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Poultry genetic resource conservation using primordial germ cells

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Abstract. The majority of poultry genetic resources are maintained *in situ* in living populations. However, *in situ* conservation of poultry genetic resources always carries the risk of loss owing to pathogen outbreaks, genetic problems, breeding cessation, or natural disasters. Cryobanking of germplasm in birds has been limited to the use of semen, preventing conservation of the W chromosome and mitochondrial DNA. A further challenge is posed by the structure of avian eggs, which restricts the cryopreservation of ova and fertilized embryos, a technique widely used for mammalian species. By using a unique biological property and accessibility of avian primordial germ cells (PGCs), precursor cells for gametes, which temporally circulate in the vasculature during early development, an avian PGC transplantation technique has been established. To date, several techniques for PGC manipulation including purification, cryopreservation, depletion, and long-term culture have been developed in chickens. PGC transplantation combined with recent advanced PGC manipulation techniques have enabled *ex situ* conservation of poultry genetic resources in their complete form. Here, the updated technologies for avian PGC manipulation are introduced, and then the concept of a poultry PGC-bank is proposed by considering the biological properties of avian PGCs.

Key words: Cryopreservation, Poultry genetic resources, Primordial germ cells, Transplantation

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Cryopreservation of animal germplasm enables sustainable and economical maintenance of genetic resources for the livestock industry and research. In mammals, an *ex situ* conservation strategy is methodologically possible by integrating key reproductive technologies such as cryopreservation of semen, ova and embryos, artificial insemination, *in vitro* fertilization, somatic nuclear transfer, and embryo transfer. Indeed, these technologies are used not only for *ex situ* preservation in domesticated animals, particularly in cattle, and experimental animals, but also for human infertility treatment. In the case of oviparous animals like birds, cryopreservation of intact embryos is the most simple as well as straightforward *ex situ* conservation strategy. However, in birds, this is impossible at present because of the large yolk-laden structure of their eggs. Although semen of some poultry such as chicken, goose, duck, and turkey can be cryopreserved successfully [1, 2], the post-thaw fertility of poultry semen lags behind other species, varying among breeds, lines, or individuals [3–5]. In the case of poultry, particularly the chicken, the current use of *ex situ* conservation is only limited to industrially as well as commercially valuable breeds or lines through the collection of frozen semen. By contrast, noncommercial breeds including indigenous breeds are exclusively maintained by *in situ* populations. However, *in situ* conservation of poultry genetic resources always carries the risk of loss owing to unexpected infectious disease outbreaks such as highly pathogenic avian influenza, and accidents. In addition to these risks, the periodic reproduction of *in situ* populations makes them costly to feed, and requires special facilities including a poultry house and farm. Moreover, cryobanking of semen is insufficient as an *ex situ* conservation strategy in birds because genes on the W chromosome and mitochondrial DNA cannot be maintained as the male is the homogametic (ZZ) sex. As an alternative, avian primordial germ cells (PGCs; Fig. 1), the first germ cell population established during early development, can be incorporated into the gonads [6] and differentiated into functional gametes following transplantation to recipient embryos [7, 8]. This technological development of avian PGC transplantation provides insight into *ex situ* conservation because PGCs enable the capture of the entire genetics of the stock. The practicality of the poultry PGC-bank is affected by the efficiency of each step of PGC manipulation, and the overall success rate in regenerating donor-derived progeny is critical to ensure an adequate effective population for genetic restoration. Because of the recent development of two innovative techniques for long-term culture of chicken PGCs *in vitro* [9, 10] and constant production of sterile chicken recipient embryos enabling the production of only donor-derived offspring [11], establishment of poultry PGC-bank programs will become more realistic. In this review, I introduce the updated technologies for avian PGC manipulation then propose the concept of poultry PGC-bank by considering the biological properties of avian PGCs.

