Cryopreservation of avian genetic material has become possible by preserving blastodermal cells or primordial germ cells. These germline cells can give rise to viable offspring via germline chimaeras. Cryopreservation of poultry semen is already possible. Thus, it is technically possible to preserve both male and female genetic material in liquid nitrogen and subsequently produce viable offspring in chickens. Proliferation of endangered avian species via germline chimaeras could increase genetic diversity. Development of new technologies such as nuclear transfer techniques in avian species is currently anticipated.

Key words: blastodermal cells, cryopreservation, germline chimaera, primordial germ cells

Introduction

Cryopreservation of avian genetic material is very important for preserving endangered avian species as well as preserving genetic stocks for poultry breeding. In chickens, cryopreservation of spermatozoa has already been achieved, but ova or fertilised eggs cannot be preserved in the same way because of their large size and yolk-laden structure. Cryopreservation of germline cells provides an alternative means for conserving both male and female genetic material. Recent development of embryo manipulation techniques (for review see Naito, 1997, 1998) made it possible to give rise to viable offspring from frozen-stored germline cells via germline chimaeric chickens. Proliferation of endangered avian species via germline chimaeras could help to increase genetic diversity because chromosomal rearrangements take place during meiosis and combination of genetically different gametes create new individuals. Moreover, it could be possible to mate male and female gametes of different generations by cryopreservation of germline cells, and this fertilisation could yield new genetic variations in avian species. Development of novel techniques for proliferation of endangered avian species is currently being explored.
Origin, migration and differentiation of avian germline cells

Germline formation in chickens can be analysed by Vasa gene expression because the Vasa gene shows germline-specific expression. From the analysis of chicken Vasa gene expression, a Vasa-positive structure is predominantly localised in the oocytes, indicating that this Vasa-positive structure is the precursory germ plasm (Tsunekawa et al., 2000). That is, the chicken germline is determined by maternally inherited factors in the germ plasm. During the early cleavage stages, Vasa protein is restrictively localised in the basal portion of the cleavage furrow, and about 30 Vasa-positive cells are scattered in the central disc of the area pellucida at stage X (Eyal-Giladi and Kochav, 1976). Similar results were obtained from an experiment of in vitro culture of blastodermal cells (Ginsburg, 1994), and a blastodermal cell transfer experiment (Naito et al., 2001). These Vasa-positive cells at stage X are called primordial germ cells (PGCs) and are recognised by anti-SSEA-1 and EMA-1 (Eyal-Giladi et al., 1981; Karagenc et al., 1996). The PGCs locate on the ventral surface of the area pellucida at stage X, and then translocate gradually to the dorsal side of the hypoblast at stages XI-XIV (Karagenc et al., 1996). By about 18 hours of incubation (stage 4, Hamburger and Hamilton, 1951) PGCs are carried anteriorly to the germinal crescent region and detected as PAS-positive cells. Then, they enter into the developing blood vascular system, circulate temporarily throughout the embryo, and finally migrate to the germinal ridges, future gonads (Nieuwkoop and Sutasurya, 1979; Kuwana, 1993; Naito, 1998).

PGCs entered into the testes start to divide actively after 13 days of incubation and differentiate into the spermatogonia (Fig. 1). At around 10 weeks after hatching, the spermatogonia start to proliferate as stem cells and produce continuously by mitotic division to yield subsequent generations of spermatogonia, and some of them differentiate into the primary spermatocytes. The primary spermatocytes then differentiate into the secondary spermatocytes via the first meiotic division and further differentiate into

![Fig. 1. The formation of spermatozoa and ova in chickens](image-url)
spermatids via the second meiotic division. Finally, they differentiate into the spermatozoa.

