9

Generation of Transgenic Poultry by Transfection of Primordial Germ Cells

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Avian primordial germ cells (PGCs) migrate via the extraembryonic blood from the germinal crescent to the developing gonads where they differentiate into spermatogonia and oogonia. PGCs thus represent a potential target cell for the introduction of foreign DNA into the avian germline. Retroviral transduction and DNA transfection of PGCs have been successfully utilized as gene transfer methods to produce transgenic offspring. The advantage of PGC methodology is the relative simplicity of the technique. A limitation of this approach is the need for second generation progeny to obtain birds carrying the transgene in all cells because the founder animal is a transgenic chimera only in gonadal tissue.

Avian Transgenesis

The development of powerful methodologies for the precise manipulation of the mammalian genome, for example knock-out mice, has intensified the effort to develop efficient methods for generating transgenic poultry. The difference in the reproductive system of birds, however, does not allow direct application of mammalian transgenic technology. Therefore, a number of different approaches have been developed for avian transgenesis (reviewed in Sang, 1994) including the use of replication-competent and replication-defective retroviral vectors, direct injection of DNA into the cytoplasm of the germinal discs, and transfection of blastodermal cells or primordial germ cells (PGCs).

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Foreign genes were first successfully introduced into the germline of chickens by using a replication-competent avian leukosis virus (ALV) (Salter *et al.*, 1986, 1987; Crittenden and Salter, 1990). Infection of embryos with wild-type or recombinant ALV produced viremic founder lines, which passed proviral DNA to F1 progeny. Expression of the subgroup A virus *env* gene from the recombinant ALV in transgenic chickens conferred resistance to ALV subgroup A infection (Crittenden and Salter, 1990). This result demonstrated the feasibility of using gene transfer as a method for developing disease resistant strains of poultry. The need, however, to develop virus-free transgenic poultry led to the development of replication-defective retroviral vectors. Microinjection of a replication-defective virus vector beneath the blastoderm of the unincubated fertile chicken egg produced male birds that contained vector DNA in their semen (Bosselman *et al.*, 1989a; Thoraval *et al.*, 1995). The vector DNA was passed on to 2–3% of the F1 offspring, demonstrating that the transgenic birds were germline chimeras. Expression of the foreign gene from the proviral DNA was detected in embryonic tissues (Bosselman *et al.*, 1989b), second-generation progeny (Briskin *et al.*, 1991), and chick embryo fibroblasts derived from transgenic embryos of F2 progeny (Thoraval *et al.*, 1995). Bosselman *et al.* (1989b) further demonstrated that embryonic expression of a transduced chicken growth hormone gene resulted in elevated levels of serum growth hormone.

Although the use of retroviral vectors has proven to be successful, this method is not preferred due to low public acceptance and the potential risk of generating replication-competent, recombinant retroviruses. Therefore, the development of non-viral methods for producing transgenic poultry has been actively pursued. Sperm-mediated gene transfer has been successfully used to introduce DNA into chickens (Nakanishi and Iritani, 1993; Squires and Drake, 1994). The direct injection of DNA into the cytoplasm of germinal discs before the first cleavage division has produced transgenic embryos (Naito *et al.*, 1991; Inada *et al.*, 1997) and adult chickens (Love *et al.*, 1994; Naito *et al.*, 1994a). Transfection or retroviral infection of blastodermal cells has also been successfully used to transfer foreign DNA into poultry (Brazolot *et al.*, 1991; Savva *et al.*, 1991; Allioli *et al.*, 1994; Bresler *et al.*, 1994; Watanabe *et al.*, 1994).

Genetic manipulation of PGCs is an alternative strategy for avian gene transfer. The advantage of this system is the simplicity of the technique relative to injection into the germinal disc or transfection and manipulation of blastodermal cells. A limitation is that the founder animal is a transgenic chimera only in gonadal tissue, thus requiring second generation progeny to obtain transgenic birds. This paper will review the methodology and successes to date using PGCs from the germinal crescent, blood or gonads as targets for gene transfer.

Origin and Characteristics of Primordial Germ Cells

Primordial germ cells originate in the epiblast of the stage X blastoderm (Eyal-Giladi *et al*., 1981). During gastrulation, the PGCs gradually translocate from the epiblast to the anteriorly migrating hypoblast and become localized in an extra-embryonic area that lies anterior and anteriolateral to the primitive streak, known as the germinal crescent (Swift, 1914; Ginsburg and Eyal-Giladi, 1986; Muniesa and Dominguez, 1990; Ginsburg, 1997). These PGCs become incorporated into the developing blood vascular system (Fujimoto *et al*., 1976b) during stages 13–16 (Hamburger and Hamilton, 1951). Circulating PGCs extravasate the blood vascular system in a region posterior to the vitelline artery and enter the neighbouring thickened epithelium of the splanchnopleure, the developing gonadal ridge (Ando and Fujimoto, 1983; Ukeshima *et al*., 1987; Hong *et al*., 1995). PGCs then migrate to the developing gonadal tissue and differentiate into spermatogonia in testes or oogonia in the ovary (Urven *et al.*, 1988).