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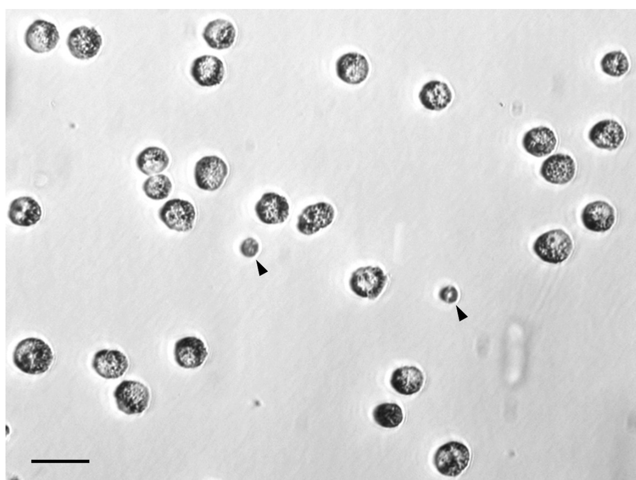

Fig. 1. Chicken primordial germ cells (PGCs) isolated from early embryonic blood. Arrow heads indicate erythrocytes. Scale bar, 20 μm.

Appropriate Timing of PGC Collection and Transplantation in Chicken

During early development, PGCs originate in a particular region of the embryo that is often located a relatively great distance from where individual germ cells will eventually reside. Unlike other species, in avian and some reptile embryos, PGCs use blood circulation for transport to the gonadal anlage. This unique biological property and accessibility of avian PGCs provides an opportunity to collect and transplant PGCs [12]. It was first reported in 1993 that transfer of chicken PGCs from early embryonic blood to the bloodstream of recipient embryos can result in transmission of the donor genotype to offspring of recipient chickens [7]. Since then, several research groups have attempted to produce germline chimeras using intravascular transplantation of PGCs, particularly in the chicken [8, 9, 11, 13–22]. Because blood circulation of avian PGCs is transient, appropriate timing of their collection and transplantation remain to be verified. Although several qualitative descriptions of the migration of avian PGCs through blood circulation have been reported [23–25], there has been very little quantitative observation. Recent findings of germline-specific molecular markers such as the chicken homologue genes *vasa*, *dead end* and *dazl* enable reliable analysis of chicken PGCs [25–27]. Therefore, the distribution and number of immunohistochemically stained chicken PGCs using anti-Vasa antibody were observed to clarify when and where chicken PGCs move from the extraembryonic region to the vasculature, and from the vasculature to the gonadal anlage [28]. The entrance of PGCs from the anterior part of the extra-embryonic region into the vascular network starts at stage 10 (Arabic numerals refer to the staging system of Hamburger and Hamilton, 1951 [29]) and is completed at stage 13. The migration of PGCs to the gonadal anlage begins at stage 15 and is completed at stage 17. However, access to developing vasculature is technically difficult until a chicken embryo reaches stage 13. In addition, the frequency of germline transmission decreases when PGCs are transferred to the chicken embryos at stage 17 or later [21, 30]. Both male and female PGCs collected from 5- to 7-day-old chicken embryos (stages 27–31) also have the ability to differentiate into functional gametes following transplantation [31, 32]. Male germ cells obtained from adult chicken testes, possibly spermatogenic stem cells, retain germline competency after transplantation to recipient embryos [33], but their competency seems lower than PGCs. In contrast to males, the germline competency of female germ cells is readily lost from 15.5 days post-incubation owing to the start of meiosis [34]. Taking these into consideration, the appropriate times for chicken PGC collection and transplantation are stages 13–14 as well as 27–31, and stages 13–16, respectively.

