The dynamic development of germ cells during chicken embryogenesis

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ABSTRACT Appropriate regulation of cell proliferation during embryogenesis is crucial for the maintenance of germness. An in-depth understanding of germ cell developmental processes may yield valuable information on germ cell biology and applied sciences. However, direct evidences about germ cell proliferation and cell cycling during avian embryonic development has not been well-studied. Thus, we explored chicken germ cell dynamics during embryonic development via flow cytometry employing a germ cell-specific anti-cVASA antibody (the chicken VASA homolog is termed CVH) and propidium iodide staining. The numbers of male germ cells increased significantly during early embryonic development, but proliferation was decreased significantly with accumulation at the G0/G1 phase after embryonic d 14 (E.14), indicating initiation of mitotic arrest in the testis. On the other hand, the number of female germ cells increased significantly throughout embryogenesis, and proliferating cells were continuously evident in the ovary to the time of hatching, although gradual accumulation of cells at the G2/M phase was also evident. 5-ethynyl-2′-deoxyuridine (EdU) incorporation analysis revealed that populations of mitotically active germ cells existed in both sexes during late embryogenesis, indicating either the maintenance of stem cell populations, or asynchronous meiosis. Collectively, these results indicate that chicken germ cells exhibited conserved developmental processes that were clearly sexually dimorphic.

Key words: Chicken, germ cells, proliferation, meiosis, mitotic arrest

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INTRODUCTION

Germ cell development and differentiation pathways during embryogenesis are crucial for the production of gametes from their precursors, and proper regulation mediated by intrinsic and extrinsic factors ensures that the gametes function normally (Richardson and Lehmann, 2010). Mitotic and meiotic arrest of early germ cells during embryogenesis, reflecting G0/G1 and G2/M pauses in males and females, respectively, allows recombination to repair genetic damage and alleviates conflicts between gametes, in turn reducing genomic mutation (Mira, 1998). Cell dynamics are tightly linked and coordinated during early germ cell differentiation (Lesch and Page, 2012).

Cell proliferation, arrest, and resumption of cell division in early-stage germ cells are largely conserved among species that produce haploid gametes (Gerton and Hawley, 2005). During early mouse embryogenesis, germ cells engage in dynamic processes including migration, proliferation, and mitotic arrest. Small populations of male primordial germ cell (**PGC**) precursors proliferate rapidly and gradually enter G1/G0 arrest during embryonic development (Tam and Snow, 1981; Byskov, 1986; Western et al., 2008). In addition, several organisms including *Caenorhabditis elegans* and *Drosophila melanogaster* share the unique physiological characteristics of male germ cell development (Cheng and Mruk, 2010). Female germ cells also undergo rapid proliferation and meiotic arrest during embryonic development. In the mouse, female germ cell ovarian numbers attain ∼25,000; the cells begin to accumulate in the G2/M phase (indicating meiotic arrest) from 13.5 d post-coitum (Borum, 1961). Molecular analysis of germ cell dynamics has revealed that fine control of cell-cycle protein expression regulates the distinct cellular processes evident in male and female cells (Western et al., 2008; Miles et al., 2010).

In avian species, germ cells transit different physiological stages during embryonic development. Germ cells begin to appear in embryonic gonads at

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Hamburger and Hamilton embryonic stage 15, and then proliferate in a sexually dimorphic manner (Hughes, 1963; Mendez et al., 2005; Nakamura et al., 2007). Previous studies reported that male germ cells in embryonic gonads proliferate gradually to embryonic d 6 (**E.6**), at which time testicular organization is apparent, and then stop proliferating until E.13, at which time proliferation resumes for the next 4 d (Swift, 1916; Mendez et al., 2005; Nakamura et al., 2007). However, female germ cells have been reported to proliferate dramatically to E.9, only to become arrested in prophase I (meiotic arrest) at this time (Hughes, 1963; Smith et al., 2008). Meiotic marker analysis including evaluation of the expression levels of synaptonemal complex proteins (**SYCPs**) defined the time of meiotic event initiation in female germ cells, and suggested that retinoic acid metabolism played a role in the embryonic urogenital system (Smith et al., 2008; Zheng et al., 2009).

Although efforts have thus been made to understand chicken germ cell development during embryogenesis; no clear, direct evidence of mitotic or meiotic arrest is available, and no proliferation profiling during embryonic development has been performed. Therefore, we explored sexual dimorphism between germ cell proliferation profiles during embryonic development using flow cytometry employing an anti-cVASA antibody (the chicken VASA homolog is termed **CVH**) and propidium iodide (**PI**) staining. Furthermore, immunohistochemical analysis using 5-ethynyl-2′-deoxyuridine (**EdU**) identified proliferating germ cells in developing embryonic gonads.

