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Kit-Ligand/Stem Cell Factor Induces Primordial Follicle Development and Initiates Folliculogenesis*

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ABSTRACT

Initiation of folliculogenesis through the induction of primordial follicle development in the ovary has an important role in determining the fertility and reproductive fitness of most mammalian species. The factors that control this critical process are largely unknown. The hypothesis tested in the current study was that kit-ligand/stem cell factor (KL) promotes the initiation and progression of primordial follicle development in the ovary. Ovaries from 4-day-old rats were maintained in organ culture for 5 and 14 days and treated with no factor (control), recombinant kit-ligand (KL), or gonadotropins (FSH and hCG). Follicles in ovarian sections were counted and histologically classified as primordial (stage 0), early primary (stage 1), primary (stage 2), transitional (stage 3), or preantral (stage 4). Fresh ovaries from 4-day-old rats contained 68% primordial follicles (stage 0) and 32% developing follicles (stages 1–4) per section. After 5 and 14 days in culture, section from control ovaries contained approximately 41% and 55%, respectively, developing follicles (stage 1–4) per section due to spontaneous development of primordial follicles. Spontaneous primordial follicle development was completely blocked by ACK-2, a c-kit antibody that blocks KL actions. This observation suggests that endogenous KL is necessary for primordial follicle development *in vitro*. After 14 days of KL treatment, sections from ovaries contained 17% primordial follicles (stage 0) and 83% developing follicles (stage 1–4) per section demonstrating a dramatic induction of primordial follicle development by KL. Gonadotropins (FSH and hCG) did not induce primordial follicle development but did increase the percentage of preantral follicles (stage 4) per section. This small increase in preantral follicles in response to gonadotropins was blocked by ACK-2 suggesting that KL may in part mediate gonadotropin actions after the initiation of primordial follicle development. Ovaries contained an average of 309 $\pm$ 10 follicles per section. The total number of follicles per section did not significantly vary between treatments suggesting that the effects of KL were not due to an alteration in follicle number (*i.e.* survival). KL appears to be one of the first factors identified to be involved in the promotion of primordial follicle development. Results suggest that KL is necessary and sufficient to induce primordial follicle development and initiate folliculogenesis. (*Endocrinology* **140:** 4262–4271, 1999)

THE FACTORS THAT control the onset of primordial follicle development in the ovary are not known. The pool of ovarian primordial follicles is established during embryonic development (*e.g.* cows, sheep, humans) or at birth (*e.g.* rodents). This pool of primordial follicles constitutes the complete supply of oocytes available to a female. Primordial follicles that initiate development are destined to ovulate or degenerate through atresia. Induction of primordial follicles to develop and grow (*i.e.* initiate folliculogenesis) is a fundamental process in ovarian biology and is essential for female reproduction. The current study investigates a factor potentially involved in this process.

Kit ligand (KL) and its receptor c-kit are essential for oocyte migration during embryonic development (1–3) and follicular development in the adult ovary (4–6). Although previous analysis of the actions of KL on follicle development (6) are likely in part to be due to actions on primordial follicle development, no direct analysis of primordial follicles has been reported. To our knowledge, previous studies have not rigorously examined the role of KL in primordial follicle development and the initial stages of folliculogenesis. KL and c-kit are the products of the Steel (Sl) and White Spotting (W) loci in mice, respectively (7–13). A number of mutations at Sl or W have been described that cause sterility due to defects in oocyte migration or follicular development. Ovaries in mice carrying steel panda (Sl^{pan}), steel t (Sl^{t}), and steel contrast (Sl^{con}) mutations contain follicles that arrest at early stages of follicular development (14–16). The pool of primordial follicles is established in these mutant mice, but initiation and progression of primordial follicle development is inhibited. These observations suggest that KL may be essential for initiation and/or progression of primordial follicle development in the ovary.

When a follicle starts to develop, its oocyte begins to synthesize RNA (17, 18), and squamous pregranulosa cells enlarge to become a single layer of cuboidal granulosa cells. Theca cells are recruited from surrounding stromal stem cells and organize into distinct theca cell layers around the follicle. Organization of theca cells around the follicle provides structural integrity and helps to establish mesenchymal-epithelial cell interactions between theca cells and granulosa cells (19). Follicular development continues as granulosa cells and theca cells proliferate and differentiate. It has been suggested that theca cells differentiate from stromal stem cells in response to a putative "theca cell organizer" produced by granulosa cells (20). Such a factor may also be important for granulosa cell-oocyte interactions involved in the initiation and progression of primordial follicle development.

