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INVITED REVIEW

Germ Cell Biology

# Germline-competent stem cell in avian species and its application

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Germ cells are the only cell type in the body that can transfer genetic information to the next generation. Germline-competent stem cells can self-renew and contribute to the germ cell lineage giving rise to pluripotent stem cells under specific conditions. Hence far, studies on germline-competent stem cells have contributed to the generation of avian model systems and the conservation of avian genetic resources. In this review, we focus on previous studies on germline-competent stem cells from avian species, mainly chicken germline-competent stem cells, which have been well established and characterized. We discuss different sources of germline-competent stem cells and recent advances for the future applications in birds.

*Asian Journal of Andrology* (2015) 17, 421–426; doi: 10.4103/1008-682X.148073; published online: 6 February 2015

**Keywords:** birds; germline-competent stem cells; primordial germ cells; spermatogonial stem cells

## INTRODUCTION

Germline competency represents the ability of cells to give rise to a functional gamete and transmit whole genetic information to the next generation. In stem cell research, this property is very crucial to be utilized for the basic researches, as well as applied biotechnology. Germline-competent stem cell has its own characteristics of stem cell potency and germline competency. Although studies on germline-competent stem cells have been mainly focused in mammals, different sources of them from other animals should be considered for the advance in animal biotechnology.

Primordial germ cells (PGCs) have many characteristics in common with embryonic stem cells (ESCs) and differences between them at the molecular level are challenging. In mouse, PGCs are capable of self-renewal, express pluripotency-related genes such as *Nanog* and *Oct4* as well as germ cell-related genes,<sup>1</sup> and are regulated by similar signaling pathways as ESCs.<sup>2</sup> In addition, mouse PGCs and spermatogonial stem cells (SSCs) can de-differentiate into ESC-like cells, which differentiate into three germ layers, form teratomas and undergo germline transmission<sup>3,4</sup> when cultured under specific conditions. In avian species, such characteristics, including germline contribution, self-renewal, expression of specific markers and differentiation ability, are common mostly in PGCs and SSCs, and partly in blastodermal cells and ESCs, which are discussed below.

In this regard, studies on germline-competent stem cells can increase our understanding of stem cell potential and differentiation. Combined with advanced biotechnologies, including germline chimera systems, transgenesis, and genome editing, germline-competent stem cells can be a powerful tool for animal biotechnology. In this review, we introduce different types of avian germline-competent stem cells,

including blastodermal cells, ESCs, PGCs and SSCs, and discuss their current research progress and future applications.

## GERMLINE-COMPETENT STEM CELLS

### *Germline-competent stem cells in animal species*

Studies on the establishment of pluripotent cell lines, production of germline chimera and transgenic animals have contributed to animal biotechnology for increasing human welfare as well as basic biology. Using germline-competent stem cells, valuable animals and endangered species can be reproduced and conserved. When combined with transgenic technologies, economic traits for animal products can be created. In mammals, several germline-competent stem cells that can contribute to the germline have been developed, such as ESCs, embryonic germ cells (EGCs) and SSCs.

Many studies have now reported the derivation of ESCs, EGCs or SSCs in various mammals including the pig,<sup>5–7</sup> cow,<sup>8–10</sup> sheep,<sup>11,12</sup> goat,<sup>13</sup> and horse.<sup>14</sup> However, excluding studies performed in the rat and mouse (laboratory animals), the germline contribution of these cells has not been fully validated despite their ability to differentiate into three germ layers *in vitro* and form teratomas *in vivo*.<sup>15–17</sup> Thus, in mammals including farm animals, somatic cell nuclear transfer (SCNT) has been used mainly for animal cloning and transgenesis rather than cell-mediated methods.<sup>18</sup>

Recently, cell reprogramming techniques *in vitro* were developed to differentiate somatic cells into pluripotent stem cells, called induced pluripotent stem cells (iPSCs).<sup>19</sup> These cells can differentiate into the three germ layers as well as into germ cells *in vitro* and *in vivo*, similarly to ESCs. Initially, iPSCs were generated from embryonic and adult somatic cells by introduction of several pluripotency-related genes such as *Oct4*, *Sox2*, *cMyc* and *Klf4*.<sup>19,20</sup> Since iPSCs can be generated

