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The Expression of c-*kit* Protein during Oogenesis and Early Embryonic Development[1]

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ABSTRACT

The c-*kit* proto-oncogene encodes a transmembrane tyrosine kinase receptor and was shown to be allelic with the white-spotting locus (*W*) of the mouse. Mutations at the *W* locus have pleiotropic effects on the development of hematopoietic stem cells, melanoblasts, and primordial germ cells. In order to elucidate the role of c-*kit* protein in gametogenesis and oocyte maturation, we have examined immunohistochemically the expression of c-*kit* in the ovaries of mice at late fetal and postnatal stages, and in early embryos. By the avidin-biotin-peroxidase (ABC) method using rat anti-mouse c-*kit* monoclonal antibody, the c-*kit* protein was detected in ovaries after the time of birth, but not before. The expression of c-*kit* was observed mainly on the surface of oocytes, but not in granulosa cells nor in interstitial regions. Oocytes of primordial to fully grown Graafian follicles showed the c-*kit* protein. When ovulation was induced by hCG, the expression of c-*kit* in ovulated unfertilized oocytes was weaker than in oocytes of Graafian follicles. In 1-cell embryos the c-*kit* protein was still observed, but with cell division its expression further decreased, and it was not detected in embryos of 4-cell, 8-cell, and morula stages. In summary, the highest expression of c-*kit* was observed on the surface of oocytes arrested in the diplotene stage of meiotic prophase. With ovulation and the resumption of meiotic maturation, its expression declined. These results suggest that the c-*kit* protein may play some role in meiotic arrest, oocyte growth, and oocyte maturation.

INTRODUCTION

A double gene dose of mutant alleles at either the dominant white-spotting (*W*) locus or the steel (*Sl*) locus affects the development of primordial germ cells, neural crest-derived melanocytes, hematopoietic stem cells, and cells of the erythroid and mast cell lineages [1–6]. There are many independent mutations at the *W* and *Sl* loci, and the different alleles vary in their degree of severity [1, 2, 7]. In spite of the similarity in the phenotypic expression of W/W^v and Sl/Sl^d mice, the underlying mechanisms are quite different. Bone marrow transplantation from congenic +/+ mice cures both mast cell deficiency and anemia of W/W^v mice [3, 5], but not those of Sl/Sl^d mice [4, 6]. Neural crest cells of +/+ embryos can differentiate into melanocytes in the skin of W/W^v embryos, but not in the skin of Sl/Sl^d embryos. Furthermore, bone marrow transplantation from anemic Sl/Sl^d mice rescues lethally irradiated +/+ mice [4], but that from W/W^v mice does not [3]. Neural crest cells of Sl/Sl^d embryos can produce melanocytes in the skin of +/+ embryos, whereas those of W/W^v embryos do not [8]. Therefore, it has been thought that an intrinsic defect in their precursor cells causes depletion of mast cells, erythrocytes, and melanocytes in W/W^v mice. In contrast, depletion of these three types of cells in Sl/Sl^d mice is due to a defect in the tissue environment rather than in the affected cells themselves. The sterility of *W* and *Sl* mutant mice is due to a defect in the proliferation and migration of primordial germ cells as they move out from the hindgut region to the genital ridge during mid-gestation [9].

It has been reported that the *W* locus is identical to the proto-oncogene c-*kit* [10–12]. C-*kit* is the cellular homologue of v-*kit*, the oncogene of the HZ4 feline sarcoma virus [13]. The c-*kit* protein is a receptor tyrosine kinase that is structurally similar to the receptors for colony-stimulating factor-1 and platelet-derived growth factor [14, 15]. The ligand for the c-*kit* protein, SLF (also called SCF, stem cell factor), has recently been cloned and shown to be encoded at the *Sl* locus [16–19]. The expression of the c-*kit* gene was observed in, but not restricted to, those populations affected by *W* mutations, including ovary, testis, placenta, bone marrow, spleen, lung, and brain [12, 14, 15]. Recently, by in situ hybridization, c-*kit* mRNA was detected in the oocytes from both immature and mature ovarian follicles [20, 21]. Therefore, to elucidate the role of c-*kit* in oogenesis and early embryogenesis, we investigated the immunohistochemical localization of the c-*kit* protein in the ovaries of mice at late fetal and postnatal stages, and in the early embryos, using rat anti-mouse c-*kit* monoclonal antibody.

