Asian-Aust. J. Anim. Sci.
Vol. 21, No. 6 : 801 - 806
June 2008

www.ajas.info

Avian Somitic Cell Chimeras Using Surrogate Eggshell Technology

Paul E. Mozdziak*, Dee Hodgson and James N. Petitte
Department of Poultry Science, Campus Box 7608/134 Scott Hall, Raleigh NC 27695, USA

ABSTRACT : A classical technique to study somitic cell fate is to employ the cross-transplantation of quail somites into a chick host. The densely stained nucleoli of the quail cells makes it possible to assess the fate of the donor quail cells in the chick host. Classical somite transplantation techniques have been hampered by the necessity of a small opening in the chick eggshell, difficulty in hatching the offspring and interspecies post-hatch graft rejection. With the advent of transgenic chicken technology, it is now possible to use embryos from transgenic chickens expressing reporter genes in somite cross-transplantation techniques to remove any possibility of interspecies graft rejection. This report describes using a surrogate eggshell system in conjunction with transgenic chick:chick somitic cell cross-transplantation to generate viable chimeric embryos and offspring. Greater than 40% of manipulated embryos survive past 10 days of incubation, and ~80% of embryos successfully cultured past 10 days of incubation hatched to produce viable offspring. (**Key Words :** Embryo, Chick, Transgenic, eGFP)

INTRODUCTION

The surgical accessibility of the chick embryo has made it a classical model to study embryonic development, and the chick embryo is an important tool that has provided much information about embryonic developmental processes. The quail-chick grafting procedure has been another important tool to learn about the developmental fate of cells in the embryo. The densely brilliant magenta nucleolar-associated heterochromatic DNA in Feuglen stained sections distinguishes the donor quail cells from host chick cells (Le Douarin, 1973a, b). Furthermore, the quail nucleolor marker is heritable making it possible to follow the developmental fate of the quail cells in a chick host.

Many significant studies about somite fate were performed using the quail-chick cross-transplantation technique, and somite cross-transplantation has been employed to describe the origin of the brachial muscles (Beresford, 1983), identify a population of cells within the somite that give rise to the muscles of the back and a second population of cells that give rise to the limb musculature (Ordahl and Le Douarin, 1992), and the fate of the first somite (Huang et al., 1997). In addition, somite transplantation can generate viable avian spinal cord chimeras, but they suffer from a demyelinating disease (Kinutani and Le Douarin, 1985; Kinutani et al., 1986), have a mapped the origin of the limb musculature (Chevallier et al., 1977; Chevallier et al., 1978; Christ and Brand-Saberi, 2002), and the origin of the pectoral muscles in the avian embryo (Beresford et al., 1978). However, a significant limitation of the quail-chick model is that viable offspring hatch at a low rate (~7.4%; Kinutani and Le Douarin, 1985). Furthermore, only 3% of the chimeric embryos survived a few weeks after hatching, and these surviving chimeric embryos develop interspecies graft rejection, while chick-chick chimeras do not develop post-hatch graft rejection (Kinutani and Le Douarin, 1985; Kinutani et al., 1986).

With the advent of transgenic chicken technology (Mozdziak et al., 2003; Mozdziak and Petitte, 2004; Chapman et al., 2005; Lee et al., 2004; Lee et al., 2005; Mozdziak et al., 2006) it is now possible to cross-transplant somitic tissue carrying reporter genes to map cell fate in the post-hatch animal following an embryonic manipulation without any problems associated with graft rejection. Furthermore, with newly developed lines of transgenic chickens, donor cells expressing fluorescent reporter genes in combination with a wild-type chick background makes it possible to follow the developmental fate of cells in ovo based upon the reporter gene expression (Chapman et al., 2005).