Enrichment of PGCs

Because the PGCs are a very small cell population during early development and comprise less than 0.02% of blood cells and approximately 2% of gonadal cells [35, 36], transfer of intact blood or gonadal cells to recipient embryos results in very low efficiency of producing germline chimeric chickens [37]. Recent investigations of several cell surface antigens of chicken PGCs such as stage-specific embryonic antigen-1 (SSEA-1) and embryonic mouse antigen-1 (EMA-1) [38] enabled enrichment of chicken PGCs by magnetic–activated cell sorting (MACS) or florescence-activated cell sorting (FACS) [36, 39]. The QCR1 antibody [40], which reacts with an epitope in PGCs of Japanese quail (*Coturnix japonica*) and common pheasant (*Phasianus colchicus*), but does not react with chicken PGCs, is available for PGC purification in these species [41, 42]. The antigen-antibody reaction-based system allows isolation of PGCs from both blood and gonads. In the future, further investigations of cell surface markers of PGCs in several avian species other than the chicken, Japanese quail, and common pheasant are anticipated. Since avian embryonic blood cells are roughly composed of a small number of PGCs and a huge number of erythrocytes, two approaches can be used to purify PGCs: one is separation of PGCs from erythrocytes by density gradient methods using Ficoll or Nycodenz [6, 35], and the other is erythrocyte lysis using an ammonium chloride-potassium buffer [43]. Among these, the Nycodenz gradient centrifugation method achieves both high recovery and purity rates of PGCs in the chicken and Japanese quail [35]. In addition to these species, availability of this method for duck (*Anas platyrhynchos*), green pheasant (*Phasianus versicolor*) and common pheasant has been confirmed (Nakamura *et al*., unpublished data). A unique PGC enrichment method from gonadal tissues was developed in the chicken using the biological property that PGCs are discharged when embryonic gonads are incubated in Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} D-PBS(–) [44]. This is the simplest method currently available to harvest chicken PGCs and is potentially applicable in various avian species. In the future, further improvement of unstable PGC recovery rate is expected.

Long-term Culture of PGCs

Mammalian PGCs can only be propagated as lineage-restricted germ cells for short periods *in vitro* [45–47]. In 2006, Etches and colleagues demonstrated that chicken PGCs can be propagated for

the long-term *in vitro* while maintaining lineage specificity and germline transmission competency [9]. Chicken PGCs isolated from embryonic blood can be expanded in a complex medium containing chicken serum, fetal bovine serum (FBS), fibroblast growth factor 2 (FGF2), and buffalo rat liver (BRL) cell-conditioned medium on a feeder of either BRL cells or Sandoz inbred mouse-derived thioguanine-resistant and ouabain-resistant (STO) fibroblasts [9]. Several research groups revealed that FGF signaling is required for chicken PGC proliferation *in vitro* [18, 48, 49]. A recent study has suggested that the membrane-bound form of chicken stem cell factor 2 (SCF2), but not the secreted form of chicken SCF1, enhanced the propagation of chicken PGCs in cooperation with FGF2 [50]. However, this ill-defined culture system cannot support efficient propagation of female chicken PGCs. More recently, McGrew and colleagues defined serum-free, feeder-free, and physio-chemically permissive culture conditions for chicken PGCs, ascertaining that FGF2, insulin, and activin are sufficient for the propagation of chicken PGCs [10]. Furthermore, a lower osmolality condition (250 mOsm/kg), one of the characteristics of the defined culture system, also enabled efficient derivation and propagation of both male and female chicken PGCs. Establishment of long-term culture systems of chicken PGCs provides the opportunity to significantly amplify donor PGCs before cryopreservation [51] as well as to manipulate the genome with subsequent cloning [52–54] in a manner similar to mouse embryonic stem (ES) cells in the chicken. Further investigations of culture conditions of non-chicken avian PGCs may help to conserve the genetic resources of wild birds *ex situ*.