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MATERIALS AND METHODS

Animals and Hormones

In all experiments, random-bred CD-1 (Charles River, Tokyo, Japan) mice housed under controlled lighting conditions (lights-on: 0700–1900 h) were used. Equine chorionic gonadotropin and hCG were purchased from Teikoku Hormone Mfg. Co., Tokyo, Japan.

Monoclonal Antibody

Rat anti-mouse c-*kit* monoclonal antibody, ACK45, was prepared and characterized as previously described [22]. The antibody was diluted 1:50 with PBS containing 0.1% BSA (Sigma Chemical Co., St. Louis, MO) and 0.1% NaN_3 (Nakarai Tesque Inc., Kyoto, Japan).

Ovaries

Postnatal ovaries were obtained from female mice at 2–3 days, 1-wk, or 3-wk of age. In one experiment, mice at 3-wk of age were primed with an i.p. injection of 5 IU eCG and 48 h later were killed by cervical dislocation and the ovaries were removed. The ovaries thus obtained were immersed in 4% paraformaldehyde (PFA) in 0.1 M PBS (pH 7.4), kept on ice for 30 min, washed in 0.1 M PBS, quickly frozen in ornithine carbamyl transferase (OCT) compound (Tissue-Tek; Miles Labs., Elkart, IN), and stored at −80°C until use.

To obtain fetal ovaries, female mice were primed at 4-wk of age with 5 IU eCG, followed 48 h later with an i.p. injection of 5 IU hCG, and mated with CD-1 males. The presence of a vaginal plug on the following morning was taken to indicate a successful mating and defined as Day 1 of pregnancy. At Day 15.5 of pregnancy, mice were killed

FIG. 1. Expression of the c-*kit* protein in the ovaries of (A, B) a murine fetus at Day 15.5 of pregnancy; (C, D) a mouse at 2 or 3 days of age; and (E, F) a mouse at 1-wk of age. A, C, E: control sections. B, D, F: sections stained by the ABC method using anti-mouse c-*kit* monoclonal antibody. The c-*kit* protein was not detected in primordial germ cells (A, B). Strong surface and weak cytoplasmic staining was detected in oocytes of primordial, primary, and secondary follicles (C–F). ×360. *Insets*: higher magnification of (B) primordial germ cells and (C, D) primordial follicles. ×480. *Arrows*: oocytes.