Traditional eggshell windowing has proven to be a

* Corresponding Author: Paul Mozdziak. Tel: +1-919-515-5544, Fax: +1-919-515-2625, E-mail: pemozdzi@unity.ncsu.edu
Received September 22, 2007; Accepted December 10, 2007

powerful tool for preparing and processing host embryos for developmental biology experiments as well as to provide an access point to deliver a viral vector to the blastoderm for generating transgenic chickens (Speksnijder and Ivarie, 2000; Andacht et al., 2004; Chapman et al., 2005). However, researchers must employ a small opening in the eggshell for access to the embryo providing a technical challenge for complex manipulation of the embryos. However, the major drawback to windowing technology is that culturing the embryos through hatching has proven to be difficult (Fisher and Schoenwolf, 1983; Fineman et al., 1986; Fineman and Schoenwolf, 1987). Although it is possible to inject fluid into somites, and culture the embryos through hatching with a 60% successful hatch rate for a stage 15 embryo (Giamario et al., 2003), the more invasive procedure of somite cross-transplantation was not attempted in the previous study. Recently, a surrogate eggshell technique was designed as an improvement (Borwornpinyo et al., 2005) over the systems of Rowlett and Simkiss (1987) and Perry (1988). Specifically, Borwornpinyo et al. (2005) reported an improvement over the previous culture systems because it employed a turkey egg shell with a large opening allowing improved embryonic access as well as improved embryonic observation. Secondly, Borwornpinyo et al. (2005) determined the optimal commercial plastic film used for seal to openings in the eggshells. Furthermore, the surrogate eggshell system has been employed to successfully generate transgenic chickens (Mozdziak et al., 2003).

The objective of these experiments was to develop somitic cell transplantation techniques within the surrogate eggshell culturing system. The main advantage of the surrogate eggshell system is to allow better access to the embryo than windowing, and improve the ability to hatch viable chicks.

MATERIALS AND METHODS

Embryos

White Leghorn Embryos expressing eGFP (Chapman et al., 2005) and wild-type embryos were incubated in a 37°C humidified incubator for 72 h to generate stage 18 embryos (Hamburger and Hamilton, 1951). The embryos expressing eGFP were generated from a homozygous flock of chickens expressing eGFP, and the generation of the lines was previously described in detail (Chapman et al., 2005).

Preparation of recipient eggshell

Surrogate eggshell procedures were based upon Borwornpinyo et al. (2005). The turkey eggs weighed between 89-105 grams. With the blunt side up, a circle was drawn using the professional COMBO CIRCLE template 977-110 (STAEDTLER®) at 41.275 mm. The Turkey eggs were washed with 70% ETOH. Using a Dremel® 300 Series High Speed Rotary Tool, the blunt end of the eggshell was removed under a laminar airflow hood to minimize contamination. Once cut, the turkey eggshells were placed cut end down into a prepared glass dish lined with paper towels and ultrapure water and covered with Saran Wrap.

Preparation of the recipient embryo

The entire contents of wild type recipient egg were removed from the shell and placed in a weigh-boat, and it was subsequently transferred into a prepared turkey eggshell. Albumin from another wild-type egg was collected and added to the turkey eggshell to raise the embryo up to the edge of the turkey eggshell (Figure 1A and B).

The embryo was observed using a Leica® MZ6 dissecting microscope (Leica Microsystems, Bannockburn, IL, USA), and using lateral illumination though a wratten 47 blue gelatin filter (Sigma, St. Louis MO). The blue filter facilitates visualization of the somites in the embryo, while it is still in the egg (Giamario et al., 2003). The vitilline membrane was removed using the 0.5 mm Etched Tungsten Micro Needle and the 0.05×0.02 mm #5 Forceps (FST®, Fine Science Tools, Foster City, CA, USA) above the somites of interest (somites #16-21). Using the forceps, the tissue containing the somites of interest was carefully etched from the embryo and extracted using the 0.5 mm Etched Tungsten Micro Needle, the forceps, and a 30 G 1/2 Precision Needle on a 3 ml syringe. The needle acts as a knife as the operator pulls the somites with the forceps.