PGCs entered into the left ovary differentiate to form the oogonia after 8 days of incubation and start to proliferate actively in the process of differentiating into the primary oocytes (Fig. 1). The primary oocytes subsequently enter the meiotic prophase upon 16 days of incubation. Differentiation then ceases temporarily at the meiotic prophase. In contrast, the right ovary ceases to develop further from 7 days of incubation onwards and degenerates during embryonic development. After hatching, the differentiation of the primary oocytes in the left ovary resumes when a hen reaches sexual maturity and starts follicle development. The primary oocyte extrudes the first polar body via the second meiotic division before ovulation and differentiates into the secondary oocyte. After ovulation, fertilisation takes place and the secondary oocyte extrudes a second polar body and becomes a mature ovum.

**Cryopreservation of PGCs**

*Production of germline chimaeric chickens*

Germline chimaeric chickens are very useful as intermediates for reconstituting frozen-stored germline cells in liquid nitrogen. The techniques for producing germline chimaeric chickens by transfer of blastodermal cells (Petitte et al., 1990; Carsience et al., 1993; Kagami et al., 1995, 1997; Specsnijder et al., 1999; Naito et al., 2001) or PGCs (Tajima et al., 1993; Naito et al., 1994a, 1998ab, 1999) has already been established (Fig. 2) and used for avian embryo manipulation (Etches et al., 1997). Avian interspecific chimaeras, such as quail-chicken chimaeras, have been successfully produced (Naito, et al., 1991; Watanabe et al., 1992, Ono et al., 1996, 1998ab), but viable offspring derived from the donor cells has not been obtained.

*Cryopreservation of stage X blastodermal cells*

Stage X blastodermal cells obtained from freshly laid eggs are pluripotent cells containing PGCs or their immediate precursor cells. Cryopreservation of the stage X blastodermal cells was attempted and produced somatic and germline chimaeric chick-
ens by transfer of frozen-thawed blastodermal cells (Naito et al., 1992; Petitte et al., 1993; Reedy et al., 1995; Kino et al., 1997).

The procedure of freezing and thawing the stage X blastodermal cells is described briefly below (Kino et al., 1997). Stage X blastoderms (BPR) were isolated from the yolk, and the area pellucida was dissected and then dissociated by trypsin treatment. The blastodermal cells were dispersed in a freezing medium (DMEM containing 30% FBS and 20% DMSO) and put into a cryotube. The cell suspension was seeded to induce ice formation at -7°C, and cooled from -7 to -35°C at 1°C/minute. The cryotube was then plunged into liquid nitrogen (-196°C). The cells were thawed by plunging into warm water (37°C).

After removing dead or broken cells, approximately 500 cells were injected into the stage X recipient embryos (WL) that were compromised by γ-irradiation and incubated until hatching. Of 52 test-mated chimaeras, five chickens (9.6%) proved to be germline chimaeras. The percentages of donor-derived offspring from these germline chimaeric chickens were 5-7%. The donor breed (BPR) was reconstituted by mating the male and female germline chimaeric chickens. On the other hand, when freshly collected blastodermal cells were transferred into the recipient embryos in the same way as the frozen-thawed blastodermal cells, 9 chickens (56.3%) out of 16 test mated chimaeras proved to be germline chimaeric chickens, and the percentage of donor-derived offspring from these germline chimaeric chickens was 30%. Thus, the germline chimaerism of the chimaeric chickens was low when frozen-thawed blastodermal cells were transferred, compared with those in freshly collected blastodermal cells. The results indicate that cryopreservation of stage X blastodermal cells can be carried out successfully for preserving avian genetic material.