Primordial germ cells are characterized by their large size (12–20 μm in diameter), large eccentrically placed nuclei and prominent, often fragmented, nucleoli (Fujimoto *et al*., 1976a). Chicken PGCs also have high glycogen content and thus are readily identified by periodic acid–Schiff base (PAS) staining (Meyer, 1960) from the time of their appearance in the germinal crescent to colonization of the gonads (Urven *et al.*, 1988). A number of antibodies that recognize mammalian PGCs (EMA-1, FC10.2, SSEA-1) have been used to identify chicken PGCs (Urven *et al*., 1988; Loveless *et al*., 1990, Karagenç *et al*., 1996). Quail PGCs do not stain with PAS (Nakamura *et al*., 1991) but can be identified with the QH-1 or QCR1 antibodies (Pardanaud *et al*., 1987; Ono *et al*., 1996). Turkey PGCs have also been shown to stain with PAS and the EMA-1 antibody (B.C. Wentworth, unpublished observations).

General Methodology for Producing Germline Chimeras by Transfer of PGCs

To generate germline chimeras, PGCs can be harvested from developing chick embryos at three different stages: (i) from the germinal crescent of a stage 5–7 chick embryo prior to migration; (ii) from the blood of a stage 13–17 chick embryo during migration; or (iii) from the developing gonad of a stage 25–27 chick embryo after migration. One hundred to 250 PGCs are detectable in the germinal crescent (Fujimoto *et al*., 1976b; Vick *et al.*, 1993b), whereas the reported number of PGCs in the blood range from one to 45 PGCs per 1.0 μl of blood (Singh and Meyer, 1967; Al-Thani and Simkiss, 1991; Vick *et al.*, 1993b; Naito *et al.*, 1994c). Thus injection of 100–200 PGCs should be sufficient to allow effective competition with endogenous circulating PGCs. Isolated PGCs are microinjected into the

blood vascular system of an appropriately staged host embryo (stage 13–17) through a small 'window' cut in the shell and shell membrane. The 'window' is sealed by a variety of methods such as surgical tape, Scotch Magic tape or a coverslip and wax. The donor PGCs circulate in the blood vascular system, from which they later exit, colonize the host gonad and differentiate into either oocytes or spermatozoa.

Clearly, success with this method is dependent upon the ability of isolated PGCs to successfully migrate to the gonadal ridge and compete with endogenous PGCs for colonization of the gonad. The founder animal should be a germline chimera, i.e. contains a mixture of germ cells derived from donor and host PGCs. Partial sterilization of the host by treatment with the drug busulphan improves the efficiency of PGC colonization of host gonadal tissue (Vick *et al.*, 1993a; Bresler *et al.*, 1994). Mating of the germline chimera to a non-transgenic animal produces non-transgenic progeny as well as transgenic progeny with the foreign DNA in all cells. Thus, second generation transgenic progeny can be readily identified by examining DNA extracted from a blood sample. The ratio of transgenic to non-transgenic second generation progeny reflects the efficiency with which donor PGCs have colonized the host gonad.

Germline Chimeras and Transgenic Poultry from Manipulated Germinal Crescent PGCs

Successful transfer of PGCs isolated from the germinal crescent to produce germline chimeras was first reported in quail (Wentworth *et al.*, 1989). This early success opened the possibility that PGCs from the germinal crescent could be genetically modified to produce transgenic poultry. Savva *et al.* (1991) first reported the introduction of foreign DNA into germinal crescent PGCs using a replication-defective reticuloendothelial virus containing the *lacZ* gene. Integration of the retroviral DNA was detected in the gonad of an 18-day embryo using a sensitive PCR–dot blot procedure. In a further study from Simkiss' group (Vick *et al.*, 1993b), germinal crescent PGCs were transduced with a defective avian leukosis virus containing the *lacZ* gene. Twenty-three per cent of the gonads (5/22) from 18-day embryos were positive by Southern blot. One male with vector DNA in his sperm was selected for breeding experiments. One out of 24 progeny sired by this male were positive for the vector DNA extracted from blood. In these studies germinal crescent PGCs were injected into the blood vascular system of recipient embryos. Injection of PGCs into the host germinal crescent was also an effective strategy. Han *et al.* (1994) reported that germinal crescent PGCs, which had been transfected with a *lacZ* containing plasmid, were still capable of migrating to the host gonads and expressing β-galactosidase activity. These results demonstrate that germinal crescent PGCs can be isolated and genetically modified while still retaining the ability to migrate and colonize the gonad of a recipient embryo.