Ultra-low Temperature Preservation of PGCs

To data, ultra-low temperature preservation of chicken PGCs has been performed using a slow-freezing method. The most widely used freezing protocol is cryopreservation of chicken PGCs in serum containing media supplemented with 10% dimethylsulfoxide (DMSO) as a cryoprotectant at a cooling rate of −1°C/min until reaching −80°C. This freezing protocol yields a recovery rate of approximately 50%, and over 85% viability of post-thawing chicken PGCs [13, 55, 56]. Several comparative studies have examined the types of cryoprotectant including their concentrations and the cooling rates to investigate a more efficient protocol [22, 55, 57, 58]. To summarize previous studies, chicken PGCs in a medium containing more than 10% serum and either 5–10% DMSO or 10% ethylene glycol as cryoprotectants at a cooling rate of −2°C/min result in higher recovery and viability of post-thawed chicken PGCs than classic freezing protocols. Commercially available serum and DMSO-based cryomedium also results in higher recovery and viability of chicken PGCs after thawing than a common freezing protocol [55]. Due to its easier availability and higher performance, commercial cryomedium particularly CELLBANKER 1 (Nippon Zenyaku Kogyo, Koriyama, Japan) has been used to cryopreserve PGCs from chicken and quail [30, 59–61]. In contrast to the slow-freezing method, a vitrification method can theoretically store cells both intracellularly and extracellularly by ice-free solidification. Although a previous study reported lower recovery and viable rates of vitrified PGCs than in frozen-thawed PGCs [56], the vitrification method could improve the recovery and viability of chicken PGCs after exploration of various vitrifying protocols.

Production of High-grade Germline Chimeras Through PGC Transplantation

The ideal host for germ cell transplantation is an infertile recipient. Indeed, several infertile animal models such as germ cell-deficient mutant mice and triploid fishes have been used widely in germ cell transplantation studies to produce only donor-derived sperm or eggs [62, 63]. To date, genetic mutants either lacking germ cells or with deficient gametogenesis have not been reported in birds. ZZZ triploid chickens develop and grow normally but are infertile [64]. ZZZ chicken embryos could potentially be used as recipients for PGC transplantation, but rarely occur. Thus, partial, if not complete, removal of endogenous PGCs prior to transplantation of donor PGCs is an effective approach to increase the efficiency of donor-derived gametes in recipient chickens. Surgical removal of tissues containing PGCs such as blastodermal cells or blood from recipient chicken embryos resulted in increased germline chimerism [65, 66]. Irradiation of chicken embryos with X-rays or gamma-rays resulted in decreased number of endogenous PGCs and thus increased the proportion of donor-derived gametes [67, 68]. However these methods failed to produce stable high-grade germline of chimeric chickens, suggesting recovery of the remaining endogenous PGCs after treatment. Busulfan (1,4-butanediol dimethanesulfonate), a DNA alkylating agent, is the most commonly used drug for removal of endogenous germ cells in mammals. In particular in adult male mice, a single intraperitoneal injection of over 40 mg/kg busulfan enables constant preparation of recipients lacking endogenous germ cells for germ cell transplantation [62, 69, 70]. Although busulfan also has sterilization effects on chicken and Japanese quail germ cells during development, the degree of germ cell depletion is variable owing to less efficient drug delivery [71, 72]. As shown in Fig. 2, Nakamura *et al*. (2008) developed a unique drug delivery method by utilizing a biological property of the chicken embryo - it lies on the top of the yolk even if the egg is rotated [73]. The principle of this method is as follows: a sustainable emulsion containing busulfan rapidly rises when it is injected into the yolk then contacts with the chicken embryos owing to a lower density than the yolk contents. This drug delivery method enabled a depletion of PGCs at a constant level. A subsequent study revealed that application of busulfan at an early time point could reduce the sterilizing effects of the residual drug on donor PGCs [74]. Finally, an experiment was attempted to transplant donor chicken PGCs to sterilized recipient embryos following administration of busulfan. Of 11 resulting recipients, seven produced only donor-derived gametes (99.5% on average of total recipients) [11]. As described above, the production efficiency of donor-derived gametes was markedly increased using sterile recipients. In the future, production of chicken lines where the germ cells are completely depleted is required for more efficient production of recipient chickens that produce only donor-derived offspring. For example, in combination with transgenesis of cultured PGCs *in vitro*, generation of Cre/loxP system-mediated germ cell-specific knock-out or ablation avian models via germline chimeras would be the best approach.