Preparation of donor somites

The donor somitic tissue was taken from an eGFP positive chick embryo (Chapman et al., 2005). The embryo was carefully removed from the eggshell, and it was placed in a weigh-boat. A ring cut from a 2.5 cm circular Whatman® ion exchange paper was placed over the blood ring of the embryo. Using sterile dissecting scissors and forceps, the embryo was removed, and placed on a sterile glass slide (Figure 1C-F). The embryo was observed using a Leica® MZ6 dissecting microscope (Leica Microsystems, Bannockburn, IL, USA) and ambient illumination. The vitelline membrane was removed using the 0.5 mm Etched Tungsten Micro Needle and the 0.05×0.02 mm #5 Forceps (Fine Science Tools, Foster City, CA, USA). Using the 0.250×0.02 mm (Fine Science Tools, Foster City, CA, USA), the tissue containing the somites of interest (somites #16-21) was carefully etched out. The tissue containing the somites were extracted using the 0.5 mm Etched Tungsten Micro Needle and the 0.05×0.02 mm #5 Forceps and 30 G 1/2 Precision Needle.

Grafting procedure and incubation

The somitic tissue taken from the eGFP-positive donor

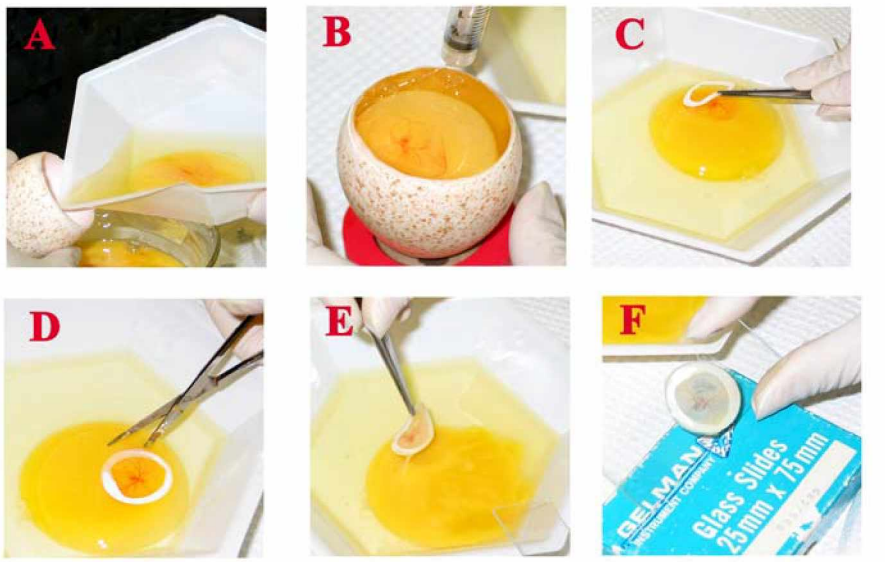

Figure 1. (A) Transfer of recipient embryo into surrogate turkey eggshell. (B) Adding additional albumin to the recipient embryo. (C) Surrounding donor embryo with filter-paper ring. (D) Isolated donor embryo from yolk. (E) Removing donor embryo from donor yolk. (F) Placing donor embryo on glass slide.

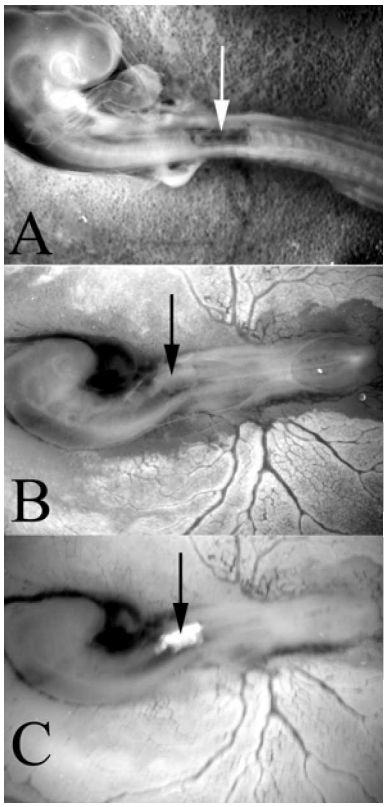

Figure 2. (A) Recipient embryo after removal of somites. White arrow indicates groove left by preparing the recipient embryo. (B) Recipient embryo with donor somites under ambient light illumination. (C) Recipient embryo with donor somites under GFP illumination. Black arrows point to donor eGFP positive somites.