Employing a novel strategy, Li *et al.* (1995) introduced foreign DNA into germinal crescent PGCs *in ovo* using ballistic transfection. Hatchlings produced by this method were raised to sexual maturity and shown to contain vector DNA in their sperm. Ten out of 45 G1 progeny were positive for the vector DNA. The vector DNA in these G1 progeny gradually disappeared as the birds matured, suggesting that the vector DNA was transmitted episomally.

Germline Chimeras and Transgenic Poultry from Manipulated Blood PGCs

Embryonic blood PGCs are a convenient source of PGCs because of the ease with which blood PGCs can be obtained and the fact that blood PGCs already exist as a single-cell suspension. In the blood of a stage 13–14 chick embryo, 0.048% of the cells are PGCs (Yasuda *et al.*, 1992). Studies that utilized transfer of retrovirally marked PGCs or interspecies transfer of PGCs have clearly demonstrated that exogenous blood PGCs can successfully target themselves to the host gonads (Simkiss *et al.*, 1989; Nakamura *et al.*, 1991; Naito *et al.*, 1994c; Ono *et al.*, 1996). In the study by Nakamura *et al.* (1991), timing of the injection was critical. When PGCs from stage 13–14 chicks were injected into stage 15–16 quail embryos, 90% of the PGCs appeared in the gonads of the host embryo. The remaining 10% were located ectopically in the head, trunk and limbs. Injection of blood PGCs into host embryos at progressively later stages of development resulted in a decreasing percentage of PGCs migrating to the gonad. When blood PGCs were injected into stage 20 host embryos only 6% of the PGCs migrated to the gonads. These results demonstrate that only a narrow window of time exists during which PGCs are competent to migrate to the developing gonads.

An early report indicated that the use of blood PGCs was not likely to produce germline chimeras. Petitte *et al.* (1991) transferred blood PGCs between marked strains of chickens. They analysed the progeny using a feather colour marker to assess the ability of donor PGCs to form functional gametes. Donor PGCs from dwarf white leghorn (DWL) embryos were injected into barred Plymouth rock (BPR) embryos. Fifty-nine male and female chicks that hatched were raised to sexual maturity and test mated with BPR fowl. All of the 3117 offspring examined showed the typical BPR phenotype and none showed the phenotype expected for a DWL $\times$ BPR.

More recent studies have shown that transfer of blood PGCs can produce germline chimeras. To concentrate PGCs from a blood sample, Yasuda *et al.* (1992) separated PGCs from blood cells using Ficoll gradient centrifugation. They reported an 80-fold enrichment of PGCs, from 0.048% to 3.9% of the cells. Using this method, Tajima *et al.* (1993) successfully generated germline chimeras following injection of 100 blood PGCs. Based

on feather colour, up to 12% of the chicks that hatched were derived from the donor PGCs. Vick *et al.* (1993b) showed that 3% of the embryos that received blood PGCs marked with a replication-defective retrovirus tested positive for the foreign DNA. One male bird was raised to sexual maturity and test mated to determine the percentage of his gametes that were derived from donor PGCs. Approximately 2% (1/56) of the progeny were derived from the donor PGCs.

Naito *et al.* (1994c) combined concentration of blood PGCs by Ficoll gradient centrifugation with depletion of endogenous PGCs by withdrawal of 4–10 μl of blood from the recipient embryo to generate germline chimeras at high frequency. Ninety-five per cent (19/20) of the birds examined by progeny testing were germline chimeras. Interestingly, when PGCs from white leghorns (WL) were transferred into BPR recipients, the average frequency of donor-derived offspring was 81% for three male chimeras and 96% for one female chimera. In contrast, the average frequency of donor-derived offspring was 23% for six male chimeras and 6% for five female chimeras when BPR PGCs were transferred into WL. Depletion of endogenous PGCs by withdrawal of blood from the host increased the frequency of donor-derived offspring from 14% to 23% in male chimeras generated by transfer of BPR PGCs into WL.