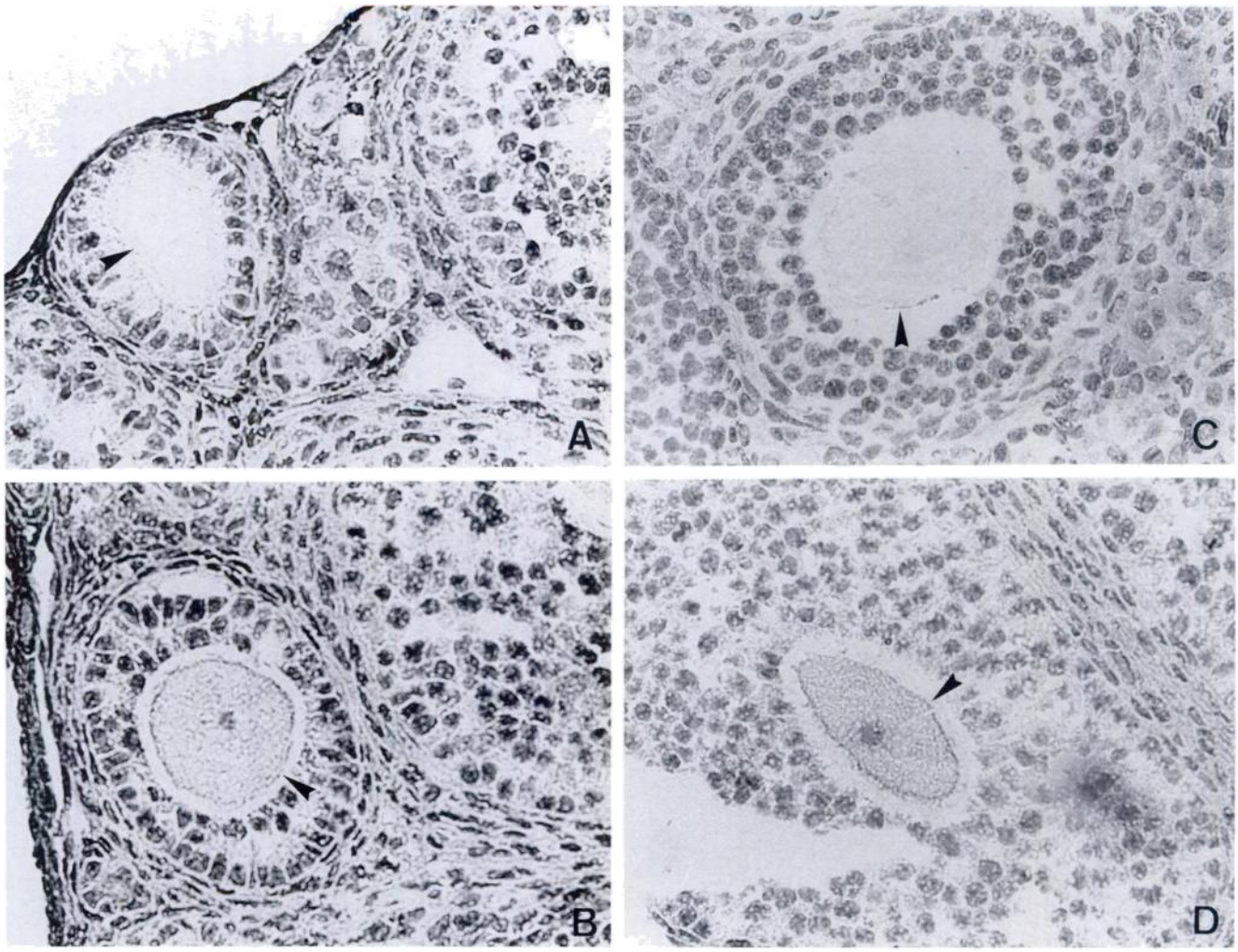

FIG. 2. Expression of the c-*kit* protein in the ovary of a mouse at 3-wk of age. A, C: control sections. B, D: sections stained by the ABC method using anti-mouse c-*kit* monoclonal antibody. Strong surface and weak cytoplasmic staining was detected in oocytes of secondary follicles (A–D). ×360. *Arrows*: oocytes.

by cervical dislocation to obtain the fetuses. Fetal ovaries with metanephroi were removed and immersed in 4% PFA in PBS, kept on ice for 30 min, washed in PBS, quickly frozen in OCT compound, and stored at −80°C until use.

Oocytes and Embryos

Female mice at 4-wk of age were primed with 5 IU eCG and killed 48 h later by cervical dislocation. The ovaries were removed and placed in PBS, and oocytes enclosed by cumulus cells were obtained by puncturing large antral follicles with fine needles. To obtain unfertilized ovulated oocytes and embryos, female mice at 4-wk of age were primed with 5 IU eCG, then 48 h later received injections of 5 IU hCG, with or without mating. They were killed by cervical dislocation at various developmental stages as shown below. Unfertilized ovulated oocytes and 1-cell embryos were collected 18 h after hCG injection by puncturing the swollen ampullae. Two-cell, 4-cell, 8-cell, and morula embryos were collected 40, 48, 55, and 70 h after hCG injection by flushing them out the ampullary ends. These oocytes and embryos were immersed in 4% PFA in PBS on ice and washed in PBS and were incubated without quenching of endogenous peroxidase activities and counterstaining.

Immunostaining of c-kit *Protein*

The immunostaining procedure was performed in cryostat sections by the avidin-biotin-peroxidase (ABC) method (Vectastain ABC kit, Vector Laboratories, Burlingame, CA). Cryostat sections 5 μm in thickness were mounted on slides (coated with 0.1% gelatin and 0.1% chromium (III) potassium sulfate), air-dried, and rinsed in 0.01 M PBS. Mounted sections were incubated with rat nonimmunized serum to minimize nonspecific staining. After excess serum was blotted from sections, they were incubated with anti-c-***kit*** monoclonal antibody for 30 min and then incubated with biotinylated anti-rat IgG antibody for 30 min at room tem-