Granulosa cells in developing follicles produce KL (4, 21), which can act on theca cells, stromal cells, and oocytes. Differentiated theca cells, undifferentiated stromal cells, and

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developing oocytes express the receptor c-kit (4, 21–23). KL has a variety of effects on isolated oocytes including the promotion of growth and maintenance of meiotic arrest (5, 24–27). In addition KL can directly stimulate proliferation and differentiated functions (*i.e.* androstenedione production) of theca cells (28). KL also stimulates the growth of ovarian stromal-interstitial cells (29). The expression patterns of KL and c-kit in the ovary, as well as the actions of KL on oocytes and theca cells, suggest that KL may be important for many stages of follicular development in the ovary.

The present study examines the ability of KL to induce primordial follicle development and initiate the early events of folliculogenesis. The current study does not address the embryonic or postnatal formation of the primordial follicles, but instead the initiation of follicle development (*i.e.* folliculogenesis). Experiments are designed to examine the actions of KL in whole-ovary organ cultures. KL is proposed to mediate epithelial-mesenchymal cell interactions between granulosa and theca cells, as well as directly stimulate oocyte functions. The hypothesis that KL may recruit theca cells from undifferentiated stromal stem cells is also discussed.

Materials and Methods

Ovary organ culture

Sprague Dawley rats were obtained from Bantin & Kingman Universal (Fremont, CA). Ovaries from 4-day-old rats were immediately placed in histochoice tissue fixative (Amresco, Solon, OH) or placed in culture. A method similar to that developed in Dr. Gerald Cunha's laboratory for organ culture of seminal vesicle and bulbourethral gland was used for ovary organ culture (30, 31). Matched pairs of ovaries were separated and one used for control cultures and the other for treated cultures. Ovaries were cultured on floating filters (0.4 μm Millicell-CM, Millipore Corp., Bedford, MA) in 0.5 ml DMEM-Ham's F-12 medium (1:1, vol/vol) containing 0.1% BSA (Sigma, St. Louis, MO), 0.1% albumax (Life Technologies, Inc., Gaithersburg, MD), 5× ITS-X (supplement containing insulin, sodium transferrin, sodium selenite, ethanolamine; Life Technologies, Inc.), and 0.05 mg/ml L-ascorbic acid (Sigma) in a four-well culture plate (Nunc plate, Applied Scientific, South San Francisco, CA). Medium was supplemented with penicillin and streptomycin to prevent bacterial contamination. Each ovary was placed in a small drop of medium (approximately 30 μl) on the floating filter. Two ovaries per floating filter were cultured at 37 C in a humidified atmosphere containing 5% CO_2 for 5 days or 14 days. Ovaries were treated with no factor (control), recombinant human kit-ligand (KL, 100 ng/ml; R&D Systems, Minneapolis, MN) or FSH (200 ng/ml; National Pituitary Agency, Baltimore, MD) and human CG (hCG, 200 ng/ml; 4010 IU/mg, Calbiochem, La Jolla, CA). Recombinant mouse kit-ligand (Sigma) was also used in some initial comparison studies. To further examine the role of KL in primordial follicle development ovaries were treated with nonimmune rat IgG (Sigma) or function-blocking antimurine c-kit (ACK-2, rat monoclonal antibody, Life Technologies, Inc.). The titer of the antibody used was approximately 1/100. Fresh media was added to each well and the ovaries were retreated on day 2 of culture. Ovaries cultured under these conditions appear healthy and do not show any signs of necrosis.

Histology

To investigate development of ovarian follicles fresh or cultured ovaries were embedded in paraffin, sectioned (7-μm sections), and stained with hematoxylin and eosin. The number of follicles at each developmental stage were counted in two serial sections from the largest cross-section through the center of the ovary. Follicles were classified (Fig. 1) as primordial (stage 0), early primary (stage 1), primary (stage 2), transitional (stage 3), or preantral (stage 4) as previously described (32). This classification is weighted toward earlier stages of follicular development. Primordial follicles are not developing and consist of an oocyte partially or completely encapsulated by flattened squamous pregranulosa cells. Early primary follicles have initiated development and contain some cuboidal (enlarged) granulosa cells. Primary follicles are identified by a single layer of cubiodal granulosa cells around the oocyte. Transitional follicles have 1–2 layers and preantral follicles have more than 2 layers of cuboidal granulosa cells. No antral follicles were observed in fresh or cultured 4-day-old rat ovaries. Multiple pole to pole sections of the ovaries were obtained for analysis. Sections were analyzed at 400× magnification under light microscopy and photographed with a Kodak DCS 420 digital camera (Eastman Kodak Co., Rochester, NY).