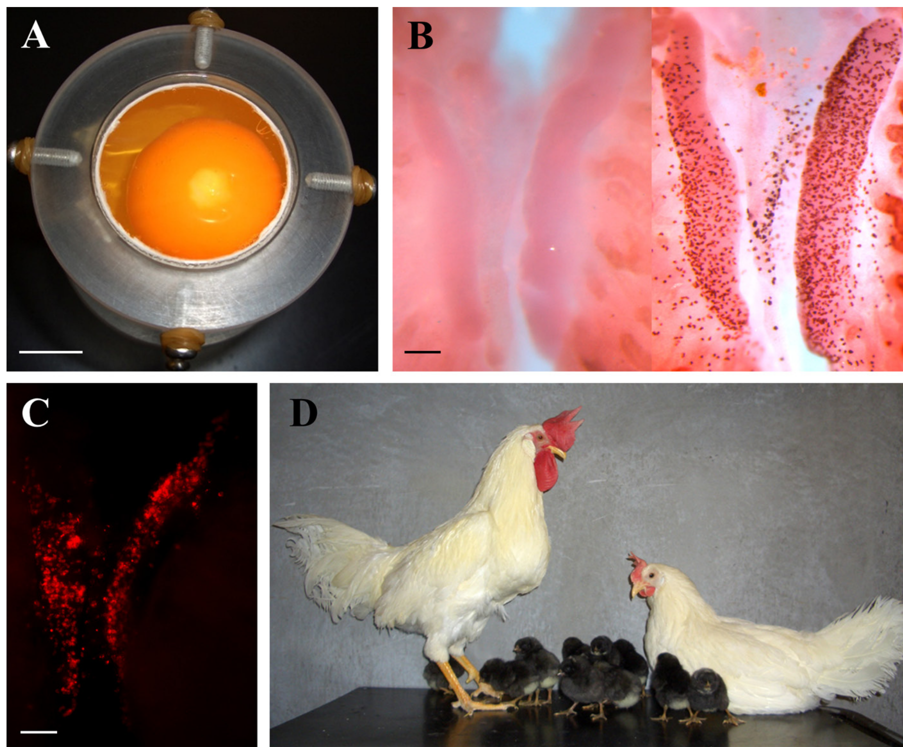

Fig. 2. Production of sterilized recipient embryos by removal of endogenous primordial germ cells (PGCs) using a unique drug delivery method and its application for PGC transplantation. (A) A sustainable emulsion containing busulfan contacts with the chicken embryos rapidly after injection into the yolk. (B) This drug delivery method allowed elimination of PGCs at a constant level. (C) Donor PGCs could repopulate with gonads of sterilized recipient embryos after transplantation. (D) Use of sterile recipients enabled efficient production of chickens that produce only donor-derived offspring. Scale bars, 1 cm (A) and 100 μm (B and C). (Revised: Nakamura *et al*., 2008; 2010)