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Received: 09 September 2014; Revised: 12 November 2014; Accepted: 09 December 2014

from adult cells, they are thought to be an alternative tool to ESCs for basic stem cell studies and human disease models. For animals in which pluripotent cell lines have not been established, iPSCs are a good alternative to animal transgenesis. In addition to mice and humans, many studies on the generation of iPSCs from mammals, including the monkey<sup>21</sup> and pig,<sup>22</sup> have been reported. Since iPSCs can be induced to differentiate into germ cells *in vitro*,<sup>23,24</sup> the establishment of iPSCs from domestic animals can be useful for animal transgenesis using germline chimera techniques. In nonmammalian species, derivation of avian iPSCs has been recently reported.<sup>25,26</sup> In particular, induced pluripotency in chicken embryonic fibroblasts resulted in a germ cell fate, suggesting different cellular mechanisms between aves and mammals.<sup>25</sup>

### Germline-competent stem cells in avian species

#### Importance of germline-competent stem cell study in avian species

The most common and powerful tool for transgenesis in mammals is pronuclear injection and SCNT techniques using unfertilized oocytes. In avian species, however, several obstacles due to the unique characteristics of avian eggs make it difficult to apply conventional systems. The cytoplasm of avian oocytes is very large compared with other species and is connected to yolk, which results in imperfect cellularization.<sup>27</sup> The yolky and large cytoplasm of avian oocyte makes it difficult to perform pronuclear injection of exogenous DNA. Due to the large amount of yolk, avian eggs have no transparency and it is difficult to identify the nucleus of oocytes. Thus, SCNT has not been possible in avians. Therefore, germline-competent stem cells-mediated transgenic systems are required.<sup>28</sup> Due to the unique developmental features of avian species, it is easy to observe and manipulate avian embryos compared with other species. Thus, germ cells and embryos at any stage can be monitored and isolated easily. It has been demonstrated experimentally that several germline-competent stem cells derived from different developmental stages can contribute to the germline and have self-renewal activity in chickens. At this time, several germline-competent stem cells such as blastodermal cells, ESCs, PGCs and SSCs have been used for germline chimera production and transgenesis in avian species (Table 1). Therefore, for practical applications, it is important to understand the origin of each cell type and the related techniques.

#### Germ cell development in avian species

In avian species, different types of germline-competent stem cells exist and can be obtained according to their developmental stages. Thus, understanding the developmental process of germ cells is required. In chickens, although PGC specification remains unclear at this time, germ plasm structures present in oocytes and cleavage

stage embryos via expression of chicken vasa homolog (CVH), which is strong evidence of the preformation mode, have been reported.<sup>29,30</sup> During primitive streak formation, PGCs migrate gradually toward the anterior region and the germinal crescent.<sup>31</sup> They incorporate into blood vessels at Hamburger, and Hamilton (HH) stages 10–12<sup>32,33</sup> and undergo circulation at HH stages 12–15 before settling into embryonic gonads.<sup>34,35</sup> The chicken PGCs then migrate toward the gonadal ridges at HH stage 17 to differentiate.<sup>34</sup> In the adult testis, spermatogonia including SSCs are located close to the basal membrane of the seminiferous tubules.

#### Blastodermal cells and embryonic stem cells

In chickens, after fertilization in the isthmus and following vigorous cell division for 20 h in the uterus, blastoderms containing approximately 40 000–60 000 cells develop at Eyal-Giladi and Kochav (EGK) stage X, which is the oviposition stage.<sup>36</sup> At EGK stage X, 1–2 cell layers are present in the area pellucida (a central region), while the area opaca (a peripheral region) remains multilayered. Although the anteroposterior embryonic axis is already formed at EGK stage X, the blastodermal cells are undifferentiated. When the cells were transplanted into the subgerminal cavity of recipient embryos at EGK stage X, somatic and germline chimeras were produced successfully, demonstrating their stem cell characteristics.<sup>37–39</sup>

Another utilization of blastodermal cells is for *in vitro* culture to generate pluripotent cell lines such as ESCs, which are derived from the inner cell mass of the blastocyst in mammals.<sup>40,41</sup> Blastodermal cells were cultured in medium containing leukemia inhibitory factor (LIF), stem cell factor, insulin growth factor 1 (IGF-1) and other factors,<sup>42,43</sup> which is conventional culture medium used for murine ESCs, to form chicken ESCs. Chicken ESCs express several stem cell markers such as stage-specific embryonic antigen-1 (SSEA-1), alkaline phosphatase and epithelial membrane antigen-1 (EMA-1).<sup>42,44,45</sup> In addition, chicken ESCs can differentiate into three germ layers and form embryoid bodies through the removal of LIF from the culture medium, and produce somatic chimeras when they are reintroduced into recipient embryos, indicative of their pluripotency.<sup>42,45</sup>