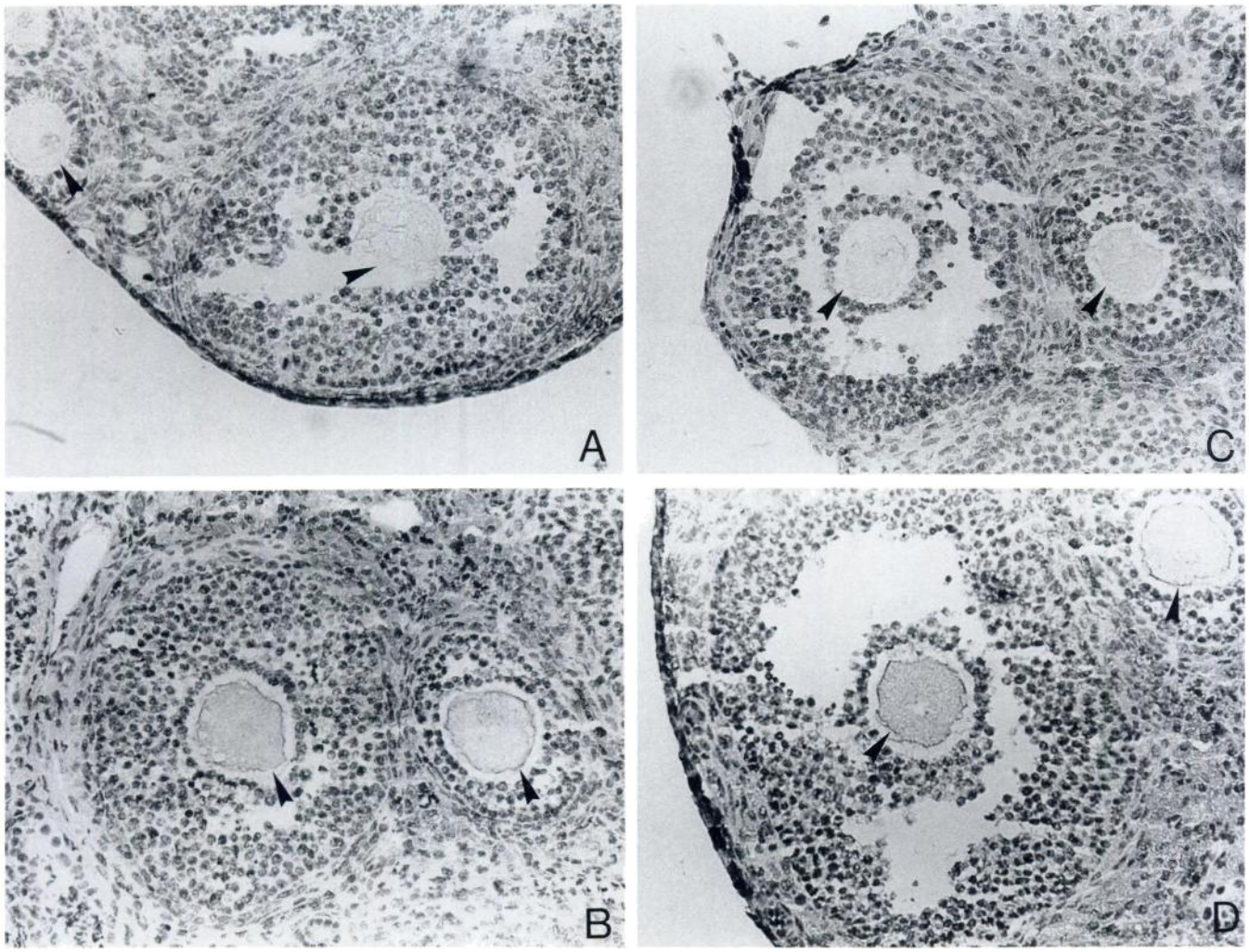

FIG. 3. Expression of c-*kit* protein in the ovary of a mouse at 3-wk of age primed with eCG. A, C: control sections. B, D: sections stained by ABC method using anti-mouse c-*kit* monoclonal antibody. Strong surface and weak cytoplasmic staining was detected in oocytes of secondary and tertiary follicles (A–D). ×180. *Arrows*: oocytes.

perature. After incubation another 30 min in methanol containing 0.3% H_2O_2 for quenching of endogenous peroxidase activities, histological sections were incubated with ABC reagent for 30 min at room temperature. After every incubation, sections were washed twice in PBS. Finally, immunoreaction products were visualized by incubating sections in a solution consisting of 0.05% diaminobenzidine tetrahydrochloride in Tris buffer solution containing 0.01% H_2O_2 for 4 min. Staining appeared as brown grains in the cell. Counterstaining was carried out with hematoxylin. Immunostaining without the primary or biotinylated secondary antibody served as control. Intestinal tissue sections were also stained as negative controls.

