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Germline Modification and Engineering in Avian Species

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Production of genome-edited animals using germline-competent cells and genetic modification tools has provided opportunities for investigation of biological mechanisms in various organisms. The recently reported programmed genome editing technology that can induce gene modification at a target locus in an efficient and precise manner facilitates establishment of animal models. In this regard, the demand for genome-edited avian species, which are some of the most suitable model animals due to their unique embryonic development, has also increased. Furthermore, germline chimera production through long-term culture of chicken primordial germ cells (PGCs) has facilitated research on production of genome-edited chickens. Thus, use of avian germline modification is promising for development of novel avian models for research of disease control and various biological mechanisms. Here, we discuss recent progress in genome modification technology in avian species and its applications and future strategies.

INTRODUCTION

Advances in biotechnology have enhanced our understanding of biological mechanisms at the molecular level. In particular, genome modification and engineering technology have contributed to numerous fields, including agriculture and medicine, as well as basic research. Genome modification and engineering are based on insertion, deletion or replacement of genes to alter the genetic information. This biotechnology can be applied to alter the genome of various cell types, including somatic, pluripotent, and germline competent cells such as sperm, ova and fertilized eggs, which transmit genetic information to the next generation. Therefore, germline modification and engineering enables production of genome-edited organisms. The primary role of genome-edited organisms is in studying biological processes in developmental biology (McMahon et al., 2012). In agriculture, genome-edited organisms have been developed for environmental threat-resistance, production of functional proteins, increasing nutritional value and improving yields, as well as unveiling biological mechanisms. Therefore, positive economic, academic and environmental effects of genome-edited livestock and plants have been reported (Tan et al., 2012). Due to completion of genome sequencing projects, the need for production of customized germline modification technology in diverse organisms has increased (Veeramah and Hammer, 2014). In the last 20 years, much effort has focused on germline modification in avian species, because aves have potential advantages in biotechnology as an animal model due to their unique embryonic development (Han, 2009). In particular, their reproductive characteristics, such as a relatively short reproductive cycle and laying over 300 eggs annually, enhance their suitability for this research area. Therefore, here we will introduce recent progress in, and discuss future strategies for, germline genome modification and engineering technology in avian species.

GERMLINE COMPETENT CELLS IN AVIAN BIOTECHNOLOGY

Germline competent cells are a unique resource used to transmit genetic information to the next generation. These include germline cell lines established by *in vitro* culture, as well as endogenous germline cells (Han et al., 2015). Since modification of germline competent cells mediated by genome modification and engineering technology can facilitate production of genome-edited organisms, this technology has been widely used in various areas of biology. Following the first report of genetically modified bacteria in 1973 (Cohen et al., 1973), several organisms with genome editing achieved via the germline—including roundworm, fruit fly, zebrafish and mouse—have been reported. These organisms have been used in basic research, such as for identification of specific gene functions, as well as in applied science, such as for disease control and mass production of functional proteins (Kaletta and Hengartner, 2006; Lieschke and Currie, 2007; Vecchio, 2015; White et al., 2013a; 2013b). In particular, embryonic stem cells (ESC) with germline competency have been utilized for production of genome-edited animals. Also, induced pluripotent stem cells (iPSC) have been used widely in regenerative medicine (Musunuru, 2013; Singh et al., 2015).