was transferred to the prepared recipient, and the somitic tissue was carefully placed in the groove left by the somitic tissue excised from the recipient embryo. Albumin was carefully removed from the egg using a 20 G needle attached to a 3 ml syringe. After visual verification that the somatic tissue was in the proper orientation, albumin was applied around the top edge of the turkey eggshell and spread with a cotton tipped applicator. The window in the turkey eggshell was covered with a 3×4 in piece of Heavy Duty Saran Premium Wrap®, which was fixed to the opening in the eggshell using a 2 inch PVC pipe and rubber bands. The turkey eggshell containing the recipient embryo was placed into a 37°C incubator overnight to all the embryos to recover from the surgery. The embryo was subsequently transferred to a 37°C humidified incubator with a turning radius of 30°, and the embryos were cultured until they were harvested for analysis.

Immunocytochemistry

Successful transplantation of the donor somatic tissue was confirmed using immunocytochemistry. Briefly, embryos were fixed in 4% paraformaldehyde in PBS, dehydrated, cleared, and embedded in paraffin. Subsequently, 10 micron thick sections were deparaffinized, and hydrated. The sections were subjected to enzymatic retrieval with alpha-chymotrypsin (1 mg/ml pH 7.8) at 37°C for 20 minutes. Slides were rinsed in PBS, and incubated overnight at 4°C with a rabbit polyclonal antibody raised against eGFP (Invitrogen, Carlsbad, CA) diluted 1:200 with PBS, 0.5% Tween, and 10% goat serum. Subsequently, the sections were incubated for 2 h with goat ant-rabbit IgG conjugated to biotin diluted 1:500 with PBS 0.5% Tween, and 10% goat serum. The secondary antibody was detected using a Vectastain kit in combination with diaminobenzidine

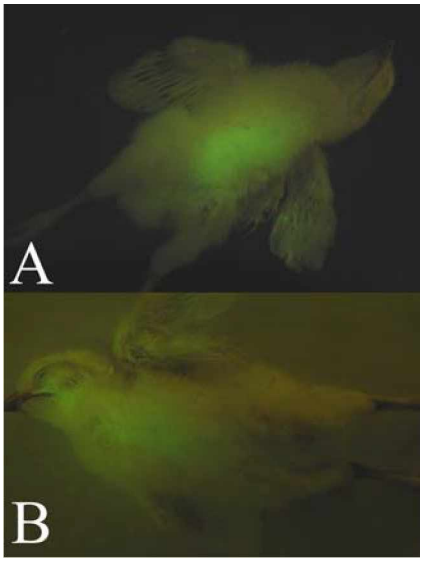

Figure 3. One day post-hatch chick under GFP illumination. Feathering overlying the left *Pectoralis thoracicus* shows GFP fluorescence.

(Vector Labs, Burlingame, CA).

RESULTS AND DISCUSSION

Firstly, successful cross-transplantation of somitic tissue was demonstrated in this study because eGFP positive somites were observed in wild-type embryos following transplantation (Figure 2), and GFP positive regions were observed in post-hatch chicks after transplantation (Figures 3 and 4). Chicks and embryos were routinely illuminated to reveal the presence of eGFP. The left *Pectoralis thoracicus* of all chicks/embryos examined consistently expressed eGFP, while eGFP was not observed in the right *Pectoralis thoracicus* of all chicks/embryos examined. The epaxial musculature was not examined in the current study. However, immunocytochemistry revealed eGFP positive myofibers in the left *Pectoralis thoracicus*, but not the right *Pectoralis thoracicus* (Figure 4). A potential improvement over the current procedures would be to employ pancreatin to digest any extraceullar matrix material from the recipient embryo or to employ an electrolytically-sharpened tungsten microscalpel to remove any adhering tissue between the neural tube and somite boundaries (Ordahal and Christ, 1997).