MATERIALS AND METHODS

Experimental Animals and Animal Care

White Leghorn (**WL**) chickens were maintained according to the standard operating protocols, and all experimental procedures were approved by the Institutional Animal Care and Use Committee of Seoul National University.

Preparation of Gonadal Cells

Chicken gonads at embryonic (E) d E.10, E.12, E.14, E.16, and E.18, and those of hatchlings, were collected with tweezers after dissection of abdomens under a stereomicroscope. For the E.10, E.12, and E.14 embryos, gonadal tissues were chopped using small scissors and then dissociated by gentle pipetting in 0.05% (w/v) trypsin solution supplemented with 0.53 mM ethylenediaminetetraacetic acid (**EDTA**; Sigma-Aldrich, St. Louis, MO). For E.16 and E.18 embryos and hatched chicks, gonadal tissues were incubated with 0.5% collagenase IV (Sigma-Aldrich) at 37°C for 10 min in a shaking incubator at 250 rpm before dissociation.

Whole Gonadal Cell and Germ Cell Counts

From E.6 to the time of hatching, the left and right gonads of each stage were dissociated into single cells, and whole gonadal cells counted using a hemocytometer. The gonadal cells were then immunostained with anti-germ-cell-specific antibody, thus a rabbit anti-CVH antibody (Lee et al., 2013), followed by staining with 4′,6-diamidino-2-phenylindole (**DAPI**; Invitrogen, Carlsbad, CA) to detect germ cells. The germ cell percentage per whole gonadal cell in an embryo was estimated by counting the number of CVH-positive cells among DAPI-positive cells under a fluorescence microscope (model TU-80; Nikon, Tokyo, Japan). Following this, germ cell numbers per embryo were estimated. Three replications were performed in all cases.

Cell Cycle Analysis

Prior to cell cycle analysis, gonadal cells were first dissociated into single cells and washed once in phosphate-buffered saline (**PBS**) and fixed in 70% ethanol for 1 d at −20°C. The fixed gonadal cells were washed once with PBS and incubated with blocking solution containing 5% goat serum and 1% bovine serum albumin (**BSA**; Sigma-Aldrich) in PBS for 1 h at 4°C. Next, the cell aliquots were incubated with an anti-CVH primary antibody (rabbit IgG) for 1 h at 4°C. After washing three times with PBS containing 0.05% Tween 20 (**PBST**), the cells were incubated with goat anti-rabbit Alexa Fluor 488 (Life Technologies, Carlsbad, CA) diluted in PBST (1:500) for 1 h at room temperature. After incubation, the cells were washed three times in PBST and dissociated in 1% BSA in PBS. For the cell cycle analysis, the isolated germ cells were treated with 10 μg/mL RNase A (Invitrogen) for 30 min at 37°C and 50 μg/mL PI (Sigma-Aldrich) for 30 min at 4°C. The cell cycle status was analyzed by FACSCalibur (Becton Dickinson and Company, Franklin Lakes, NJ) and data were analyzed using FACSDiva and Modfit LT cell-cycle analysis software (Verity Software House, Topsham, ME).

5-Ethynyl-2′-deoxyuridine (EdU) Incorporation

To ascertain the proliferation status of the germ cells, EdU (Life Technologies) incorporation analysis was conducted to detect S-phase synthesis, as described previously (Lee et al., 2013). After careful removal of the eggshell and shell membrane, EdU diluted in dimethylsulfoxide (**DMSO**; Sigma-Aldrich) was injected into the extra-embryonic blood vessels of E.10–E.18 embryos (10 μL of 10 mM EdU) or blood vessels of hatched chicks (100 μL of 1.6 M EdU) 4 h before subsequent analysis. After injection, the eggs were sealed with Parafilm and incubated at 37.5°C and 60–70% relative humidity.