Cryopreservation of PGCs isolated from embryonic blood

PGCs circulate in the bloodstream of embryos before colonising the germinal ridges, and they are referred to as cPGCs (circulating PGCs). Cryopreservation of cPGCs in liquid nitrogen was first accomplished by Naito et al. (1994b). The procedure is briefly described below (Fig. 3). Blood was collected from the embryos (WL or BPR) at stages 13-15 and the pooled blood was dispersed in cHanks’ solution (modified Hanks’ solution containing 10% FBS). The cPGCs were concentrated by Ficoll density gradient centrifugation (Yasuda et al., 1992). The cPGC-rich fraction was then washed with cHanks’ solution and the collected cPGCs were dispersed in a freezing medium (cHanks’ solution containing 10% DMSO) at 4°C. The prepared cell suspension was put into a cryotube, which was placed in a freezing vessel and stored at -80°C for 3 hours. During freezing, the temperature of the freezing vessel decreased by 1°C/minute. The cryotube was then plunged into liquid nitrogen and stored for 4-5 months. The cryotube was taken out of liquid nitrogen and immediately plunged into water at 4°C. After thawing, the cPGCs were washed with cHanks’ solution. The viability of the frozen-thawed cPGCs was 94.2%, and 100% for freshly collected cPGCs, as measured by the Trypan blue exclusion method.

The collected cPGCs (100 cells) were transferred into the bloodstream of partially sterilised recipient embryos (BPR or WL, stages 14-15) whose blood was taken 4-5
hours before cPGC injection. The manipulated embryos were transferred into large recipient eggshells and cultured until hatching (Perry, 1988; Naito et al., 1990). The hatched chicks were raised until maturity and mated with BPR. The frequency of production of germline chimaeric chickens was 90% (9/10), and viable offspring derived from the frozen-thawed cPGCs were obtained at the frequencies of 0.9–26%. Also, viable male and female offspring derived from both male and female frozen-thawed cPGCs were produced by mating the male and female germline chimaeric chickens. Thus, cPGCs isolated from embryonic blood were successfully preserved in liquid nitrogen and subsequently gave rise to viable offspring via germline chimaeric chickens.

Cryopreservation of PGCs isolated from embryonic gonads

PGCs migrate to the germinal ridges after circulating embryonic blood, and the germinal ridges differentiate to testes or ovary. Embryos incubated for 5 days contain PGCs in their indifferent gonads, which are referred to as gPGCs (gonadal PGCs). These gPGCs still have the ability to re-enter the germinal ridges when they are injected into the bloodstream of recipient embryos (Chang et al., 1995), and give rise to viable offspring via germline chimaeric chickens (Chang et al., 1997).

Cryopreservation of gPGCs in liquid nitrogen was reported by Tajima et al. (1998). A brief description of the procedure is as follows. Fertilised eggs (WL) were incubated for 5 days and embryos were isolated from the yolk. The gonads were dissected from the embryos and sliced by a needle, and the cells were dissociated by trypsin treatment. The collected cells were dispersed into a freezing medium (MEM containing 10% FBS and 10% DMSO) at 5°C and then put into a cryotube, which was placed in the freezing vessel and incubated at -80°C for 3 hours. The temperature of the freezing vessel decreased by 1°C/minute during freezing. The cryotube was then plunged into liquid nitrogen and stored for 3–4 months. The tube was taken out of liquid nitrogen and immediately plunged into water at 4°C. After thawing, the gPGCs were washed with MEM.

One hundred gPGCs, as judged by their morphology, were transferred into the
bloodstream of partially sterilised recipient embryos (BPR) and incubated until hatching. The hatched chicks were raised until maturity and mated with BPR. The frequency of production of germline chimaeric chickens was 75\% (3/4) and the percentages of donor-derived offspring from these germline chimaeric chickens were 3.2–18.1\%. The results show that gPGCs isolated from the gonads of 5-day incubated embryos were successfully preserved in liquid nitrogen and subsequently reconstituted viable offspring via germline chimaeric chickens.

Quail gPGCs isolated from embryonic gonads can also be preserved in liquid nitrogen (Chang et al., 1998). Quail gPGCs isolated from the gonads of 5-day incubated embryos were dispersed in a freezing medium (M199 containing 10\% FBS and 10\% DMSO) and preserved in liquid nitrogen for up to 5 months in the same way as those of chickens (Naito et al., 1994b; Tajima et al., 1998). After thawing, the quail gPGCs were transferred into the bloodstream of recipient embryos and produced germline chimaeric quail. The percentages of donor-derived offspring from the two germline chimaeric quail were 2.4\% and 2.5\%. The results indicate that the quail gPGCs could be stored in liquid nitrogen, and that they retained the ability to differentiate into functional gametes.