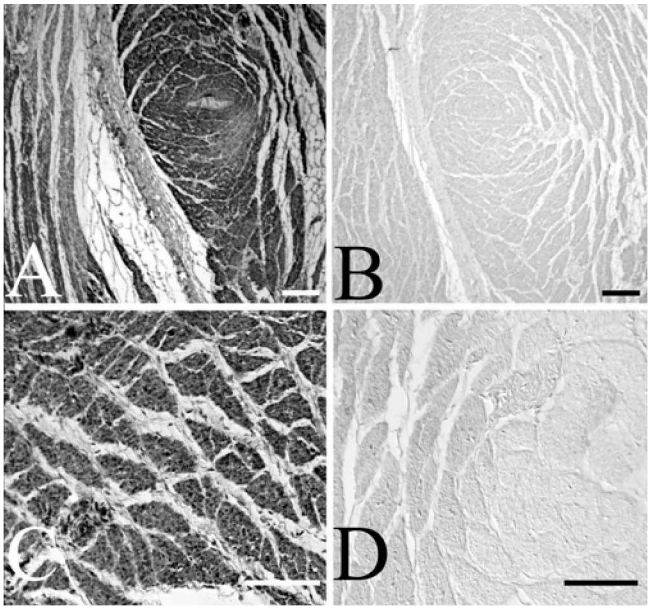

Figure 4. Immunocytochemical analysis of an embryo at 20 days of incubation following somite surgery. eGFP was detected with a rabbit polyclonal antibody (Invitrogen, Carlsbad CA) in combination with Goat-Anti Rabbit IgG conjugated to biotin. (A) Left *Pectoralis thoracicus* of the embryo following somite transplantion. (B) *Right Pectoralis thoracicus* of the same embryo following somite transplantation. (C) eGFP positive embryonic *Pectoralis thoracicus* (tissue from eGFP positive transgenic embryos). (D) Wild-type embryonic *Pectoralis thoracicus* immunostained for eGFP (negative control). Dark brown staining indicates eGFP, and it appears homogenous across the sections (A and C). Scale bars represent 100 microns.

The focus of these experiments was to achieve survival of the embryos following somite:somite cross-transplantation through 10 days post-surgery, which provides the opportunity for the embryo to complete pattern formation, and establish the basic body plan. Approximately 42% of the embryos survived past 10 days of somite grafting (Table 1), which corresponds to an approximately equivalent percentage of survival following injection into the somite (41%; Giamario et al., 2003) or injection of retrovirus into the blastoderm (Harvey et al., 2002; Mozdziak et al., 2003). However, it must be noted that the procedures employed for the somitic tissue transplantation are significantly more invasive than microinjection procedures. It should also be noted that 23% of the embryos

Table 1. Post-grafting embryo survival

	Embryos surgically manipulated	Days post-grafting			
		2 days	3 days	5 days	>10 days
Number viable of embryos[1]	79	61	61	47	33
Number of dead embryos[2]	0	18	18	32	46
% Live embryos[3]	100	77	77	60	42
% Dead embryos[4]	0	23	23	40	58

[1] Number of embryos viable at each day post-graft. [2] Number of dead embryos at each day post-graft.
[3] Percentage of live embryos at each day post-graft. [4] Percentage of dead embryos at each day post-graft.

died over the first two-days post-grafting suggesting that the grafting procedures resulted in a significant amount of mortality likely resulting from improper graft placement or graft movement during the initial incubation period. Of the embryos that remained viable after the initial two days after graft placement, greater than 50% (33 of 61) survived beyond ten days of incubation. Somites were visualized using illumination through a written blue 47 filter instead of using classical India Ink staining in combination with ambient light. It is possible that the absence of India Ink promotes survival of the embryos following the surgical procedures (Giamario et al., 2003). Overall, the current procedures are a viable way to perform somite transplantation experiments.