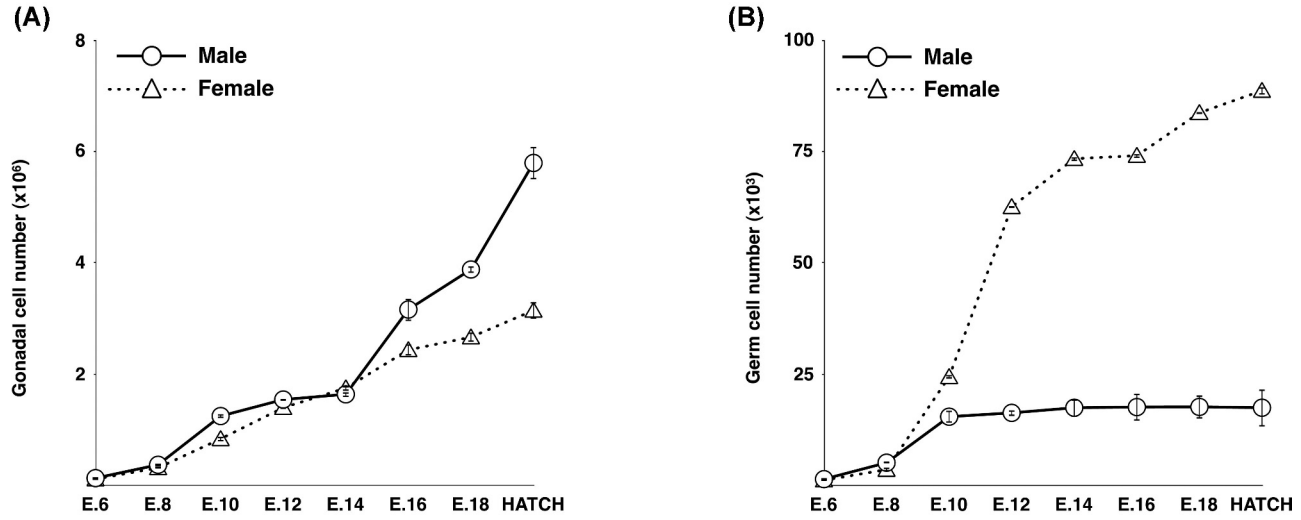

Figure 1. Changes in the numbers of all gonadal and germ cells during embryonic development. (A) The numbers of all gonadal cells in both sexes from embryonic d 6 (E.6) to hatching. (B) The numbers of germ cells in both sexes from E.6 to hatching. Open circles with solid lines indicate males, and open triangles with dashed lines females.

Table 1. The numbers of whole gonadal cells and germ cells during embryonic development in both sexes.

	E.6	E.8	E.10	E.12	E.14	E.16	E.18	HATCH
Gonadal cell number (Male)	126,400 ± 7,760[a]	366,233 ± 30,435[b]	1,243,000 ± 33,808[c]	1,540,000 ± 14,730[d]	1,634,333 ± 47,815[d]	3,160,000 ± 206,639[e]	3,880,000 ± 60,828[f]	5,796,666 ± 295,014[g]
Gonadal cell number (Female)	108,300 ± 7,143[a]	311,033 ± 3,523[b]	832,666 ± 46,715[c]	1,393,333 ± 15,275[d]	1,746,667 ± 49,328[e]	2,433,333 ± 100,166[f]	2,663,333 ± 83,266[g]	3,143,333 ± 149,778[h]
Germ cell number (Male)	1,380 ± 79[a]	5,078 ± 426[b]	15,429 ± 420[c]	16,289 ± 153[c]	17,438 ± 510[d]	17,589 ± 515[d]	17,629 ± 276[d]	17,450 ± 894[d]
Germ cell number (Female)	1,138 ± 75[a]	3,530 ± 40[a]	24,388 ± 1,368[b]	62,623 ± 686[c]	73,440 ± 2,074[d]	74,138 ± 3,051[d]	83,766 ± 2,618[e]	88,763 ± 4,223[f]

Data represent the means ± SDs of triplicate replications.
Different letters ([a-h]) in each row indicate significant differences at $P < 0.05$.

Immunohistochemistry

Embryonic gonads were dissected from E.10–E.18 embryos and hatched chicks. The dissected gonads were fixed in 4% paraformaldehyde (Sigma-Aldrich) overnight and washed in PBS. They were then immersed in 5% sucrose in PBS, 30% sucrose in PBS, and 30% sucrose in Tris-buffered saline overnight at 4°C, respectively. After embedding in optimal cutting temperature compound (Sakura Finetek, Torrance, CA) and frozen in liquid nitrogen, they were cryosectioned (thickness, 5 μm) at −28°C. Cryosections were dried on a slide warmer at 55°C for 20 min and rinsed in PBS for 20 min at room temperature. To detect incorporated EdU, cryosections were stained using a Click-iT EdU detection kit (Invitrogen) according to the manufacturer's protocols. After blocking, the sections were incubated with anti-CVH antibody overnight at 4°C. The sections were then treated with secondary antibody, goat anti-rabbit Alexa Fluor 488 (Life Technologies), for 1 h at room temperature. Sections were then mounted with Prolong Gold anti-fade reagent with DAPI and visualized using a fluorescence microscope.

Statistical Analysis

Statistical Analysis System (**SAS**) software was used for analysis of all numerical data. The effects of all treatments were compared using the least-square difference test. The analysis of variance module of the SAS package was used to determine the significance of the main effects. The level of significance between groups was set at $P < 0.05$ and indicated as different letters.