**Differentiation of donor PGCs in the gonads of mixed-sex germline chimaeric chickens**

When endangered avian species are proliferated using germline chimaeras, the combination of sex in donor cells and recipient embryos seems to be important. In avian species, the male is the homogametic sex (ZZ) producing spermatozoa with the Z chromosome, and the female is the heterogametic sex (ZW) producing ova with either the Z or W chromosome. Recent development of a molecular sexing technique in avian species by analysing the W chromosome-specific repeating sequences (Mizuno et al., 1993; Clinton, 1994; Petitte and Kegelmeyer, 1995) has made it possible to identify the sex of donor cells and recipient embryos and to produce mixed-sex germline chimaeric chickens. If PGCs transferred into opposite-sex recipient embryos can differentiate into functional gametes, viable offspring derived from donor PGCs can be produced efficiently via mixed-sex germline chimaeric chickens.

**Transfer of Stage X blastodermal cells**

Same-sex and mixed-sex germline chimaeric chickens have been produced by transfer of stage X blastodermal cells (Kagami et al., 1995, 1997, 2002). In these experiments, male or female donor blastodermal cells were transferred into same or opposite-sex recipient embryos, and same-sex and mixed-sex germline chimaeric chickens were produced. Both same-sex and mixed-sex germline chimaeric chickens produced donor-derived offspring efficiently. Since stage X blastodermal cells contain PGCs or their immediate precursor cells (Karagenc et al., 1996), it is clear that PGCs or their immediate precursor cells at stage X have the ability to differentiate into both male and female gametes, irrespective of their genetic sex, giving rise to viable offspring via germline chimaeric chickens.

On the other hand, stage X blastodermal cells derived from the central disc, the marginal zone and the area opaca were transferred into recipient embryos (Naito et al.,
Germline chimaeric chickens were produced efficiently (46.7%, 7/15) when the blastodermal cells derived from the central disc were transferred into recipient embryos of the same-sex, whereas no germline chimaeric chickens were produced when the blastodermal cells derived from the marginal zone or the area opaca were transferred into recipient embryos of the same-sex (0/12). Germline chimaeric chickens were also produced by transfer of blastodermal cells derived from the central disc (6.7%, 1/15), marginal zone (10.0%, 1/10), or area opaca (11.1%, 1/9) into recipient embryos of the opposite-sex. Germline chimaerism of the chimaeric chickens produced by transfer of blastodermal cells derived from the central disc was higher in the same-sex germline chimaeric chickens than in the opposite-sex germline chimaeric chickens. Thus, the ability to differentiate into functional gametes of the donor blastodermal cells in the same or opposite-sex recipient gonads was not the same.

Transfer of a mixed population of male and female PGCs isolated from embryonic blood

Mixed-sex germline chimaeric chickens have also been produced by the transfer of PGCs, obtained from embryonic blood at stages +/−/+/−/+/−, into recipient embryos (Tajima et al., 1993; Naito et al., 1994ab, 1998ab). Donor PGCs in these experiments were collected from the pooled blood of both male (ZZ) and female (ZW) embryos; therefore, the male:female ratio of the donor PGCs was expected to be equal.

When PGCs are transferred into female recipient embryos, PGCs bearing ZZ chromosomes differentiate into ova bearing Z chromosomes, and PGCs bearing ZW chromosomes normally differentiate into ova bearing Z or W chromosomes. The male:female ratio of donor-derived offspring from female chimaeric chickens was therefore expected to be 3:1 if all ova were functional. The results of the experiment (Naito et al., 1994a, 1998b) show that the male:female ratio of the donor-derived offspring from the female germline chimaeric chickens did not deviate significantly from 1:1. The difference in sex ratio between the observed and the expected results suggests that not all the PGCs bearing ZZ chromosomes can differentiate into functional ova in the recipient ovary.