#### Primordial germ cells

Primordial germ cells are precursors of functional gametes, sperm or eggs. PGCs in most animal species originate from early developmental stages through two different mechanisms; the preformation and induction modes.<sup>29</sup> In chickens, PGCs have been thought to originate through the preformation mode by maternally inherited determinants such as CVH.<sup>30</sup> At HH stage 4, when the primitive streak is fully developed, PGCs gather in the germinal crescent, which is the anterior marginal region between the area opaca and area pellucida.<sup>35</sup> The PGCs then circulate in the extraembryonic blood vessels until they settle in the genital ridges at HH stage 17.<sup>34</sup> After embryonic day 6, when gonadal sex differentiation occurs, the PGCs undergo sex-specific differentiation.<sup>46</sup> In this regard, until embryonic day 6, PGCs can be isolated from different embryonic tissues such as the germinal crescent at HH stage 4, extraembryonic blood vessels at embryonic day 2.2 and embryonic gonads at embryonic day 5–6. To enrich of the isolated PGCs, fluorescence-activated cell sorting and magnetic-activated cell sorting using PGC-specific antibodies have been used in avian systems.<sup>47,48</sup>

Since only a limited number of PGCs can be obtained from embryos, it is important to establish long-term *in vitro* culture systems for PGCs. Recent studies demonstrated the establishment of such systems for chicken PGCs using germline transmission efficiency.<sup>49–51</sup> It is demonstrated that basic fibroblast growth factor (bFGF) via MEK/ERK signaling plays a crucial role in the survival and long-term

**Table 1: Different sources of germline-competent stem cells in avian species**

Cell type	Source and origin	Germline transmission efficiency	References
Blastodermal cells	Stage X embryos	+	37–39
ESCs	Blastodermal cells	±	42,45
EGCs	PGCs	±	63,64
PGCs	Blood vessels at embryonic day 2.2 Gonads at embryonic day 5.5	+++	49,51,65,66
SSCs	Juvenile and adult testis	+	53,80

ESCs: embryonic stem cells; EGCs: embryonic germ cells; PGCs: primordial germ cells; SSCs: spermatogonial stem cells

proliferation of chicken PGCs.<sup>49</sup> In addition, cultured PGCs express PGC-specific markers and exhibit telomerase activity, migratory activity, and germline contribution, indicating that germ cell integrity can be maintained even after long-term culture.<sup>49,51</sup> In this regard, in avian species, PGCs are the most powerful type of germline-competent stem cell for avian biotechnology.

#### **Spermatogonial stem cells**

After entering embryonic gonads, PGCs undergo sex-specific differentiation: spermatogenesis in males and oogenesis in females. In male chickens, PGCs enter mitotic arrest as prospermatogonia at embryonic day 8, and spermatogonia proliferate after hatching.<sup>46</sup> SSCs, self-renewing cells in the testis that can produce sperm continuously, are classified into four types in avians based on their morphological characteristics: a dark type A (Ad), two pale A types (Ap1 and Ap2) and dark type B spermatogonia.<sup>52</sup> Among these, the Ad spermatogonia are thought to be SSCs.

In chicken, SSCs can be isolated from juveniles at 3–4 weeks or from adult testes. The proportions of SSCs are approximately 0.4% in juvenile testis and 0.03% in adult testis.<sup>53</sup> When chicken testicular cells were cultured in medium containing LIF, bFGF and IGF-1, they formed colony structures and expressed germ cell-specific markers such as SSEA-1/3/4, integrin alpha 6, integrin beta 1 and EMA-1. In addition, these SSCs differentiated into embryoid bodies in LIF-free medium, indicative of their stem cell potency.<sup>53</sup>

### **APPLICATION OF GERMLINE-COMPETENT STEM CELLS**

#### ***Germline chimera and transgenic birds***

##### **Blastodermal cells and embryonic stem cells**

In many studies on germline chimera and transgenic birds, researchers have targeted blastodermal cells or blastoderms at EGK stage X. Compared with PGCs or SSCs, blastodermal cells can easily be obtained and manipulated from fertilized eggs. When retroviral or lentiviral vectors were introduced into EGK stage X embryos, transgene expression was observed in various tissues (including germ cells) after further development, indicative of the contribution of blastodermal cells to somatic and germ cells.<sup>54,55</sup> In addition, when isolated blastodermal cells were transplanted into the subgerminal cavity of EGK stage X embryos, somatic and germline chimeras were produced.<sup>37,39</sup> However, compared with the somatic chimerism rate, germline transmission efficiency of blastodermal cells was too low.