Achieving viable hatched chicks following embryonic manipulation has been a difficult goal to achieve. For example, Kinutani and Le Douarin (1985) only successfully hatched 7.4% of their manipulated embryos, but these chicks eventually rejected the grafted tissue. Following injection into somites of Stage 10-15 embryos, up to 60% of the manipulated embryos hatch successfully (Giamario et al., 2003), and following blastoderm injection of retrovirus, approximately 30% of the embryos successfully hatch (Harvey et al., 2002; Mozdziak et al., 2003). An advantage of the techniques is that it is possible to hatch and study the chicks following the somatic tissue manipulation.

In the current study, only 6 of the 33 embryos surviving past 10 days of incubation were purposely incubated to hatching and 5 of the 6 embryos (~83%) successfully hatched with no signs of abnormalities. The other 27 embryos were killed for histochemical analysis of cell fate. One chicken was maintained through 8 weeks of age with no signs of abnormalities. Therefore, it is possible to hatch chicks following somitic manipulations without the problem of species-specific graft rejection (Kinutani and Le Douarin, 1985).

The focus of this manuscript is to present an alternative to windowing for somite cross-transplantation or somatic tissue cross-transplantation studies. The advantage of the modified technology is that the embryo is more readily available for manipulation, that the wide window may make it possible to follow the eGFP labeled cells through the developmental processes, that the embryos survive past 10 days of incubation, and it is possible to hatch chicks following the manipulation. Lastly, it is possible to perform somatic cross-transplantation studies without the post-hatch graft rejection reported in the offspring from quail:chick chimeras (Kinutani and Le Douarin, 1985; Kinutani et al., 1986).

ACKNOWLEDGMENTS

Support provided in part by funds under project number NC06590 (PEM) of North Carolina State University. Supported in part by National Research Initiative Competitive Grant no. 2005-35206-15241 from the USDA Cooperative State Research, Education, and Extension Service (PEM).

REFERENCES

Andacht, T., W. Hu and R. Ivarie. 2004. Rapid and improved method for windowing eggs accessing the stage X chicken embryo. Mol. Reprod. Dev. 69:31-34.

Beresford, B. 1983. Brachial muscles in the chick embryo: the fate of individual somites. J. Embryol. Exp. Morphol. 77:99-116.

Beresford, B., C. Le Lievre and M. P. Rathbone. 1978. Chimaera studies of the origin and formation of the pectoral musculature of the avian embryo. J. Exp. Zool. 205:321-326.

Borwornpinyo, S., J. Brake, P. E. Mozdziak and J. N. Petitte. 2005. Culture of chicken embryos in surrogate eggshells. Poult. Sci. 84:1477-1482.

Chapman, S. C., A. Lawson, W. C. Macarthur, R. J. Wiese, R. H. Loechel, M. Burgos-Trinidad, J. K. Wakefield, R. Ramabhadran, T. J. Mauch and G. C. Schoenwolf. 2005. Ubiquitous GFP expression in transgenic chickens using a lentiviral vector. Development 132:935-940.

Chevallier, A., M. Kieny and A. Mauger. 1977. Limb-somite relationship: origin of the limb musculature. J. Embryol. Exp. Morphol. 41:245-258.

Chevallier, A., M. Kieny and A. Mauger. 1978. Limb-somite relationship: effect of removal of somitic mesoderm on the wing musculature. J. Embryol. Exp. Morphol. 43:263-278.

Christ, B. and B. Brand-Saberi. 2002. Limb muscle development. Int. J. Dev. Biol. 46:905-914.

Fineman, R. M. and G. C. Schoenwolf. 1987. Animal-model-dysmorphogenesis and death in a chicken-embryo model. Am. J. Med. Genet. 27:543-552.