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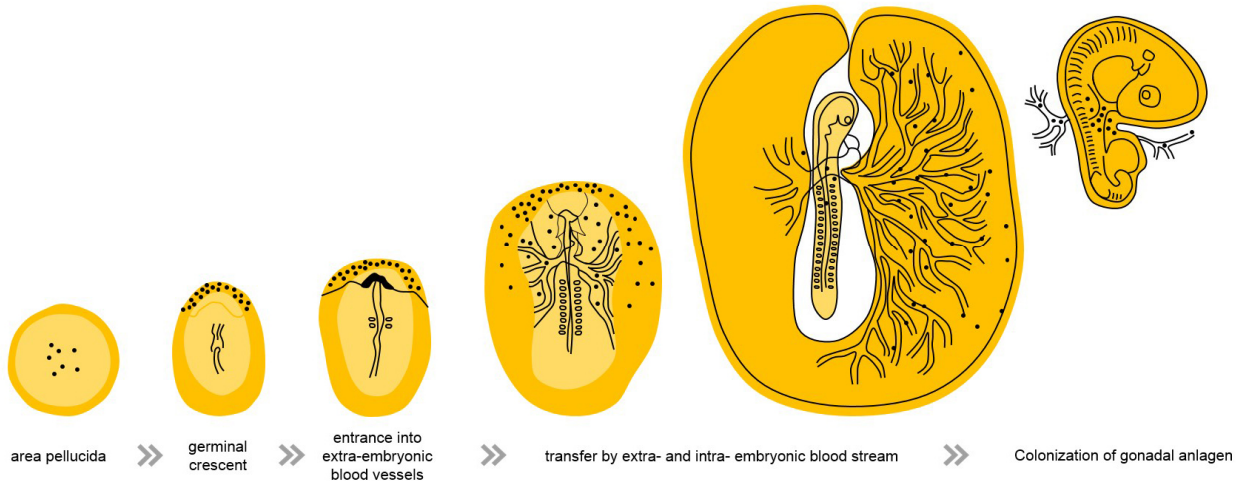

Fig. 1. Chicken PGC migration and settlement during embryonic development. Avian PGCs are dispersed at stage X and move to the germinal crescent at HH stage 4. They then undergo circulation via extra-embryonic blood vessels until settlement in embryonic gonads at HH stage 17. The figure is modified from Nieuwkoop and Sutasurya(1979).

In avian species, several types of germline competent cell have been introduced. Chickens lay eggs composed of 40,000-60,000 cells, known as stage X blastoderms, in which the cells actively proliferate following incubation under optimal conditions (Eyal-Giladi and Kochav, 1976). A number of researchers have suggested that blastodermal cells at stage X maintain an undifferentiated status similar to mammalian ESCs derived from blastocysts. However, the germline transmission efficiency of blastodermal cells transplanted into stage X recipient embryos was relatively low (0.003-42.5%), despite efforts to increase it, such as gamma-ray irradiation or short-term culture of the blastodermal cells (Carsience et al., 1993; Pain et al., 1996; Petitte et al., 1990).

To overcome the low germline transmission efficiency of blastodermal cells, primordial germ cells (PGCs), the precursors to germ cells, derived from various embryonic stages have been used in avian species (Chang et al., 1997; Han et al., 2002; Naito et al., 1994; Ono et al., 1998; Park et al., 2003a; 2003b; Tajima et al., 1993). Avian PGCs have a unique development system in terms of origin, specification, proliferation, and differentiation (Tsunekawa et al., 2000). Avian PGCs are dispersed at stage X immediately after oviposition and move to the germinal crescent at Hamburger and Hamilton (HH) stage 4 (Hamburger and Hamilton, 1992). Then, the PGCs enter the circulation via extra-embryonic blood vessels until settling in embryonic gonads at HH stage 17 (Fig. 1) (Nieuwkoop and Sutasurya, 1979). Previous works reported that PGCs isolated from each developmental stage show higher germline transmission efficiency (11.3-95.8%) than that of blastodermal cells when introduced into the bloodstream of recipient embryos (Naito et al., 1994; Tajima et al., 1993).

Testicular cells also have germline competency in avian species. The seminiferous tubules of recipient roosters transplanted with testicular cells produced donor-testicular cell-derived chicks. This system is considered an effective method for germline transmission because of the reduced time required for production of the next generation. However, it showed low germline transmission efficiency (0.4-0.9%) (Lee et al., 2006). Therefore, technologies that can enhance germline transmission efficiency—such as elimination of recipient germ cells or purification and establishment of germline competent cell populations from donor roosters—are required (Kanatsu-Shinohara et al., 2012; Lim et al., 2014; Nakamura et al., 2010; Park et al., 2010).

ESTABLISHMENT OF AVIAN GERMLINE COMPETENT CELL LINES

Long-term *in vitro* culture of germline competent cells without loss of their germline transmission ability is essential for germline modification and its applications, as is isolation and transplantation of the cells themselves. The technology not only enhances understanding of the specific mechanisms of germ cell maintenance, development and differentiation but also provides sources for production of genome-edited animals. *In vitro* proliferation of germline competent cells—including ESCs, embryonic germ cells (EGCs), PGCs and spermatogonial stem cell—in mammals has been reported, and these cells have been widely used in modern biology (Evans and Kaufman, 1981; Kanatsu-Shinohara et al., 2003; Matsui et al., 1992; Resnick et al., 1992).