In another approach, when chicken ESCs were transplanted after *in vitro* transfection, transgene-expressing chimeric chickens were produced.<sup>56</sup> Despite several efforts with *in vitro* culture of chicken ESCs, germline transmission capacity was extremely low and has not been repeated experimentally so far.<sup>57</sup> One possible reason is that chicken germ cell and somatic lineages are already segregated at EGK stage X, according to the presence of the CVH protein and germplasm-like structures in chicken oocytes and cleavage stage embryos,<sup>30</sup> such that an insufficient number of PGCs among blastodermal cells exists to contribute to the germline. In addition, chicken ESCs showed a reduction in germline potency during culture, but then reacquired potency when exogenous CVH was overexpressed,<sup>58</sup> indicating the conventional culture condition used for ESCs cannot induce germ cell fate. Thus, to utilize blastodermal cells or ESCs from the chicken, greater detail on germ cell formation is required.

##### **Primordial germ cells**

In avian species, PGCs are more commonly used to produce the germline chimera than ESCs, SSCs and other germline-competent

cells because of their germline unipotency and easy access. Especially, germline chimeras in chicken and quail have been produced using blood PGCs and gonadal PGCs after transplantation into the recipient embryonic blood vessels.<sup>48,51,59–62</sup> Since other pluripotent stem cell lines such as ESCs and EGCs in chicken showed too low germline competency, PGCs are thought to be an optimal tool for a stable avian transgenesis.<sup>42,63,64</sup>

The establishment of long-term culture techniques for PGCs has resulted in remarkable advances in avian biotechnology. Using nonviral transfection of cultured PGCs, transgenes were expressed continuously and strongly in the transgenic chickens for generations.<sup>65–67</sup> More recently, gene knockout chickens were produced by genome editing on PGCs using transcription activator-like effector nucleases (TALENs) and homologous recombination.<sup>68,69</sup> In particular, because TALEN knockout chickens are nontransgenic, they can be widely used for agricultural practical applications.<sup>68</sup> Thus, the next goal in avian biotechnology will be the development of elaborate and efficient genome editing techniques for model animals.<sup>68</sup> One possible method is the Clustered Regularly Interspaced Short Palindromic Repeats-associated (CRISPR)-Cas system.<sup>70,71</sup> Because the CRISPR-Cas system is more efficient for the insertion, deletion or modification of target genes compared with TALENs, it requires further investigation before application in avian systems.