Fineman, R. M., G. C. Schoenwolf, M. Huff and P. L. Davis. 1986. Animal-model-causes of windowing-induced dysmorphogenesis (neural-tube defects and early amnion deficit spectrum) in chicken embryos. Am. J. Med. Genet. 25:489-505.

Fisher, M. and G. C. Schoenwolf. 1983. The use of early chick-embryos in experimental embryology and teratology-improvements in standard procedures. Teratol. 27:65-72.

Giamario, C., J. N. Petitte and P. E. Mozdziak. 2003. Hatchability of chicken embryos following somite manipulation. Biotechniques 34:1128-1130.

Hamburger, V. and H. L. Hamilton. 1951. A series of normal stages in the development of the chick embryo. J. Morphol. 88:49.

Harvey, A. J., G. Speksnijder, L. R. Baugh, J. A. Morris and R. Ivarie. 2002. Consistent production of transgenic chickens using replication-deficient retroviral vectors and high-throughput screening procedures. Poult. Sci. 81:202-212.

Huang, R., Q. Zhi, C. P. Ordahl and B. Christ. 1997. The fate of the first avian somite. Anat. Embryol. (Berl) 195:435-449.

Kinutani, M., M. Coltey and N. M. Le Douarin. 1986. Postnatal development of a demyelinating disease in avian spinal cord chimeras. Cell 45:307-314.

Kinutani, M. and N. M. Le Douarin. 1985. Avian spinal cord chimeras. I. Hatching ability and posthatching survival in homo- and heterospecific chimeras. Dev. Biol. 111:243-255.

Le Douarin, N. 1973. A biological cell labeling technique and its use in experimental embryology. Dev. Biol. 30:217-222.

Le Douarin, N. M. 1973. A feulgen-positive nucleolus. Exp. Cell Res. 77:459-468.

Lee, H. T., K. S. Chung, D. W. Han, K. Whang, K. S. Roh, B. C. Koo, M. S. Kwon, C. K. Lee, Y. T. Heo, T. Kim and Y. M. Lee. 2004. Germ-line transmission of pseudotyped retroviral vector in chicken. Asian-Aust. J. Anim. Sci. 17:27-32.

Lee, C. H. Yan, B. Xiao, P. Trefil, S. Liu, Y. Kim and X. Wu. 2005. Production of transgenic chimeric chickens using blastodermal cells. Asian-Aust. J. Anim. Sci. 18:158-164.

Mozdziak, P. E., S. Borwornpinyo, D. W. McCoy and J. N. Petitte. 2003. Development of transgenic chickens expressing bacterial beta-galactosidase. Dev. Dyn. 226:439-445.

Mozdziak, P. E. and J. N. Petitte. 2004. Status of transgenic chicken models for developmental biology. Dev. Dyn. 229:414-421.

Mozdziak, P. E., Q. Wu, J. M. Bradford, S. L. Pardue, S. Borwornpinyo, C. Giamario and J. N. Petitte. 2006. Identification of the lacZ insertion site and beta-galactosidase expression in transgenic chickens. Cell Tissue Res. 324:41-53.

Ordahl, C. P. and B. Christ. 1997. Avian somite transplantation: A review of basic methods. Methods in Cell Biology, Vol. 52. 52:3-27.

Ordahl, C. P. and N. M. Le Douarin. 1992. Two myogenic lineages within the developing somite. Development 114:339-353.

Perry, M. M. 1988. A complete culture system for the chick-embryo. Nature 331:70-72.

Rowlett, K. and K. Simkiss. 1987. Explanted embryo culture - *invitro* and *inovo* techniques for domestic-fowl. Br. Poult. Sci. 28:91-101.

Speksnijder, G. and R. Ivarie. 2000. A modified method of shell windowing for producing somatic or germline chimeras in fertilized chicken eggs. Poult. Sci. 79:1430-1433.