 29, 181-186

Tajima A, Naito M, Yasuda Y, Kuwana T (1993) Production of germ line chimera by transfer of primordial germ cells in the domestic chicken. *Theriogenology* 40, 509-519

Thoraval P, Lasserre F, Coudert F, Dambrine G (1994) Somatic and germline chicken chimeras obtained from brown and white leghorns by transfer of early blastodermal cells. *Poul Sci* 73, 1897-1905