In avian species, trials of long-term *in vitro* culture systems have been reported. ESCs derived from stage X blastodermal cells using stem cell factor, fibroblast growth factor (FGF) and leukemia inhibitory factor were established. The cells showed expression of pluripotency marker genes, including POU domain class 5 transcription factor 3 (*POUV*), SRY (sex determining region Y)-box 2 (*SOX2*), Nanog homeobox (*NANOG*), and Kruppel-like factor and the ability to differentiate into three germ layers, similar to murine ESCs, although the cells showed low germline transmission efficiency (Jean et al., 2013; Pain et al., 1996; Petitte et al., 2004).

In vitro culture of EGCs derived from gonadal PGCs has also been investigated. We reported previously successful establishment of EGCs derived from gonadal PGCs of HH stage 28 embryos *in vitro*; the cells showed several ESC characteristics—including migration, Periodic Acid–Schiff (PAS) and Stage-Specific Embryonic Antigen-1 (SSEA-1) antibody reactivity and

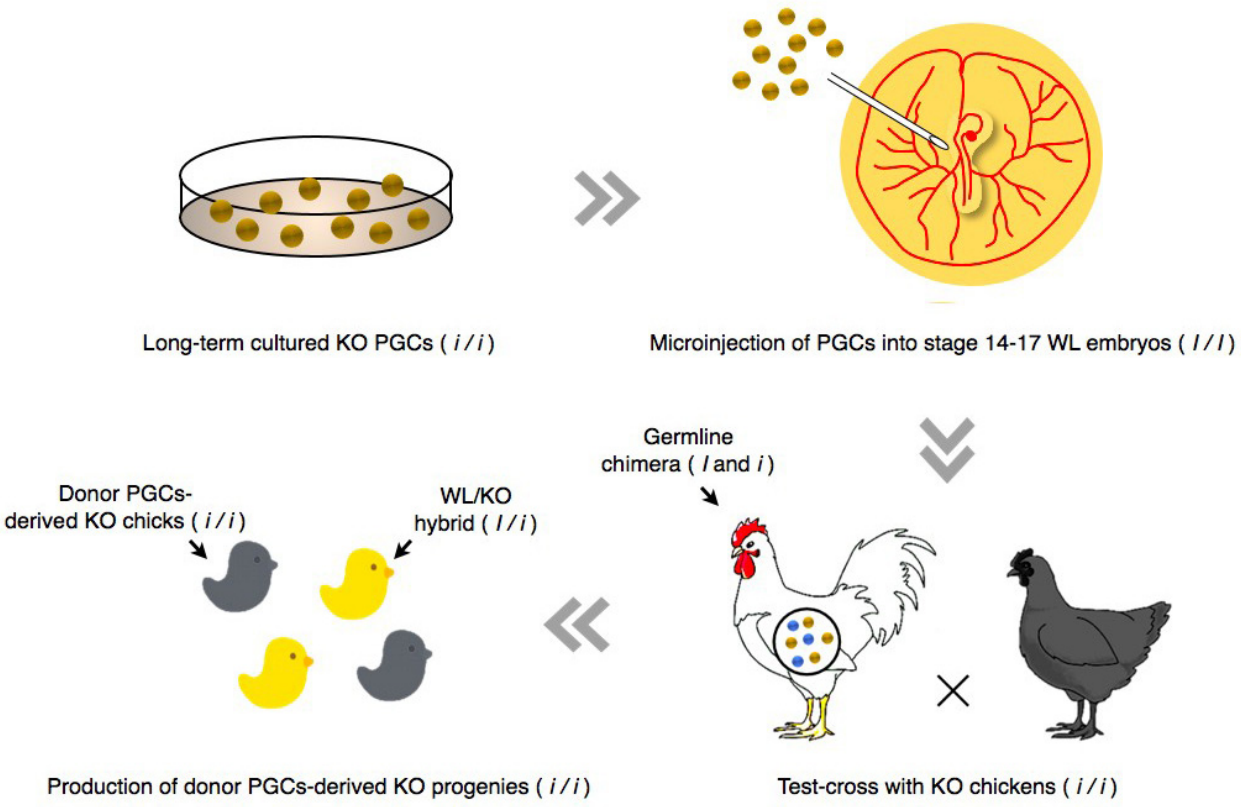

Fig. 2. Schematic representation of germline chimera production using long-term cultured, germline-competent PGCs. Long-term cultured PGCs derived from Korean Oge (KO) (*i/i*; black feather) are transplanted into blood vessels of stage 14-17 White Leghorn (WL) embryos (*I/I*; white feather). Sexually matured germline chimeras (*I* and *i*) are crossed with KO (*i/i*). Feather color distinguishes donor PGC-derived progeny (*i/i*) from the WL/KO hybrid (*I/i*).

germline competency—when transplanted into recipient embryos (Park et al., 2003a). The cells showed higher germline transmission capacity (1.5-25.0%) than that of ESCs derived from stage X blastodermal cells.

Both ESCs and EGCs, however, still showed low germline transmission efficiency. To overcome this problem, researchers used *in vitro* PGC cultures. Park et al. (2003b) reported that PGCs isolated from embryonic gonads (stage 28) and cultured for 5 or 10 days showed relatively high germline competency (0.9-56.5%). Recently, McGrew and our group reported a chicken PGC *in vitro* culture system using basic FGF medium. The PGCs proliferated in an unlimited manner *in vitro*, expressed germness-related genes—including chicken vasa homolog, deleted in azoospermia-like, *POUV, SOX2, NANOG* and v-myc avian myelocytomatosis viral oncogene homolog (*C-MYC*)—and exhibited telomerase activity and unique migratory characteristics when injected into recipient embryos. Furthermore, compared with other germline competent cell lines, they showed markedly higher germline transmission ability when transplanted into recipient embryos (12.5-82.6%) (Fig. 2) (Choi et al., 2010; Macdonald et al., 2010).

DIRECT GERM CELL ENGINEERING FOR GERMLINE MODIFICATION