RESULTS

Proliferation of Germ Cells in Both Sexes during Embryonic Development

To define the proliferation profiles, the number of germ cells in both sexes during embryonic development was calculated. In males, the total number of whole gonadal cells in an embryo significantly increased from E.6 to hatching (Figure 1A and Table 1). Conversely, the number of male germ cells labeled with CVH antibody showed similar proliferation profiles from E.6 to E.10 to those of whole gonadal cells, but growth was

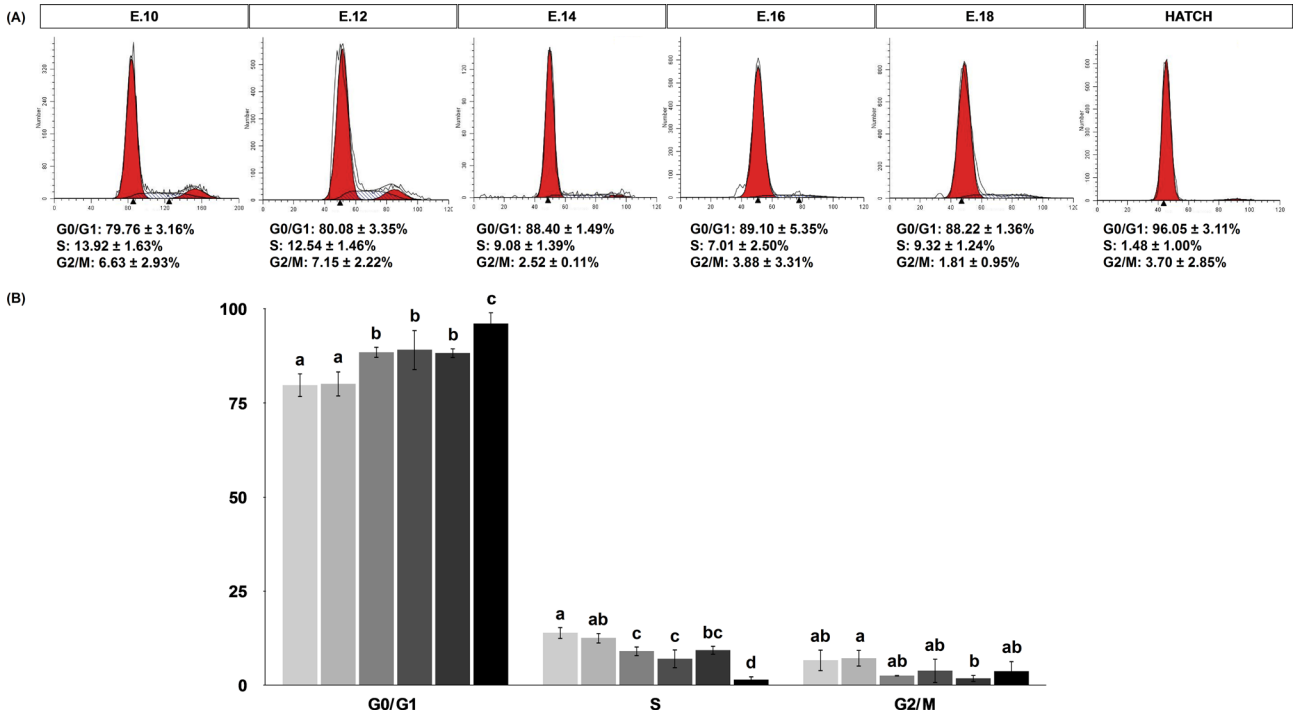

Figure 2. Cell-cycle developmental profiles of male germ cells from E.10 to E.18 (embryos) to hatching. (A) Flow cytometric analysis of cell cycle distributions of male germ cells from embryonic d 10 (E.10) to hatching. The percentages of cells in each cell cycle phase are shown (means ± standard deviation [SD]). (B) Accumulation of male germ cells in the G0/G1 phase during embryonic development, associated with a reduced proportion of germ cells in the S and G2/M phases. The different superscripts denote significantly different ($P < 0.05$) means in terms of cell cycle status. Bars indicate the SDs of mean triplicate analyses.

retarded from E.10 to hatching (Figure 1B). According to the estimated data, the number of male germ cells increased significantly from E.6 to E.14 but not thereafter (Figure 1B and Table 1). In females, the total number of whole gonadal cells in the embryo gradually increased until hatching, as was the case in males (Figure 1A). However, the number of female germ cells increased significantly during all embryonic stages; growth retardation was evident only from E.14 to E.16. Notably, the number of female germ cells increased dramatically from E.8 to E.14 (from 3,530 cells at E.8 to 73,440 cells at E.14); male germ cells did not exhibit this burst (Figure 1B and Table 1). Thus, more female than male germ cells were evident at hatching (88,763 female and 17,450 male germ cells), although no significant difference was evident between the numbers of gonadal or germ cells in either sex at early development stages (E.6 to E.8; Figure 1A and 1B). Collectively, the results suggest that germ cell proliferation in embryonic chicken gonads was sexually dimorphic.