Androstenedione production

Ovaries were placed in culture on floating filters and immediately treated with no factor (control), 100 ng/ml KL, or gonadotropins (200 ng/ml FSH + 200 ng/ml hCG) in the presence of nonimmune IgG or ACK-2 antibody. After 2 days in culture, fresh medium was added and the cells were retreated. Cultures were terminated on day 5. Media from days 2–5 were collected and assayed for androstenedione using the RSL ^{125}I androstenedionne kit (ICN , Costa Mesa, CA). The sensitivity of the assay is 0.01 ng/ml androstenedione. Data were expressed as ng androstenedione/ovary.

Statistical analysis

All data were analyzed by a JMP 3.1 statistical analysis program (SAS Institute, Inc., Cary, NC). Effects of control culture conditions, KL, gonadotropins (FSH + hCG) and the ACK-2 antibody on follicle development were analyzed by a one-way ANOVA. Observed significance probabilities of 0.05 (Prob > F) or less were considered evidence that an ANOVA model fits the data. Follicles were counted and characterized (developmental stage 0–stage 4) on two serial sections from each treatment in a total of four different experiments (n = 8). In some cases, follicles were counted in half of a section and multiplied by a factor of 2. Follicles were assessed blindly in all experiments. In addition, results were confirmed independently by two individuals. At least 14,000 total follicles were counted and classified and data were expressed as % follicles per section ± SEM. Within each developmental stage, significant differences between each treatment were determined using the Tukey-Kramer HSD (honestly significant difference) test, which protects the significance tests of all combinations of pairs (33, 34). The Tukey-Kramer test is recommended for multiple comparisons of all pairs (35).

Results

Primordial follicle development in freshly collected 4-day-old rat ovaries was characterized. Follicles were counted and classified as undeveloped primordial follicles (stage 0), early primary follicles (stage 1), primary follicles (stage 2), transitional follicles (stage 3), and preantral follicles (stage 4) using a previously described procedure (32). This classification is weighted toward early developing follicles and is shown in Fig. 1. Sections of fresh 4-day-old rat ovaries contained 68% primordial follicles (stage 0), 12% early primary follicles (stage 1), 11% primary follicles (stages 2) and 9% transitional and preantral follicles (stages 3–4). No antral follicles were observed in freshly collected 4-day-old rat ovaries. Primordial follicles were generally observed in the cortex (outer region) of the ovaries and larger preantral follicles were observed in the medulla (central region) of the ovaries (Fig. 2). The pattern of undeveloped primordial follicles observed in the 4-day-old rat ovarian cortex is similar to prepubertal, mouse, bovine, and human ovaries (unpublished observation). The characterization of follicle development in 4-day-old rat ovaries demonstrated that at this developmental period a large number of undeveloped primordial follicles (*i.e.* 68% per section) are available for analysis. These 4-day-old rat ovaries were used in the *in vitro* system to study the