Genome modification of germ cells based on culture systems has provided opportunities not only to investigate the functions of specific genes in germ cells but also to develop novel animal models for human disease (Saitou and Yamaji, 2012). Development of disease models, especially for cancer, can provide enhanced understanding of the mechanisms of proliferation, metastasis and apoptosis of cancer cells (Cheon and Orsulic, 2011). In addition, mass production of pharmaceutical proteins in genome-edited organisms mediated by direct germ cell engineering has considerable economic and societal benefits (Dove, 2000; Sijmons et al., 1990).

After development of somatic nuclear cell transfer (SCNT) technology in sheep (Wilmut et al., 1997), direct germline modification using SCNT has been investigated in several organisms for the purposes of efficient farm animal production, mass production of drugs, regenerative medicine and conservation of genetic resources (Ogura et al., 2013). SCNT is at present the most effective method for producing genome-edited animals and research into germ cell biology in mammalian species.

Direct germ cell engineering in avian species focused initially on blastodermal cells for production of transgenic animals. After the first report of production of transgenic chickens mediated by retroviral infection of stage X embryos, production of transgenic avians has been based primarily on viral infection systems (Kamihira et al., 2009; Lillico et al., 2007; Salter et al., 1986). However, genome engineering of blastodermal cells using viral infection systems has several limitations, including low germline

transmission ability, silencing of the expression of the inserted gene, and unexpected phenomena due to random transgene integration (Kamihira et al., 2005; Mizuarai et al., 2001).

PGCs, which are unipotent and differentiate only into germ cells, have been emphasized as an alternative resource for germline modification. In 2006, direct germline modification mediated by electroporation of linearized DNA into cultured chicken blood PGCs was reported. The results suggested that PGCs are a promising cell type for germline modification (van de Lavoir et al., 2006). Establishment of a long-term culture system for chicken PGCs using basic FGF-containing medium (Choi et al., 2010; Macdonald et al., 2010) was followed by production of transgenic chickens via transplantation of cultured PGCs (Macdonald et al., 2012; Park and Han, 2012; Park et al., 2015). Furthermore, several studies based on long-term PGC culture systems have aimed to identify specific genetic and epigenetic mechanisms in PGCs, which is difficult in other species (Lee et al., 2011; Rengaraj et al., 2011; 2014).