Cell Cycle Analysis of Chicken Germ Cells during Embryonic Development

Cell cycle analysis was performed by flow cytometry to determine whether chicken germ cells displayed a male-female difference regarding the cell cycle status, based on their DNA contents. Male germ cells had a high proportion of G0/G1 phase and a low proportion of S- and G2/M-phase cells during all developmental stages (Figure 2A). The proportion of germ cells at G0/G1 phase increased significantly after E.14, indicating initiation of germ cell accumulation at the G0/G1 phase. At this time, the proportion of cells in the S phase decreased significantly without any significant change in the proportion of cells in the G2/M phase (Figure 2B). Upon hatching, most male germ cells accumulated in the G0/G1 phase (96.05 ± 3.11%), indicating mitotic arrest of such cells (Figure 2A).

During embryonic development, female germ cell proliferation differed notably from that of male germ cells. At E.10, E.12, E.14, and E.16, female embryos contained relatively high levels of germ cells in the G0/G1 phase and relatively few in the G2/M phase. At E.16 and thereafter, the proportions changed dramatically. At E.16 and E.18, the proportion of germ cells in the G0/G1 phase decreased significantly from 63.17 ± 8.67 to 45.8 ± 10.37%, and the proportion in the G2/M phase increased significantly from 23.56 ± 8.74 to 48.51 ± 9.38%, indicating that meiotic arrest was in play. At hatching, 80.19 ± 4.90% of female germ cells accumulated at the G2/M phase but 15.14 ± 4.90% of female germ cells remained in the G0/G1 phase, suggesting that such cells underwent meiotic arrest in an unsynchronized manner (Figure 3A). In addition, cell cycle analysis showed that female germ cells consistently remained in the S phase during embryonic development, although a significant decrease in the proportion of such cells was evident from d E.12 to hatching (Figure 3B).

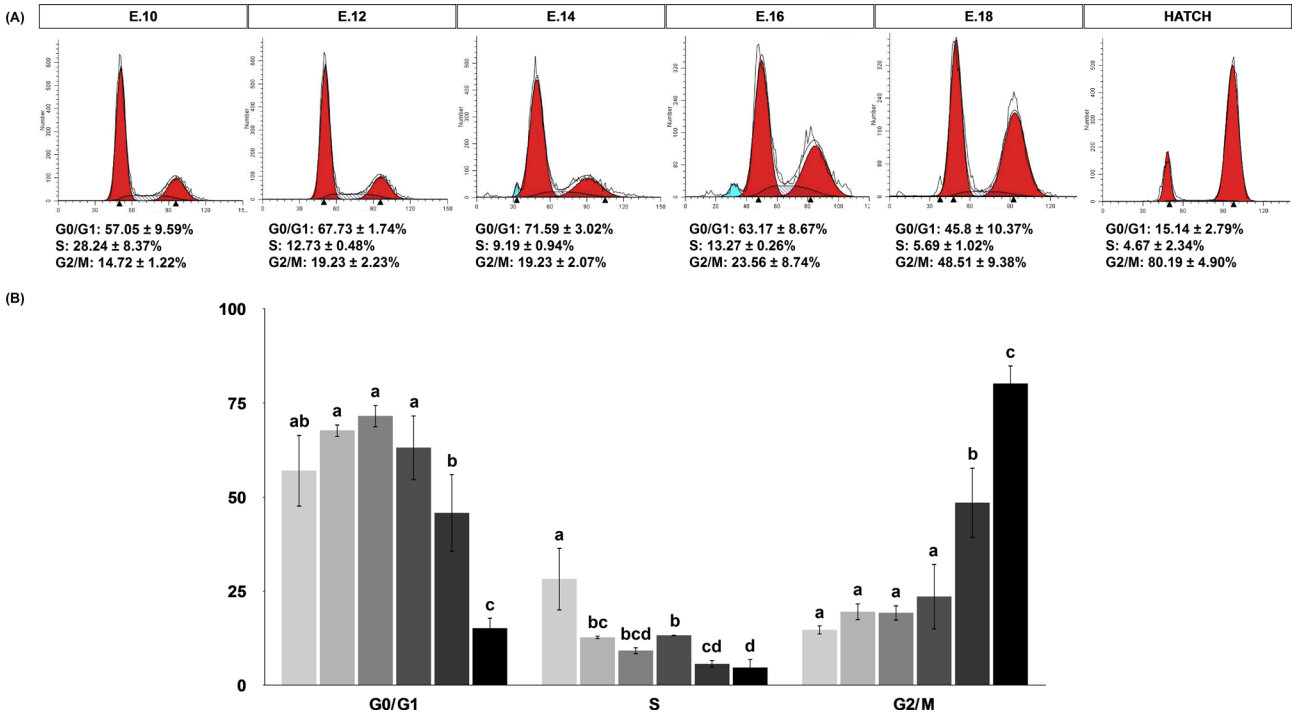

Figure 3. Cell-cycle developmental profiles of female germ cells from E.10 to E.18 (embryos) and at hatching. (A) Flow cytometric analysis. The percentages of cells in each cell cycle phase are shown (means ± SDs). (B) Accumulation of female germ cells in the G2/M phase was observed during embryonic development, associated with reduced proportions in the G0/G1 and S phases. The different superscripts denote significantly different ($P < 0.05$) means in terms of cell cycle status. Bars indicate the SDs of the means of triplicate analyses.