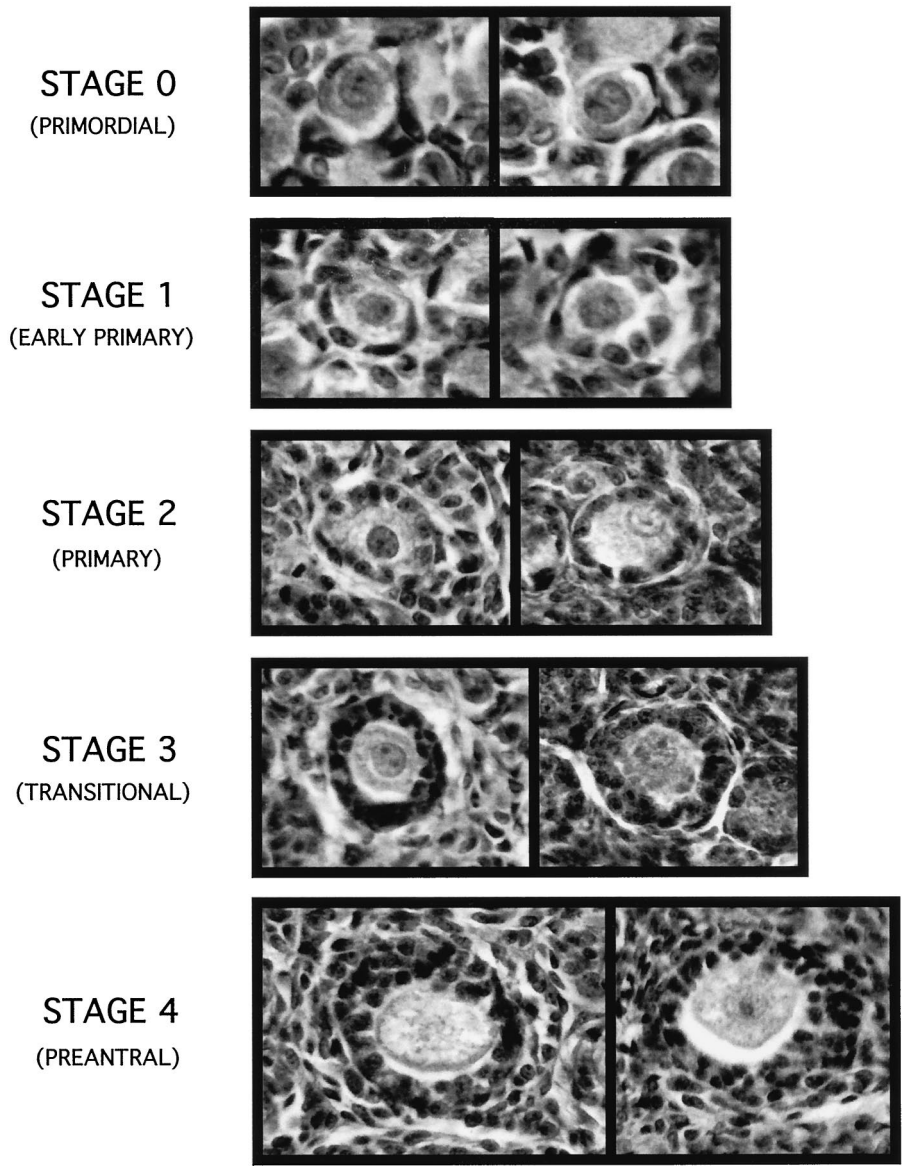

FIG. 1. Freshly collected postnatal 4-day-old ovaries were sectioned (7 μM), stained with hematoxylin and eosin, and photographed using a Kodak DCS420 digital camera. Two representative follicles are shown at each stage (stages 0 to 4). Classification of follicles was determined by morphology as described in *Materials and Methods*. Primordial follicles (stage 0) are not developing and consist of an oocyte partially or completely encapsulated by flattened squamous pregranulosa cells. Early primary follicles (stage 1) have initiated development and contain some cubiodal (enlarged) granulosa cells. Primary follicles (stage 2) are identified by a single layer of granulosa cells around the oocyte. Transitional follicles (stage 3) have 1–2 layers and preantral follicles (staage 4) have more than two layers of cubiodal granulosa cells. Magnification is approximately 400×.

factors involved in the initiation of primordial follicle development.

Whole ovaries were dissected from 4-day-old rats and cultured on floating filters as described in *Materials and Methods*. After 5 days in culture, ovaries appeared healthy and contained many healthy follicles (Fig. 2). The total number of follicles per section did not vary significantly under any culture condition examined (Fig. 3). Under control conditions, a small but significant reduction in the percentage of primordial follicles (stage 0) per section was coupled to an increase in the percentage of early primary follicles (stage 1) (Fig. 4). The morphology of these follicles are shown in Fig. 4. No change was observed in the percentage of primary (stage 2), transitional (stage 3), or preantral (stage 4) follicles in control cultures compared with fresh ovaries (Fig. 5). These results demonstrate that primordial follicles in rat ovaries can spontaneously initiate development to early primary follicles (stage 1) *in vitro*. This whole ovary organ culture system was used to examine the ability of KL to induce primordial follicle development.