When PGCs are transferred into male recipient embryos, PGCs bearing ZW chromosomes differentiate into spermatozoa bearing Z or W chromosomes. Since spermatozoa bearing W chromosomes seem to be produced very rarely (Simkiss et al., 1996; Tagami et al., 1997; Abinawanto et al., 1998) and appear to be non-functional (Kagami et al., 1995), only spermatozoa bearing Z chromosomes can give rise to viable offspring. Donor PGCs bearing ZZ chromosomes differentiate normally into spermatozoa bearing Z chromosomes. The male:female ratio of donor-derived offspring from male germline chimaeric chickens, therefore, is expected to be 1:1, assuming all the spermatozoa bearing Z chromosomes are functional. In this case, however, it is impossible to evaluate the capability of development and differentiation of PGCs bearing ZW chromosomes into spermatozoa in the testes compared with that of PGCs bearing ZZ chromosomes.

Transfer of PGCs of known sex

Recently, same-sex and mixed-sex germline chimaeric chickens were produced by
transfer of PGCs whose sex was identified, and examined to determine whether PGCs obtained from embryonic blood could differentiate into functional gametes and give rise to viable offspring in opposite-sex recipient embryos and chickens (Naito et al., 1999). When the sex of the donor PGCs and the recipient embryo were the same, 68.2% (15/22) of male chimaeric chickens and 62.5% (10/16) female chimaeric chickens produced donor-derived offspring. When the sex of donor PGCs and the recipient embryo were different, 22.2% (4/18) of male chimaeric chickens and 11.1% (2/18) of female chimaeric chickens produced donor-derived offspring. The rates of donor-derived offspring from the germline chimaeric chickens were 0.6-40.0% in male donor and male recipient, and 0.4-34.9% in female donor and female recipient. However, the rates of donor-derived offspring from the germline chimaeric chickens were 0.4-0.9% in male donor and female recipient, and 0.1-0.3% in female donor and male recipient. The presence of W chromosome-specific repeating sequences was detected in the sperm samples of male chimaeric chickens produced by transfer of female PGCs. These results indicate that PGCs isolated from embryonic blood can hardly differentiate into functional gametes giving rise to viable offspring in the gonads of opposite-sex recipient embryos and chickens. Thus, the sex of donor PGCs and recipient embryo should be the same when endangered avian species are proliferated via germline chimaeras produced by transfer of PGCs isolated from embryonic blood.

Combination of chicken breeds in donor cells and recipient embryos

The efficiency of obtaining donor-derived offspring from chimaeric chickens was high when the donor was WL and recipient was BPR (Naito et al., 1994a). Similar results were obtained when germline chimaeric chickens were produced by the transfer of stage X blastodermal cells. The combination of a WL donor and a BPR recipient produced W-bearing spermatozoa more efficiently than the reverse combination (Kagami et al., 2002). Perhaps the apparent dominance of WL over BPR in the competition of germ cell proliferation in the recipient gonads reflects the difference in the egg laying performance between WL and BPR. Endangered avian species usually show low performance in egg production. When endangered avian species are proliferated by the transfer of PGCs into high performance chickens such as WL, the efficiency of producing donor-derived offspring will be low. In order to overcome this difficulty, it is necessary to develop a technique for replacing recipient PGCs completely with donor PGCs. Production of inter-specific avian chimaeras would also become very important for the proliferation of endangered avian species.

Cryopreservation of spermatozoa

In chickens, cryopreservation of spermatozoa has already been achieved using glycerol as a cryoprotective agent (Lake and Stewart, 1978), and the techniques of semen freezing have been extensively reviewed by Hammerstedt (1994). At present, development of a simpler technique for semen preservation, such as a non-glycerol method, is expected.