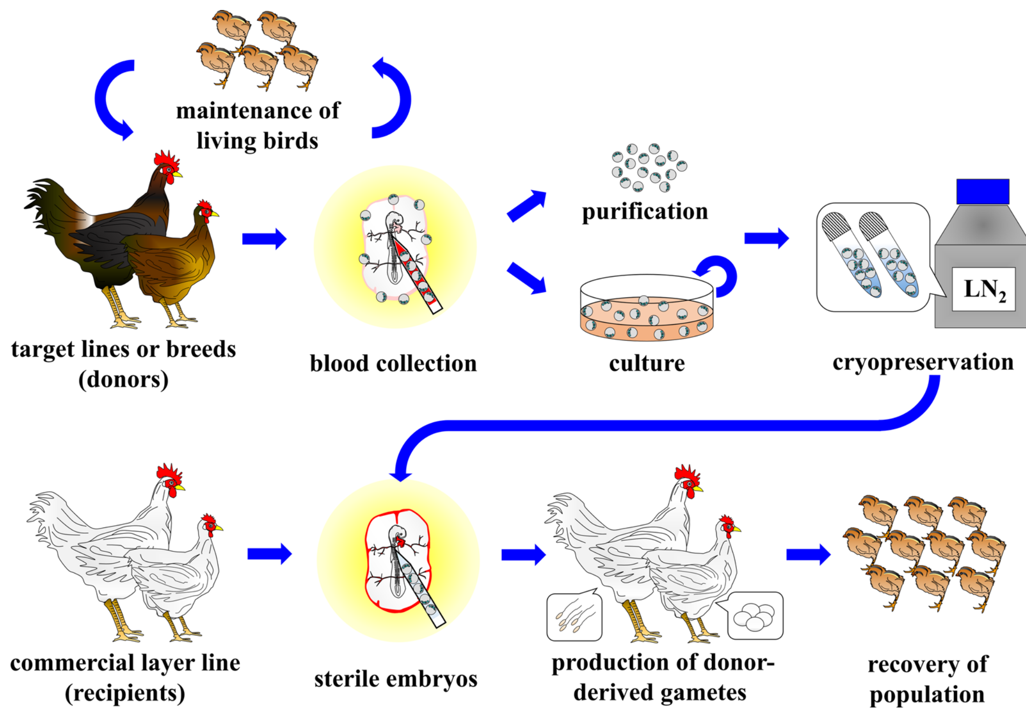

Fig. 3. Outline of a poultry PGC-bank program.

Conclusions and Perspectives on a Potential Poultry PGC Bank

In this review, recent technical advances regarding avian PGC manipulation for use in poultry PGC-bank programs have been introduced. As shown in Fig. 3, the procedures of *ex situ* conservation of poultry genetic resources consist of five steps; 1) collection of embryonic tissues containing PGCs from target lines or breeds, 2) purification or culture of PGCs, 3) PGC storage in liquid nitrogen, 4) PGC transplantation to sterilized recipient embryos, and 5) recovery of populations by mating of male and female recipients. To date, successful long-term culture of PGCs from various chicken breeds including indigenous breeds has been reported [9, 18, 22, 49]. Therefore, at least in the chicken, the major advantage of PGCs for use as a source of gene banking is that PGCs obtained from a single donor embryo would be amplified significantly *in vitro* prior to cryopreservation. In addition, transplantation of post-thaw PGCs to sterile recipients of a commercial layer line would be rapidly and significantly multiplied, recovering the population size from only a small flock of recipients.

The most important aspect of gene banking is the collection of germplasm without losing the present wealth of genetic diversity. To recover a population whilst maintaining sustainable genetic diversity from the germplasm repository, it will be necessary to conserve the germplasm from at least 13 individuals of each sex (ideally 25 individuals of each sex) [75]. Thus, the collection of PGCs from fertilized embryos obtained from various combinations of males and females in each population would be ideal for poultry gene banking. From an industrial point of view, cryopreservation of PGCs could be used as back-up for commercially or industrially important poultry lines or breeds that have been selected for a long time in case of they are lost as a consequence of pathogen outbreaks, genetic problems, breeding cessation, or natural disasters. Additionally, PGC cryobanking enables a dramatic reduction of costs associated with maintenance of live birds.