Ovarian organ cultures were treated with no factor (control), recombinant human KL (100 ng/ml), or a combination of FSH and hCG (200 ng/ml each). Human recombinant KL and mouse recombinant KL in excess of 50 ng/ml were found to have similar actions on the 4-day-old rat ovaries in culture. Because most primordial follicles were present in the ovarian cortex (outer region), the effects of KL on primordial follicle development were primarily observed in this outer region. After 5 days of culture, KL dramatically decreased the percentage of undeveloped primordial (stage 0) follicles and increased the percentage of early primary (stage 1) and primary follicles (stage 2) per section (Figs. 4 and 5). Interestingly, over 50% of the follicles per section in KL-treated ovaries had initiated development (*i.e.* stage 1–stage 4 follicles) (Fig. 6a). Treatment with gonadotropins did not induce primordial follicle development but did inhibit the sponta-

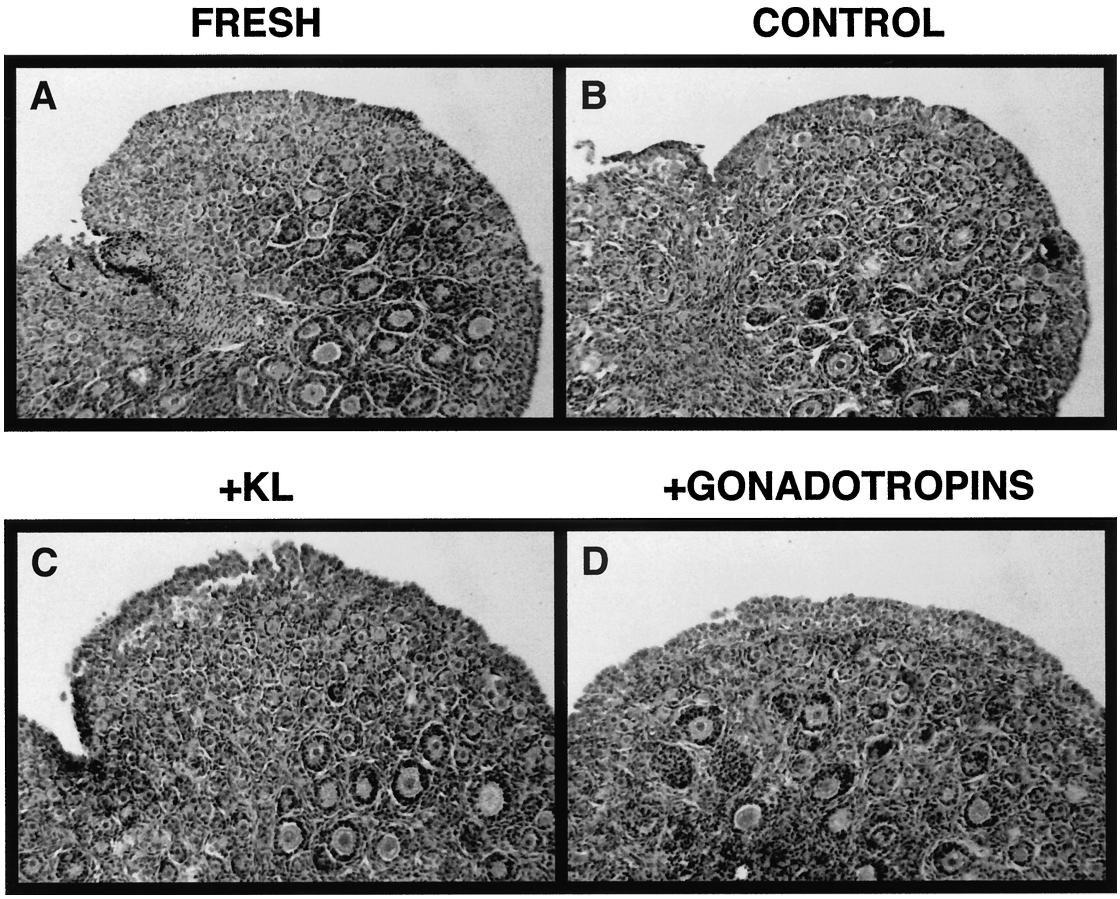

FIG. 2. Histology of 4-day-old rat ovaries at low magnification. Ovaries were prepared fresh (A) or cultured for 5 days (B–D). Ovaries were sectioned (7 μM), stained with hematoxylin and eosin, and photographed using a Kodak DCS420 digital camera. Ovaries contained a mixture of primordial (stage 0) to preantral follicles (stage 4) and appeared remarkably similar at low power magnification. A, Fresh 4-day-old ovaries; B, untreated (control) ovaries cultured for 5 days; C, cultured ovaries treated with kit-ligand (KL); D, cultured ovaries treated with gonadotropins (FSH + human CG). Magnification is approximately 40×. Data are representative of at least 8 different experiments performed in duplicate.