Future technologies
It is now expected that new technologies for the preservation and proliferation of avian species will be developed. These new technologies will make it possible to use somatic cells for avian preservation.

**Nuclear transfer in chickens**

Recent development of the somatic cell cloning technique in mammals (Wilmut *et al.*, 1997) will make it possible to clone avian species by somatic cell nuclear transfer and give rise to viable offspring. In avian species, there are, at present, two strategies for the use of a somatic cell nucleus that gives rise to a viable offspring.

The first strategy is nuclear transfer into a newly ovulated ovum. Chicken ova can be obtained from the infundibulum or magnum of the oviduct of hens. The ova obtained from the infundibulum, just after ovulation, are very fragile and manipulation and culture in vitro and *in vivo* are not easy. The manipulated ova are transferred to the oviduct of recipient hens and could give rise to viable offspring (Tanaka *et al.*, 1994). The ova obtained from the anterior portion of the magnum can be manipulated and the manipulated ova can be cultured until hatching (Naito *et al.*, 1995). The ova obtained from the posterior portion of the magnum can also be manipulated and cultured until hatching (Perry, 1988; Naito *et al.*, 1990). Development in culture of the manipulated ova becomes easy as the ova are obtained from the magnum at later stages, but the duration of retaining the developmental ability of the ova is unknown. When nuclear transfer is carried out in chickens, ova are obtained from the oviduct and enucleated by irradiation by UV, soft X-ray or γ-ray. The nuclei derived from cultured cells are then injected into the blastodisc, activated by electric stimuli or chemicals, and cultured *in vitro* until hatching. This procedure seems to be one of the most promising strategies for producing cloned chickens.

The second strategy is nuclear transfer of PGCs with somatic cell nuclei. If the nucleus of a PGC can be replaced by a somatic cell nucleus, it is expected that the somatic cell nucleus can enter into the germline of chickens and give rise to viable offspring via germline chimaeric chickens. Since a system for producing germline chimaeric chickens by the transfer of PGCs has already been established (Naito *et al.*, 1994b), PGCs will be a suitable vehicle for introducing somatic cell nuclei into the germline of chickens. The physical procedure for enucleation of the nucleus from PGC and the transfer of the somatic cell nucleus into the enucleated PGC will be difficult due to the fragility of PGCs. Alternatively, compromising the nucleus of the PGC and then fusing this PGC with the somatic cell would be a novel strategy for nuclear transfer in chickens. After the transfer of the fused cells into recipient embryos, it is expected that the nucleus of the PGC will disappear from the fused cells during embryonic development and, as a result, the PGC nucleus will be replaced with the somatic cell nucleus. It is expected that offspring derived from somatic cells could be produced by this method, although cloned chickens will not be produced due to recombination during meiosis.

**Other possible methods**

Cryopreservation of germline cells (blastodermal cells, primordial germ cells, sperm cells, etc.) in liquid nitrogen has become possible, and the blastodermal cells or PGCs can be reconstituted as viable offspring via germline chimaeric chickens. Embry-
onic stem cells derived from blastodermal cells (Pain et al., 1996), or embryonic germ cells derived from PGCs (Park and Han, 2000), could contribute to the preservation of genetic resources in avian species. In attempts to proliferate endangered avian species, \textit{in vitro} fertilisation by microinjection of spermatozoa into a newly ovulated ovum may help to produce viable offspring. Furthermore, many of the genes involved in germ cell formation are known, \textit{e.g.}, Oskar, Vasa, Tudor, Bicoid, Caudal, Hunchback, Nanos, etc. In avian species, homologues of such genes would be present and involved in germ cell formation. If these genes can be manipulated appropriately, somatic cells might be induced to differentiate into germ cells.

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of primordial germ cells or their precursors in stage X blastoderm of chickens and their ability to differentiate into functional gametes in opposite-sex recipient gonads. Reproduction, 121 : 547–552. 2001.