Immunohistochemical Analyses of Chicken Germ Cells during Embryonic Development and Hatching

We performed immunohistochemical staining using anti-EdU and -CVH antibodies to identify the distributions of proliferating gonadal germ cells during embryonic development. EdU- and CVH-co-immunostaining cells were common in embryonic gonads throughout the embryonic development of both sexes (Figures 4 and 5). In males, EdU- or CVH-positive germ cells were dispersed over the entire embryonic gonad early in embryonic development (E.10 and E.12). At later stages (from E.14 to hatching), CVH-positive germ cells became localized to the future seminiferous tubules whereas EdU-positive cells were dispersed over the entire gonads during embryonic development. EdU- and CVH-doubly positive cells were evident throughout embryonic development; most such cells were singly dispersed in embryonic gonads (Figure 4).

In females, on the other hand, CVH-positive germ cells were localized to the gonadal cortex during embryonic development, but EdU-positive cells were dispersed throughout the gonad. EdU- and CVH-doubly positive cells were evident even at the early stages of embryonic development, as was also true in males. However, in females, most EdU- and CVH-doubly positive cells were in cysts from E.12 to the time of hatching. Approximately 3–7 EdU- and CVH doubly positive cells were noted in each developing cyst (Figure 5).

DISCUSSION

During germ cell development, strict controls of cell cycle events, such as proliferation and the induction of meiosis, are required at the appropriate times to establish the formation of functional gametes. Under aberrant regulation, germ cells undergo excessive apoptosis, formation of testicular germ cell tumors or cause infertility (Jorgensen et al., 2013). The mechanisms of germ cell development are strongly conserved in the animal kingdom (Gerton and Hawley, 2005). Sex differentiation is initiated during embryonic development, while the onset of meiosis occurs in females but not in males during the embryonic phase, with the involvement of several genes in gonadal differentiation, both functionally and structurally (Smith and Sinclair, 2004). In chickens, germ cell development has a mechanism similar to that in mammals, in terms of the developmental processes and molecular expression profile (Smith et al., 2008; Zheng et al., 2009; Yu et al., 2013). Nonetheless, no direct evidence of mitotic and meiotic arrest, or proliferation profiling after sexual differentiation, has been reported in chicken germ cells. Thus, we investigated the sexually dimorphic proliferation profile of germ cells during chicken embryonic development.

First, the proliferation profiles of gonadal and germ cells during chicken embryonic development were investigated using the germ cell-specific anti-CVH antibody. The numbers of gonadal cells increased significantly during embryonic development in both sexes, as previously reported (Figure 1A) (Swift, 1916;