Blood PGCs have been successfully cultured on a feeder layer of stromal cells derived from the germinal ridge (Chang *et al.,* 1995a). PGCs were first labelled with a fluorescent dye to distinguish them from endogenous PGCs. A 4.8-fold increase in the number of labelled PGCs was reported along with a 3.9-fold increase in endogenous PGCs after 4 days in culture. This increase was only observed when the PGCs were grown on stromal cells derived from 5-day (stage 27) embryos and not with chick embryo fibroblasts. PGCs isolated from the blood of 2-day-old chick embryos have also been successfully stored in liquid nitrogen for 4–5 months (Naito *et al.,* 1994b). Frozen–thawed PGCs retained 94% viability and were still capable of producing viable offspring. These results indicate the potential for cryopreservation of PGCs as a possible approach to the conservation of genetic material in avian species.

Using a simpler approach, Watanabe *et al.* (1994) transfected blood PGCs *in vivo*. A cationic liposome–DNA complex containing the *lacZ* gene was injected into a stage 10–15 chick embryo. Twenty-four hours after injection, β-galactosidase activity was detected in blood cells, endothelial cells and endocardium cells of the heart in all embryos examined. Examination of other embryos 2–3 days after injection of the DNA–liposome complex revealed β-galactosidase activity in 4% of the embryos injected with a RSV–*lacZ* construct and 40% of the embryos injected with a β-actin–*lacZ* construct. The mean number of β-galactosidase-positive PGCs per embryo was 0.2 and 2.1, respectively. These results indicate that endogenous PGCs were successfully transfected *in vivo* and that the β-actin promoter is more active in PGCs than the RSV promoter. We have similarly

found that injection of a DNA–liposome construct into stage 15 turkey embryos transfected host embryonic tissues and blood PGCs. Vector DNA could be detected by PCR in 43% (10/23) of the heart samples and 21% (4/19) of the gonadal samples (B.C. Wentworth *et al.*, unpublished observations). These encouraging results demonstrate that *in vivo* transfection of blood PGCs is a potentially efficacious method of generating germline chimeras. This method would be advantageous because of its simplicity. The efficiency, however, is a critical issue since all progeny of the founder animal need to be screened for the foreign DNA. In contrast, in transfection studies using donor PGCs from marked strains, only donor-derived progeny are analysed for the presence of foreign DNA because they can be identified phenotypically.

Germline Chimeras and Transgenic Poultry from Manipulated Gonadal PGCs

In the 5-day (stage 25–27) chick embryo most of the PGCs have completed their migration to the gonads and have initiated a stage of rapid proliferation. One advantage to culturing PGCs derived from the gonad is the elimination of the need to prepare a separate feeder layer of cells for the PGCs. The somatic gonadal cells typically form a layer of stromal cells on which the PGCs proliferate. Allioli *et al.* (1994) cultured PGCs isolated from the gonads of 5-day-old chick embryos and transfected the cultured PGCs with a replication-defective retroviral vector containing the β-galactosidase gene. The number of PGCs increased with days in culture and more than half of the PGCs were positive for β-galactosidase activity. These results demonstrated that gonadal PGCs could proliferate *in vitro* and express a foreign gene.

Chang *et al.* (1995b) also showed that cultured chick PGCs isolated from a stage 27 chick embryo could still migrate and proliferate in a host embryonic gonad. The transplanted PGCs had undergone at least three to seven cell divisions. In a subsequent study, Chang *et al.* (1997) reported that transfer of gonadal PGCs resulted in 6/60 recipients that were germline chimeras. Both male and female germline chimeras produced progeny derived from donor PGCs at a frequency of 1.3–3.1%. Like blood PGCs, gonadal PGCs can also be stored in liquid nitrogen for up to 3 months without loss of the ability to form germline chimeras (Tajima *et al.*, 1996). These results demonstrate that PGCs from the gonads of stage 25–27 chicken embryos, which have long since passed beyond the normal migration stage *in vivo*, are still capable of migrating to and colonizing the embryonic gonad of a stage 15–17 recipient embryo.

Similarly, our earlier work indicated that PGCs from the indifferent gonads of equivalent stage 27 turkey embryos could be cultured and transfected *in vitro* to generate transgenic F1 offspring (Wentworth *et al.*, 1995). Analysis of