RESULTS

c-kit *Protein in Fetal and Postnatal Ovaries*

In fetal ovaries of mice at Day 15.5 of pregnancy, there were many primordial germ cells undergoing mitosis. As shown in Figure 1, A and B, no expression of c-*kit* protein was detected.

In the ovaries of mice 2 or 3 days of age, there were many primordial and primary follicles and a few secondary follicles. Oocytes showed strong surface staining and weak cytoplasmic staining. There was no specific staining in granulosa cells or interstitial regions (Fig. 1C,D).

In the ovaries of mice 1-wk of age, an increased number of secondary follicles and primary follicles were observed. Oocytes showed strong surface staining and weak cytoplasmic staining. There was no specific staining in granulosa cells or interstitial regions (Fig. 1E,F).

c-kit *Protein and Effects of eCG Treatment*

In the ovaries of mice 3-wk of age, there were many secondary follicles, a few primary follicles, and a few tertiary follicles. Similar to the ovaries of mice 1-wk of age, oocytes showed strong surface staining and weak cytoplas-

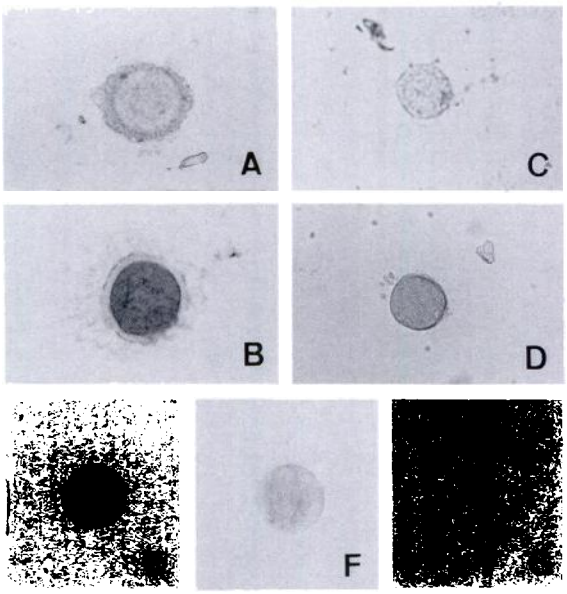

FIG. 4. Expression of c-*kit* protein in oocytes and embryos. A, C: control staining. B, C: oocytes. E, F, G: embryos stained by the ABC method using anti-mouse c-*kit* monoclonal antibody. A, B: oocytes from an ovary primed only with eCG. C, D: ovulated unfertilized oocytes. E: 1-cell embryo. F: 2-cell embryo. G: 4-cell embryo. An ovulated unfertilized oocyte exhibited weaker staining than an oocyte in the ovary primed only with eCG before ovulation (A–D). After fertilization, in 1-cell embryos, the c-*kit* protein was still observed (E), but with cell division, it further decreased (F), and it was not detected in 4-cell embryos (G). ×90.

mic staining. There was no specific staining in granulosa cells or interstitial regions (Fig. 2).

When 3-wk old mice were primed with 5 IU eCG, which induces follicular growth, many secondary and tertiary follicles and a few primary follicles were observed in the ovaries. Similar to oocytes of mice without eCG treatment, oocytes showed strong surface staining and weak cytoplasmic staining. There was no specific staining in granulosa cells or interstitial regions (Fig. 3).

c-kit *Protein in Oocytes and Embryos*

When ovulation was induced by hCG, ovulated unfertilized oocytes were stained more weakly than oocytes in ovaries primed only with eCG before ovulation (Fig. 4, A–D). After fertilization, the c-*kit* protein was still observed in 1-cell embryos (Fig. 4E), but with cell division it decreased (Fig. 4F) and was not detected in 4-cell (Fig. 4G) and 8-cell embryos.