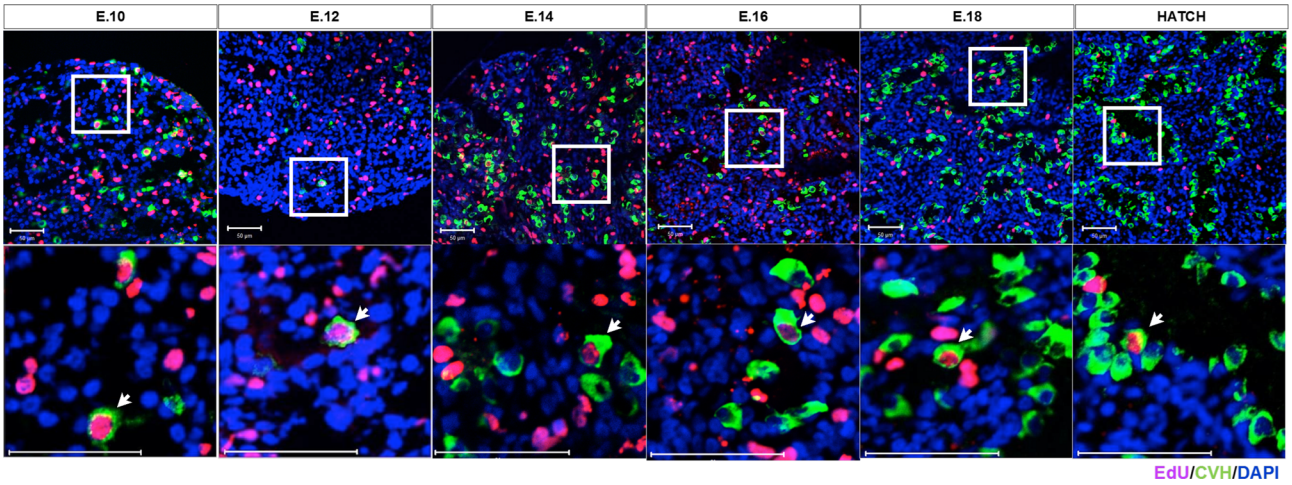

Figure 4. The distributions of CVH-positive and EdU-incorporating cells in male gonads from E.10 to E.18 and after hatching. Proliferating germ cells during embryonic development that were doubly positive for the chicken vasa homolog (CVH) (green) and 5-ethynyl-2′-deoxyuridine (EdU) staining (pink) are shown. 4′,6-diamidino-2-phenylindole (DAPI; blue) was used to detect proliferating germ cells in gonads. The boxed regions in the upper panels are shown at higher magnification in the lower panels. Arrows indicate single proliferating male germ cells. Scale bars = 50 μm.

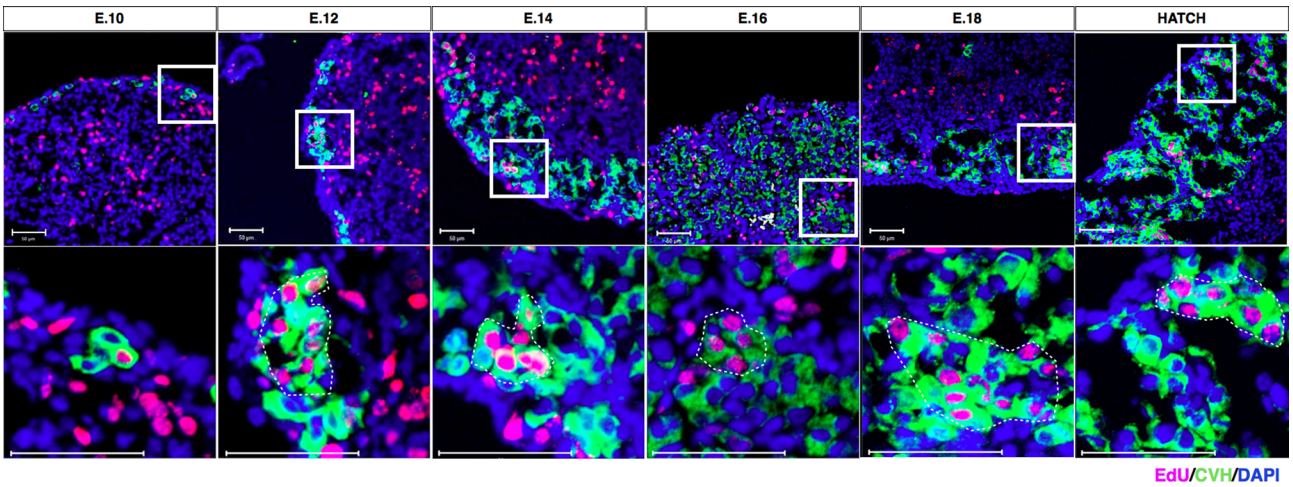

Figure 5. The distributions of CVH-positive cells and EdU-incorporating cells in female gonads from E.10 to E.18 (embryos) and in hatched chicks. Some germ cells proliferating during embryonic development were doubly positive for CVH (green) and EdU staining (pink). DAPI was used to detect proliferating germ cells in gonads. The boxed regions in the upper panels are shown at higher magnification in the lower panels. Dashed lines indicate proliferating female germ cells that formed cysts. Scale bars = 50 μm.

Mendez et al., 2005). The germ cell proliferation profile revealed that male germ cells underwent significant proliferation from E.6 to E.14 (to attain approximately 1,400–17,000 cells) and these numbers then did not increase significantly from E.14 to hatching (Figure 1B and Table 1). Early studies on germ cell proliferation reported that male germ cells ceased to proliferate at the time of testicular organization (E.6) (Swift, 1916; Mendez et al., 2005), but we detected a significant increase in the number of male germ cells at later stages (Table 1). Also, our estimate of the number of male germ cells is much lower than that of a previous study (approximately 17,000 vs. 30,000 at E.14), even though the cited authors counted only the number of germ cells in the left gonad (Mendez et al., 2005). Germ cell counts were earlier affected by morphological criteria (cell diameter and shape, and the locations of the nucleus and the Baliani body). We employed an antibody-based counting technique.