subsequent generations of our transgenic turkeys show that the vector DNA has persisted into the F3 generation. Surprisingly, the vector DNA appears to be transmitted as an episome by pedigree analysis, Southern blot analysis, and plasmid rescue studies (E.A. Wong *et al.*, unpublished observations). Transmission of the vector DNA does not follow normal Mendelian inheritance as would be expected for DNA that has integrated into the genome (Fig. 9.1). In pedigree 168, an untested founder female was mated with semen pooled from 12 transgenic and non-transgenic F0 males. The pooled semen tested positive by PCR for the transgene. Six F1 progeny were tested, of which two, no. 204 and no. 206, were positive by PCR for the transgene in DNA extracted from blood. Mating of positive F1 hen no. 204 and negative hen no. 205 with PCR-positive, pooled semen from 54 F1 males generated F2 progeny, all of which were positive by PCR or genomic Southern blot analysis. Further mating of Southern blot-positive male no. 1340 to negative female no. 1292 produced all positive F3 progeny. This last mating clearly demonstrated that the foreign DNA could be transmitted through the male germline. In addition, the pattern of inheritance was consistent with episomal transmission and not stable integration of the foreign DNA. In support of this hypothesis, Southern blot analysis of genomic DNA digested with a restriction enzyme that cleaves the vector DNA once produced only a single band that co-migrated with linear vector DNA. No junction fragments diagnostic of an integration event were detectable. Finally, vector DNA could be rescued from undigested genomic DNA by transformation of *Escherichia coli.* The rescued plasmid was identical to the plasmid used for transfection by restriction mapping and partial DNA sequencing. Presently it is not known by what mechanism the vector DNA replicates episomally in the transgenic turkeys. It is possible that an avian origin of replication has been fortuitously included in the construct or a cryptic origin of replication is being used.

Evidence for episomal persistence of foreign DNA has been observed during the generation of transgenic birds after cytoplasmic microinjection of DNA into one-cell fertilized eggs (Sang and Perry, 1989), sperm-mediated gene transfer (Nakanishi and Iritani, 1993; Squires and Drake, 1994), and *in vivo* ballistic transfection (Li *et al*., 1995). Perhaps the presence of numerous microchromosomes in avian species creates an environment that allows episomal replication of foreign DNA molecules.

Episomal transmission of transgenes has been observed in other transgenic animals. Extrachromosomal replication of DNA injected into the cytoplasm has been reported for the nematode (Stinchcomb *et al*., 1985), sea urchin (McMahon *et al*., 1985), *Drosophila* (Steller and Pirrotta, 1985), *Xenopus* (Etkin *et al*., 1984) and fish (Guyomard *et al*., 1989). In mice, inclusion of putative origins of replication from polyoma virus or the *c-myc* gene resulted in extrachromosomal replication of the transgene (Rassoulzadegan *et al*., 1986; Sudo *et al*., 1990). The episomal plasmid was shown to be transmitted in a non-Mendelian fashion to the F1 and F2 generations through both eggs and sperm (Rassoulzadegan *et al*., 1986) or only eggs (Sudo *et al*., 1990).

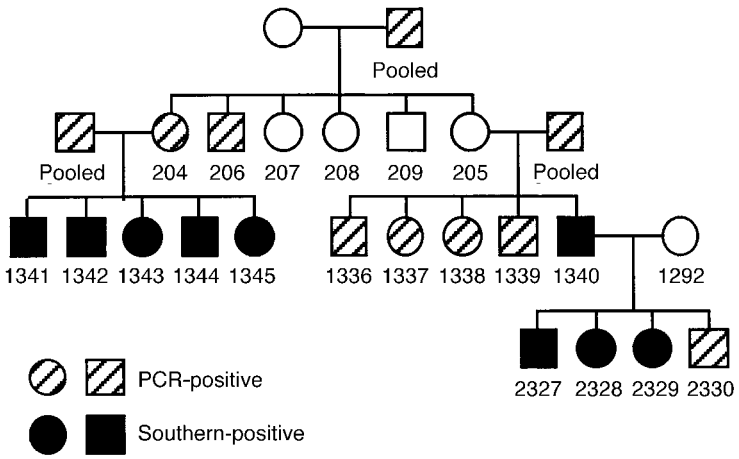

Fig. 9.1. Transmission of foreign DNA in pedigree 168. Females are represented by circles and males are represented by squares. Hatched or filled symbols indicate that the DNA samples were positive by PCR analysis only or by both PCR and genomic Southern blot analyses, respectively. Open symbols indicate that the DNA samples were negative by PCR analysis. The founder female was not tested.

The encouraging progress that has been made in the development of a number of techniques for transferring DNA into poultry opens the exciting possibility for manipulating the avian genome. Two potential applications of avian gene transfer technology involve the development of poultry that are disease resistant or that produce heterologous proteins in eggs. The ability to culture PGCs for days without loss of the capability to form a germline chimera will allow the application of powerful gene targeting methodologies that have been developed for murine embryonic stem cells. What remains to be determined is whether extended selection for PGCs with targeted integration events will adversely affect the ability of PGCs to migrate and colonize the host gonad. With all of these tools in hand, avian transgenesis is about to enter an exciting phase.

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