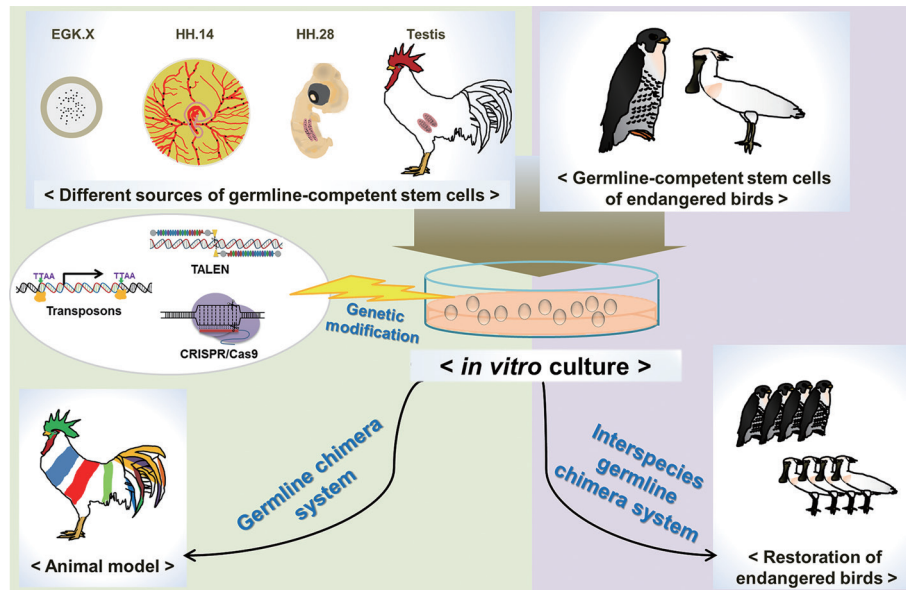
#### **Spermatogonial stem cells**

To produce transgenic birds using blastodermal cells, ESCs or PGCs, it is important to wait until the founder's sexual maturation and to produce a large number of founders for enrichment and analysis of donor-derived offspring. Thus, although studies on avian SSCs are limited compared with blastodermal cells and PGCs, utilization of SSCs can be a time-efficient alternative. When testicular cells were transplanted into recipient testes, they could produce donor-derived offspring.<sup>72,73</sup> In addition, when freshly isolated or *in vitro* cultured SSCs were transplanted into blastoderms at EGK stage X or embryonic blood vessels at 53–54 h, they could produce donor-derived offspring, indicating that later-stage germ cells maintain their developmental unipotency *in vivo*.<sup>74</sup> One major obstacle of SSCs for avian biotechnology is their low germline transmission efficiency. Despite this, the transgenic efficiency of offspring derived from transplanted SSCs is high. Thus, studies on the enrichment of exogenous SSCs in recipient testes and maintenance of their potency during culture are required. On the other hand, another candidate can be oogonial stem cells (OSCs) which are present in adult ovary. After the first studies on mammalian OSCs were reported at 2004,<sup>75–77</sup> isolation and characterization of them has been actively studied. However, there is no report on OSCs in avian species, so far. Therefore, investigation of reliable markers of avian OSCs should be studied in the future.

#### **Interspecies germline chimera for restoration of endangered species**

Endangered species, which increase every year, are an important and global problem that must be addressed. Although animal cloning using nuclear transfer has been developed in mammals, it is not applicable to avian species. Thus, an interspecies germline chimera system is a powerful tool for the conservation of avian genetic resources. At this time, many studies have been conducted on the production of interspecies germline chimeras by transplantation of PGCs, blastodermal cells and SSCs, including quail-to-chicken,<sup>78–80</sup> turkey-to-chicken,<sup>81</sup> duck-to-chicken<sup>82</sup> chicken-to-duck<sup>83</sup> and chicken-to-guinea fowl.<sup>84</sup> However, the production of donor-derived progeny was too low<sup>82</sup> or absent,<sup>78,79,81</sup> mainly due to physiological





**Figure 1:** Application of germline-competent stem cells-mediated methods in avian species. Germline-competent stem cells derived from various sources can be applied to animal model systems and the conservation of endangered birds.

differences between species such as reproduction cycle and egg size. Recently, donor-derived progeny of wild birds, including pheasant<sup>85</sup> and Houbara bustard<sup>86</sup> using PGCs and gonadal cells, respectively, were produced successfully using the interspecies germline chimera system. Thus, for applications in other endangered birds, culture methods of germline-competent stem cells for diverse species should be investigated. In addition, physiological aspects such as donor cell sex and differences between species should be considered.<sup>87</sup>

## CONCLUSIONS

Recent advances in long-term *in vitro* culture of germline-competent stem cells and germline chimera production in the chicken combined with genome editing have highlighted the need for an avian species as an animal model<sup>68,69</sup> (Figure 1). In addition, the production of interspecies germline chimera via germline-competent stem cells has become a powerful tool for the restoration of endangered avian species<sup>83,85,87</sup> (Figure 1). Since several types of germline-competent stem cells can be obtained from different developmental stages in birds, optimal *in vitro* culture systems for different types of germline-competent stem cells should be established in future studies. Indeed, the presence and function of germline-competent stem cells are closely associated with the potency of the cells. In other words, once they emerge, understanding why and how they acquire their potency is important to regulate and maintain these cells *in vitro*. Therefore, studies on the underlying mechanisms of germline-competent stem cells formation and maintenance in avian species can contribute to the utilization of avian species as animal model systems and the conservation of avian genetic resources.

## AUTHOR CONTRIBUTIONS

JYH conceived of the study, and JYH, HCL and TSP participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

## COMPETING INTERESTS

All authors declare no competing interests.

## ACKNOWLEDGMENTS

This work was supported by a grant from the Cooperative Research Program for Agriculture Science and Technology Development (Project No. PJ010390012014 and Project No. PJ01053301) of the Rural Development Administration, Republic of Korea.

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