PROGRAMMABLE GENOME EDITING FOR GERMLINE MODIFICATION

Despite the efficiency of transgenic technology using long-term culture of PGCs in avian species, random integration remains limited due to use of transposon vector systems (Macdonald et al., 2012; Park and Han, 2012). Therefore, there is a need for precise genome targeting. In 2013, homologous recombination-mediated immunoglobulin gene knockout in chickens was reported; however, the germline transmission efficiency was extremely low (< 0.002%) (Schusser et al., 2013). Since homology-directed repair (HDR) occurs less frequently than nonhomologous end joining (NHEJ) in nature (Mali et al., 2013), promotion of HDR by repression of NHEJ machinery may be an alternative method for precise genome replacement mediated by homologous recombination (Maruyama et al., 2015).

On the other hand, our group adopted transcription activator-like effector nucleases (TALEN), a programmable genome editing (PGE) technology, to produce ovalbumin gene knockout chicken. TALEN breaks double-stranded genomic DNA and induces NHEJ (Christian et al., 2010). NHEJ occurs more frequently than HDR; therefore, TALEN shows higher efficiency in terms of genome mutation than that of homologous recombination mediated genome editing. As expected, the efficiency of genome-edited chicken production was improved markedly (< 10.4%), and precise genome editing was confirmed without plasmid residue (Park et al., 2014).

PGE technologies—including zinc finger nucleases, TALEN and clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (CRISPR/Cas9)—are versatile tools for genome editing in various organisms (Kim and Kim, 2014). Although there are concerns regarding induction of off-target mutations than can cause chromosomal rearrangements—including deletions, inversions and translocations—the specificity of the nucleases has been increased based on experimental evidence and computational analysis (Koo et al., 2015).