neous development that occurred in control cultures (Figs. 4 and 5). In addition, a slight increase in the percentage of preantral follicles (stage 4) was observed in gonadotropin-treated ovaries, suggesting that these later stage follicles are gonadotropin dependent. More dramatic effects of KL were observed after 14 days of culture (Fig. 6b). Over 83% of the follicles per section initiated development after 14 days of KL treatment. The ability of KL to promote development of early primary (stage 1) and primary follicles (stage 2) suggests that KL is sufficient to initiate primordial follicle development in the ovary.

ACK-2 is an antimurine c-kit antibody (rat monoclonal) that recognizes the extracellular domain of the c-kit receptor and strongly inhibits the actions of KL *in vivo* and *in vitro* (36–38). Ovary organ cultures were treated with normal rat IgG (*i.e.* control) or ACK-2 to examine the role of endogenous KL on primordial follicle development. ACK-2 completely blocked the spontaneous follicle development that occurred in control cultures (Fig. 7a). The percentage of primordial (stage 0) and early primary follicles (stage 1) were identical in freshly collected ovaries and ovaries cultured in the presence of ACK-2. The titer of ACK-2 antibody used was approximately 1/100. This inhibition of follicle development by ACK-2 suggests that endogenous KL is necessary for the spontaneous development of primordial follicles in these cultures.

ACK-2 partially blocked follicle development in KL-stimulated ovaries (Fig. 7b). In gonadotropin-treated ovaries, ACK-2 did not affect the percentage of primordial (stage 0), early primary (stage 1), primary (stage 2), or transitional follicles (stage 3) per section. However, the ACK-2 c-kit antibody did inhibit the small increase in preantral follicles (stage 4) that occurred in response to FSH and hCG (Fig. 7c). The ability of ACK-2 to inhibit preantral follicle development in response to gonadotropins suggests that KL is also necessary for preantral follicular development.

Development of early stage follicles (*i.e.* stage 1–4) involves differentiation and organization of theca cells around follicles and enlargement of squamous granulosa cells. During follicle development, theca cells differentiate and begin to produce increasing amounts of androstenedione. To study potential recruitment and differentiation of theca cells, androstenedione production was examined in the ovary organ cultures. Ovaries were cultured in the presence of normal rat IgG or ACK-2 c-kit antibody and treated with no factor (control), recombinant KL, or gonadotropins for 5 days. Me-

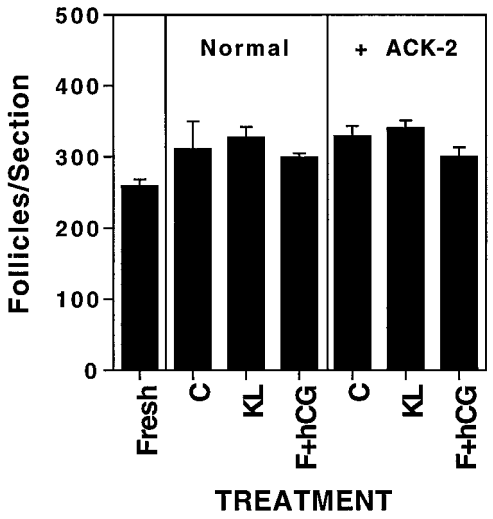

FIG. 3. Total follicle number per section in 4-day-old rat ovaries. Ovaries were prepared fresh or cultured in the presence of rat IgG (Normal) or function-blocking c-kit antibody, ACK-2 (+ ACK-2). Ovaries were sectioned (7 μM), stained with hematoxylin and eosin, and photographed using a Kodak DCS420 digital camera. Follicles were counted in the largest cross-section per ovary (*i.e.* pole to pole). No significant differences were observed between fresh, normal cultured, and +ACK-2 cultured ovaries as determined by the Tukey-Kramer HSD test. Follicles were counted in two serial sections from each ovary and data were expressed as the mean ± SE from four different experiments (n = 8). Fresh, Fresh 4-day-old ovaries; C, control; KL, kit ligand; and F + hCG, FSH + human CG.