In summary, the expression of the c-*kit* protein was clearly detected in oocytes after birth when oocytes were arrested at the diplotene stage of prophase of the first meiosis. It was maintained on the surface of oocytes of primordial to fully grown Graafian follicles, irrespective of eCG treatment. But after ovulation and resumption of meiotic maturation, the c-*kit* protein decreased and became undetectable in 4-cell embryos.

DISCUSSION

Oocytes enter meiosis during the late fetal stage and become arrested at the diplotene stage of meiotic prophase at the time of birth. Oocytes of nonatretic follicles are maintained at this stage and resume meiotic maturation only after a gonadotropin surge [23]. The first change associated with the resumption of meiotic maturation is the breakdown of the germinal vesicle (GVBD) [24]. However, when oocytes are isolated from antral follicles and cultured ex vivo, they resume meiotic maturation spontaneously [25]. This observation has led to the hypothesis that intrafollicular substances inhibit the resumption of meiosis until the preovulatory gonadotropin surge. Various molecules have been proposed to play an important role in the regulation of meiotic maturation. It is inhibited by oocyte maturation inhibitor [26, 27], cAMP [28, 29], and purines [30, 31], and promoted by epidermal growth factor [32] and lectins [33]. In addition, the cumulus cells that are in close contact with the oocytes via gap junctions may play a critical role in inhibiting or promoting meiotic maturation [34, 35]. In the present study, the expression of c-*kit* protein, a receptor for SLF, was detected by immunohistochemical methods in oocytes that entered meiosis and became arrested at the diplotene stage of meiotic prophase. During follicular growth, it was detected on the surface of oocytes from primordial to fully grown Graafian follicles. These results suggest that the c-*kit* protein may be involved in meiotic arrest and follicular growth.

Since in vitro and in vivo studies have demonstrated the importance of close cell-to-cell interaction for the receptor, c-*kit* protein, to be activated [36, 37], a pressing need of future research is to determine whether SLF is present in the cumulus or granulosa cells.

Primordial germ cells of mice can first be seen in the 7.5-day-old embryonic allantois [38, 39]. They move caudally from the yolk sac to the genital ridge. Most of them reach the developing gonad by Day 12 of pregnancy, during which they proliferate from the initial population of 10–100 cells to 2 500–5 000 cells [38]. After migrating to the genital ridge, they proliferate for only 2–3 days and enter meiosis. At Day 18 of pregnancy, some oocytes are seen at the diplotene stage of meiotic prophase, and most of them have reached this stage at the time of birth [39]. The defect of gametogenesis in *W* and *Sl* mutant mice is thought to result from a failure in the proliferation and migration of primordial germ cells [9]. By in situ hybridization, the expression of c-*kit* mRNA has been detected in cells at the genital ridge of 12.5-day-old embryos [20]. However, the c-*kit* protein was not detected in primordial germ cells of 15.5-day-old embryos in the present study (Fig. 1A,B). It remains to be elucidated whether the discrepancy between the expression of c-*kit* mRNA and protein may result from a down-regulation of c-*kit* protein, a difference in assay sensitivity, or some other cause.

In response to the gonadotropin surge, the oocytes of fully grown follicles resume meiotic maturation, initially manifested by GVBD, and are ovulated. During preparation of this manuscript, Manova et al. [21] reported that by indirect immunofluorescence using a rabbit immune serum, the c-*kit* protein was detected in primordial and growing oocytes collected from pronase-digested ovaries and persisted during growth and ovulation and through the 2-cell stage of embryogenesis, disappearing thereafter. However, in the present study using a monoclonal antibody, the expression of the c-*kit* protein in the ovulated unfertilized oocytes was weaker than that in the oocytes of fully grown follicles primed only with eCG before ovulation (Fig. 4, A–D). After fertilization, in 1-cell embryos, the c-*kit* protein was still observed (Fig. 4, E), but with cell division it further decreased (Fig. 4F) and was not detected in 4-cell embryos (Fig. 4G). These observations suggest that the c-*kit* protein may play an essential role in oocyte maturation and early embryonic development. Further investigation is required to elucidate the exact function of the c-*kit* protein and its ligand, SLF, during oogenesis and early embryogenesis.

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