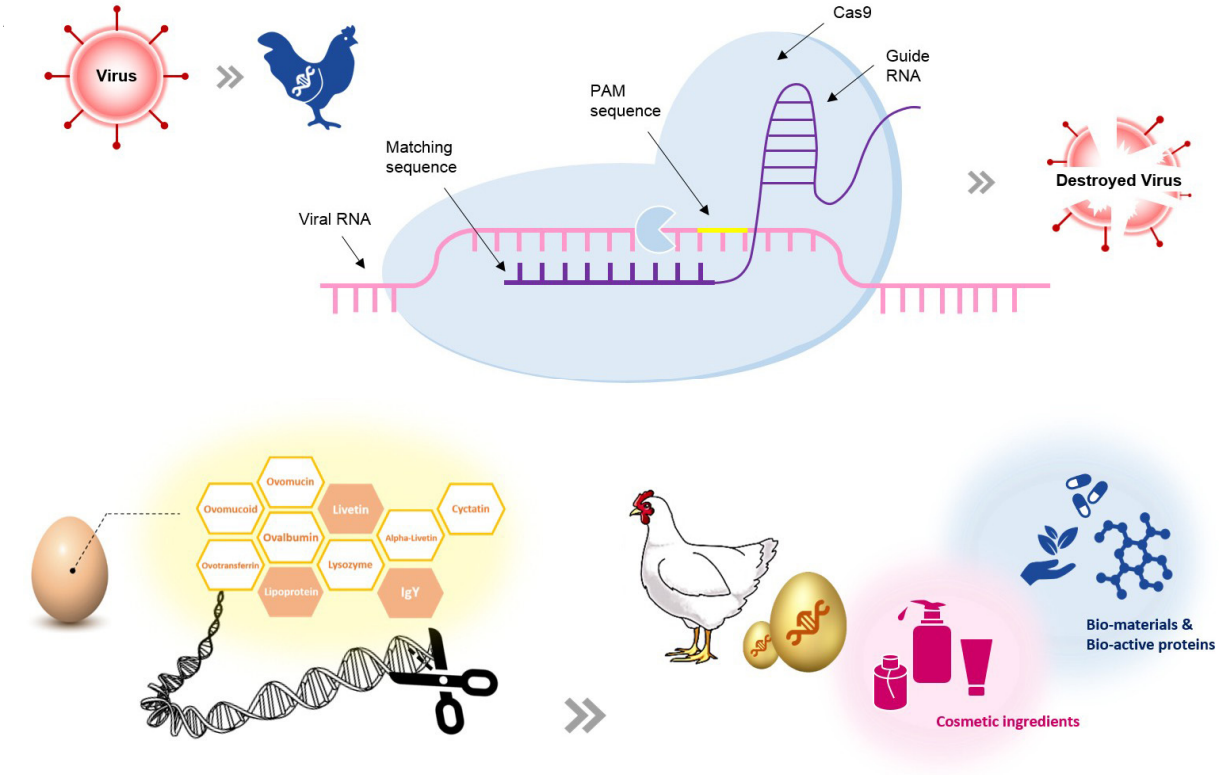

Fig. 3. Applications of genome-edited chickens in avian influenza (AI) resistance and egg protein modification. (A) CRISPR/Cas9 system-mediated viral RNA recognition and degradation. Transgenic chickens expressing CRISPR/Cas9 elements specifically targeting AI viral RNA segments exhibit AI resistance. (B) Genome modification and engineering of egg protein-coding genes. Eggs laid by genome-edited chickens could be used for production of functional proteins.

FUTURE STRATEGIES IN AVIAN GERMLINE MODIFICATION

Avian species are needed as model animals as well as industrial resources. However, few genome-edited aves have been reported due to the lack of an efficient system that can be applied to avian species. Recent reports of development of transgenic chickens and/or genome-edited chickens showed their potential for various applications (Macdonald et al., 2012; Park and Han, 2012; Park et al., 2014; 2015; Schusser et al., 2013). Therefore, highly efficient and precise genome editing technology will maximize the utilization of the chicken as an animal model as well as a valuable resource in industry.

One example is genome-edited avians used for disease control. At present, not only global climate change but also disease pandemics threaten the lives of wild animals. In particular, epidemic avian influenza (AI) mediated by migratory birds is a major cause of death among avian species, and mutations can enable this virus to infect other animals, including humans (Schrauwen and Fouchier, 2014). Diverse approaches have been used to preserve avian genetic resources from potential threats. However, no definitive method has been presented. Germline modification and engineering represent a novel approach to establish disease-resistant avian lines. Indeed, genome-edited chickens expressing shRNA specifically targeting viral RNA polymerase activity resulted in reduced AI viral transmission (Lyall et al., 2011). This suggests germline genome modification of avian species to be an alternative method for addressing viral disease pandemics. Also, the recently developed CRISPR/Cas9 system, which recognizes and cleaves single-stranded RNA (Fig. 3), and inducible expression of viral proteins using the *piggyBac* system are expected to be utilized for production of disease-resistant avian lines (O'Connell et al., 2014; Park and Han, 2012). Germline modification will be utilized not only for avian diseases but also for human diseases, such as cancer. In particular, chicken ovarian cancer has histological, morphological and molecular similarities with human ovarian cancer that make it a suitable model for the latter (Hawkridge, 2014). Development of novel avian disease models using germline modification technology could contribute to our understanding of ovarian cancer at the molecular level.

Another promising application of germline modification is alteration of egg protein composition. Chicken egg white is composed of major proteins—such as ovalbumin, ovotransferrin, ovomucoid, ovomucin, and lysozyme—and small quantities of bio-functional proteins (Stevens, 1991). Application of PGE techniques using germ cell manipulation and transgenic systems to regulate the composition of egg white enables production of protein-modulated eggs. These can have various properties, such as increased nutrient levels, reduced allergenicity, production of high levels of antimicrobial substances, and increased concentrations of bioactive materials (Fig. 3). PGE technologies, including TALEN and CRISPR/Cas9, have been applied to develop a high-yield egg-bioreactor, which can produce useful materials by regulating the production of natural proteins. Furthermore, many valuable proteins can be produced using this system; *e.g.*, high-cost growth factors, recombinant vaccines, hormones, and industrial materials, such as recombinant spider silk-like protein. The combination of traditional animal farming and the latest biotechnology may yield benefits to industry.

CONCLUSION

Germline modification will play an essential role in future biotechnology. Despite the wide range of applications of birds as animal models, only a few genome-edited animals have been reported to date compared with other species. State-of-the-art genome editing technology using long-term cultured PGCs has been applied to avian species only recently. Efficient and precise PGE technologies combined with long-term PGC culture systems will open a new era in avian biotechnology and have considerable benefits for the poultry industry.

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