To clarify germ cell proliferation, cell cycle status was investigated with the aid of flow cytometry, which revealed that male germ cells entered G0/G1 phase from E.14, at which time a reduced proportion were in S phase. We found that that most male germ cells were in G0/G1 at hatching (Figure 2). This indicated that the pause in male germ cell proliferation commencing at E.14 was attributable to a reduced proportion of germ cells in S phase (Figure 1B). Considering the definition of mitotic arrest, germ cell accumulation at G0/G1 during embryonic development reflects such arrest (Western et al., 2008). Compared to the mouse, the mitotic arrest of chicken male germ cells was prolonged (mitotic arrest is maintained from only E.12.5 to E.14.5

in the mouse). However, the gradual and unsynchronized entry into mitotic arrest exhibited by the mouse was conserved in the chicken.

Female gonadal cells proliferated actively throughout embryonic development, as did male cells (from E.6 to hatching) (Figure 1A) even after entry into meiotic arrest (approximately 1,100–89,000 germ cells) (Figure 1B and Table 1). Rapid proliferation of cells in the left female gonad was previously reported, in agreement with our results (Mendez et al., 2005). However, our results differ greatly from those of earlier works in terms of the numbers of female germ cells. An early study on female germ cell proliferation found that cell numbers increased greatly from E.9 to E.17, to attain 680,160, and decreased thereafter (Hughes, 1963). Another study reported that 500,749 female germ cells were present at E.17 (Erickson, 1974). Mendez et al. did not record the number of female germ cells present at E.17, but found that such cells increased in number continuously from E.8 to E.14, to attain approximately 10,000–200,000 (Mendez et al., 2005). As mentioned above, these between-study differences are attributable to the methods used to count germ cells; we employed an antibody-based technique.

Cell cycle analysis of female germ cells showed that the proportion in G2/M increased significantly from E.16 to hatching, with significant decreases apparent in the proportions in the G0/G1 and S phases (Figure 3). Previous reports indicated that SYCP3 (a mitotic marker) mRNA and protein expression were first detected in female chicken gonads in d E.8 and E.15.5, respectively; and histological analyses of female germ cells revealed that pre-leptotene oocytes containing packed chromatin in their nuclear centers were first detected at E.13 (Hughes, 1963; Smith et al., 2008; Zheng et al., 2009). Collectively, these results suggest that initiation of meiosis by female germ cells commences on E.13 and continues gradually even after hatching, resulting in cell accumulation in the G2/M phase (Figure 1B and Table 1). In the mouse, female germ cells continue to proliferate (to attain ~25,000 cells), most of which remain in diplotene I for 2 d (E.13.5 to E.15.5) (Borum, 1961; Miles et al., 2010). Our results suggest that the proliferation profiles of chicken germ cells during embryonic development are strongly consistent (being similar to those of the mouse), exhibiting distinctive characteristics especially in terms of timing and the extent of regulation.

Next, we used EdU incorporation data to show that mitotically active germ cells were present in both male and female gonads during embryonic development. Single mitotically active male germ cells were present on the basement membranes of seminiferous tubules from d E.16 to hatching (Figure 4), indicating that these were primitive diploid germ cells or putative spermatogonial stem cells (Phillips et al., 2010). However, several questions concerning the identity of mitotically active female germ cells remain. Mitotically active female germ cells were present in ovarian cysts from after meiosis onset to the time of hatching (Figure 5), suggesting that the relatively high proportion of germ cells in S phase allows cell numbers to continuously increase (Figures 1B and 3B). Furthermore, the results suggest that the meiotic arrest of female germ cells was gradual, even after hatching. Previous reports suggested that meiotic arrest was complete on the d after hatching; this was termed "delayed" meiosis (Hughes, 1963; Byskov, 1986).

On the other hand, cyst development may constitute evidence of the veracity of an alternative hypothesis. In insects, both male and female germline stem cells (**GSCs**) undergo meiosis throughout the entire life cycle, via cyst formation (de Cuevas et al., 1997). The existence of cysts may be critical for GSC maintenance in mice, although this remains controversial (Lei and Spradling, 2013). Further experiments are needed to confirm the identity of mitotically active germ cells in the ovaries of hatchlings, including analysis of marker expression and tracking of cell fate after hatching.

CONCLUSION

In this study, sexually dimorphic germ cell proliferation profiles were analyzed in terms of DNA content, and via visualization of mitotically active germ cells during chicken embryonic development. The results showed that chicken possess consistent mechanisms and distinctive patterns of germ cell development compared to mammalian species. To gain a full understanding of the unique germ cell development in chicken embryos, further research will focus on identification of the molecular signaling networks leading to germ cell development in the gonads. Specifically, distinct signaling pathways are involved in the maintenance of germness and the differentiation capacity of early-stage germ cells, for example PGCs. Finally, this study on chicken germ cell proliferation and cell cycle status provides comprehensive insight into the biology of germ cells in avian species.

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