dia from the days 2–5 collection was assayed for androstenedione accumulation by RIA. Under control conditions, approximately 1 ng adrostenedione/ovary was detected in the media (Fig. 8). ACK-2 did not alter basal levels of androstenedione production in control cultures. Interestingly, KL had no effect on the production of androstenedione in the presence or absence of ACK-2 (Fig. 8). Gonadotropins (*i.e.* FSH and hCG) dramatically stimulated production of androstenedione by ovary organ cultures (Fig. 8). This stimulation of androstenedione production by FSH and hCG was likely due to stimulatory actions on large preantral follicles (stage 4) because only large follicles (*i.e.* stage 4 or later) normally express gonadotropin receptors and produce steroids (39, 40). Interestingly, the inhibitory actions of ACK-2 increased androstenedione production by gonadotropin-stimulated ovaries (Fig. 8).

Discussion

Results demonstrate that KL can induce primordial follicle development in 4-day-old rat ovary organ cultures. Approximately 250 to 350 total follicles per ovarian cross-section (*i.e.* pole to pole) were observed in fresh and cultured ovaries. Neither the culture conditions or treatments altered total follicle number. Whole ovaries were covered with a small drop of medium and cultured on floating filters for 5–14 days. This simple organ culture method was developed to parallel ovarian follicle development *in vivo*. Ovaries maintained structural organization and appeared healthy *in vitro*. Freshly collected ovaries contained approximately 68% primordial follicles (stage 0), 23% early primary and primary follicles (stages 1–2), and 9% transitional and preantral follicles (stages 3–4) per cross-section. Ovaries cultured under control conditions had similar follicle development as the fresh ovaries with 60% primordial follicles (stage 0) per cross-section. Ovaries that were cultured for 5 days and treated with KL contained approximately 45% primordial (stage 0) follicles, 45% early primary, and primary follicles (stages 1–2) and 10% transitional and preantral follicles (stages 3–4) per cross-section. The total number of follicles did not change suggesting the effects of KL were not due to an increased survival of follicles during organ culture. Therefore, KL induced an increase in primordial follicle development.

When 4-day-old ovaries were placed in culture for 5 or 14 days, some primordial follicles proceeded to develop. This spontaneous initiation of primordial follicle development suggests that primordial follicles were actively developing *in vivo* and continued to develop *in vitro*. Alternatively, there may be inhibitory signals for primordial follicle development *in vivo* that were no longer present *in vitro*. Wandji *et al.* also reported spontaneous initiation of primordial follicle development in cultured ovarian cortex fragments from embryonic cows (41) and baboons (42). In the current study, spontaneous initiation of primordial follicle development was blocked by ACK-2, a c-kit monoclonal antibody that inhibits the actions of KL on the receptor c-kit. Although this is a rat monoclonal antibody to mouse c-kit, ACK-2 has been shown to cross-react in other species such as rat (27). Observations suggest that KL may be necessary for initiation of primordial follicle development.

The number of primordial follicles that initiated development was significantly increased by KL. This induction of primordial follicle development by KL treatment was partially inhibited by ACK-2. Total inhibition of primordial follicle development in response to KL may be limited in part by access of ACK-2 to the organ culture. Other factors that may contribute to the partial inhibition were that the optimal titer of antibody was not used to suppress the actions of exogenously added KL.

Initiation of primordial follicle development primarily occurs after the onset of puberty but can be observed prepubertally and during infancy when ovulation has not yet started (43, 44). Primordial follicles continue to initiate development until menopause (*i.e.* in the human) when the pool of primordial follicles is diminished. Interestingly, primordial follicle development continues in the adult even during pregnancy when follicles do not ovulate (45–48). These observations suggest that the factors that control initiation of primordial follicle development are important at various stages of reproductive development. This study is the first to suggest that KL may be sufficient to initiate primordial follicle development. The expression and actions of KL in the ovary during these various stages of reproductive development remain to be elucidated.

Gonadotropins (*i.e.* FSH and LH) have been shown not to influence the initiation of primordial follicle development (49–51). However, gonadotropins are critical for preantral follicles (stage 4) to continue development (40). Preantral follicles (stage 4) express gonadotropin receptors and are completely dependent on normal levels of FSH and LH to develop. In the current study, treatment with gonadotropins