PGC collection sites should be considered in accordance with the situation. Collection of PGCs from developing gonads has the advantage of recovering a large number of PGCs and ensuring a prolonged period for collection. However all embryos which have the potential to hatch out under ordinary circumstances must be sacrificed for gonadal PGC collection. Therefore, the practical use of gonadal PGCs would be restricted if the availability of fertilized embryos is not limited, such as the case with industrially as well as commercially valuable poultry breeds or lines. However, the availability of fertilized embryos in most noncommercial breeds such as indigenous, rare and/or endangered breeds is restricted by several factors such as small population sizes, low egg production, and seasonal breeding. A unique biological property of avian PGCs that temporally circulate thorough the vascular system would provide the opportunity to combine the reproduction of living birds with collection of PGCs. Indeed, of the 88 fertilized embryos of Gifujidori used for PGC collection, 12 survived to sexual maturity with normal reproductive capacity. Subsequently, six Gifujidori offspring were successfully regenerated (6% of total offspring) by mating male and female recipient chickens that had received frozen-thawed Gifujidori PGCs [59]. In this method, drawing blood from donor embryos involves simple windowing procedures. It was also demonstrated that this procedure is available for combined preservation of living birds with PGCs in Japanese quail, green pheasant, and common pheasant (Nakamura *et al*., unpublished data).

In fish, male germ cells (type A spermatogonia) and female germ cells (oogonia) that are transplanted to the opposite sex of recipients can differentiate into functional eggs and sperm [63, 76]. Although chicken PGCs can differentiate into functional gametes in the gonads of opposite sex recipients, the efficiency is very low [77]. Histological analysis suggested that female PGCs in male chicken gonads are capable of passing through the first and second meiotic divisions, but rarely complete spermatid elongation [78]. For gene banking purposes, male and female PGCs should be collected separately and cryopreserved, as efficient germline transmission of PGCs requires that the sex of the donor be matched to the sex of the recipient.

If avian PGCs can differentiate into functional gametes in the gonads of xenogeneic recipients, PGC transplantation could be a powerful tool for *ex situ* conservation of wild birds including endangered species. In avian species, xenogeneic germline transmission between phylogenetically different genera (common pheasant to chicken), family (chicken to guinea fowl (*Numida meleagris*)) or order (duck to chicken, and houbara bustard (*Chlamydotis undulata*) to chicken) was reported only in males, but not in females [42, 79–82]. In future, detailed histological analysis is needed to evaluate the ability of xenogeneic gametogenesis by transfer of donor PGCs carrying a reporter gene to sterilized interspecific recipient embryos.

Recently, Nakamura *et al*. (2013) reported that cryopreservation of PGCs and subsequent production of functional gametes following transplantation is also feasible in Japanese quail in which the semen has not been cryopreserved [61]. More recently, a practical trial reported that successful transport of a chicken breed by shipment of cryopreserved PGCs using a dry shipper [30]. In such a situation, it should be possible to run poultry PGC-bank programs that conduct a collection, cryopreservation, depositing, and distribution service. Based on the recent technical advances in poultry PGC manipulation described here, collection, freezing and storage of PGCs from both industrial and indigenous poultry breeds has begun as part of the National Institute of Agrobiological Sciences (NIAS) Genebank projects (NIAS, Tsukuba, Japan). At present, the NIAS Genebank contains 15 chicken breeds, including eight indigenous breeds that are designated as natural treasures of Japan (Gifujiori, Hinaidori, Jitokko, Koeyoshi, Kurokashiwa, Satsumadori, Toumaru and Yakido), and three Japanese quail lines (Nakamura *et al*., unpublished data). To date, successful transplantation of freeze-thawed immature ovarian tissues to a juvenile recipient has been performed in the Japanese quail [83]. Because of its high production efficiency of donor-derived eggs in recipients, immature poultry ovarian tissues could also be a source for gene banking of female germplasm. Cryopreservation of PGCs together with frozen stocks of semen and immature ovaries will ensure preservation of poultry genetic resources economically and semi-permanently.

Final Notes

My name means “the bright falcon”. The falcon (*Falco peregrinus japonensis*) is classified as vulnerable (i.e., species facing a high risk

of extinction in the wild) by the Japanese Ministry of Environment Red List. PGC culture and xenotransplantation technologies might save falcons from extinction. I do not know whether this idea can be realized, but I intend to establish germ cell manipulation technologies that are universally available across species, to allow conservation of genetic resources of various animal species.

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