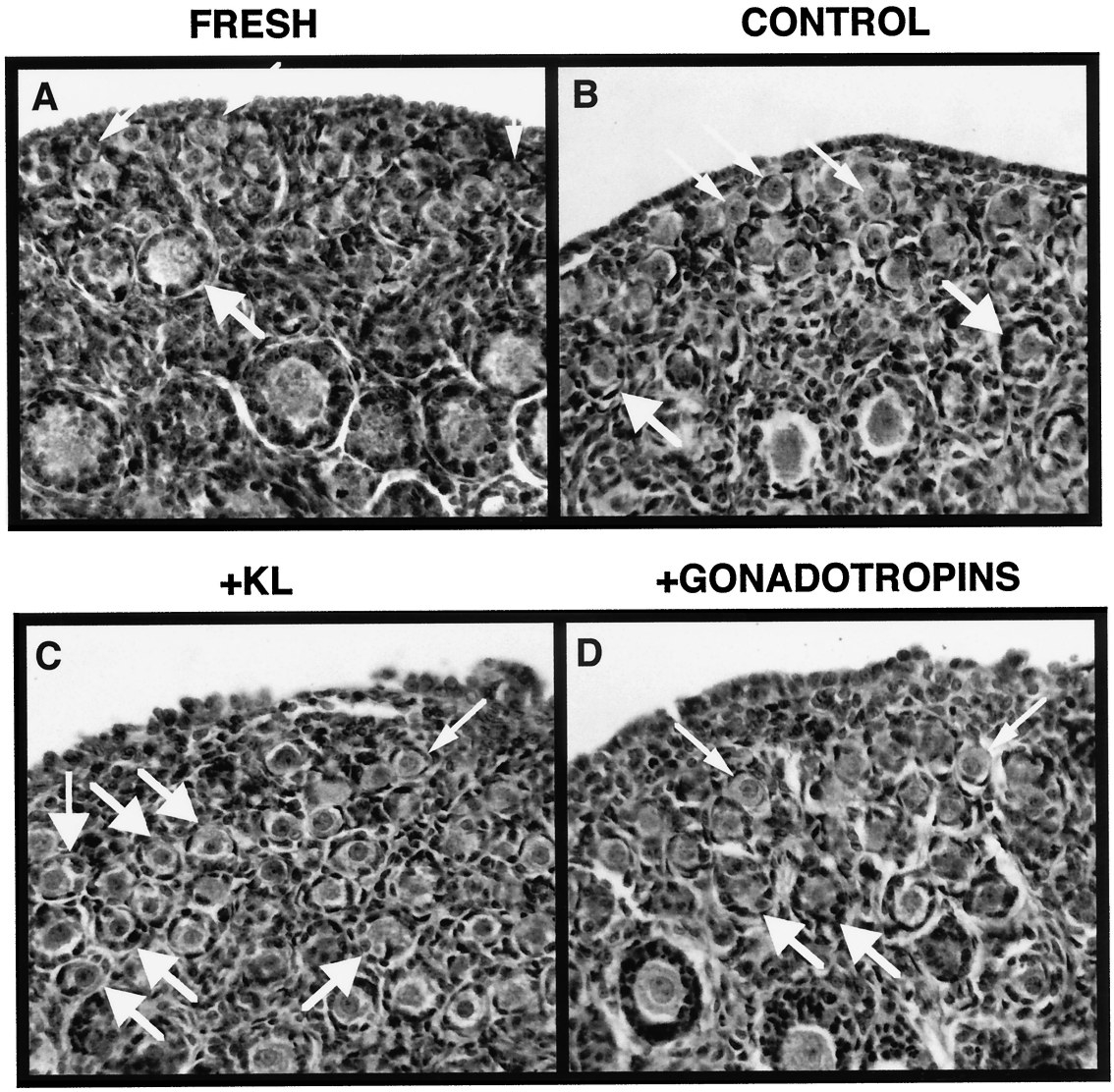

FIG. 4. Cortex of 4-day-old rat ovaries at high magnification. Ovaries were prepared fresh (A) or cultured for 5 days (B–D). Ovaries were sectioned (7 μM), stained with hematoxylin and eosin, and photographed using a Kodak DCS420 digital camera. Follicles were classified as described in *Materials and Methods*. *Small arrows* indicate undeveloped primordial follicles. *Large arrows* indicate developing follicles (from stages 1–4). A, Fresh 4-day-old ovaries; B, untreated (control) ovaries cultured for 5 days; C, cultured ovaries treated with kit-ligand (KL); D, cultured ovaries treated with gonadotropins (FSH + human CG). Magnification is approximately 200×. Data are representative of at least four different experiments performed in duplicate.

resulted in a small increase in the percentage of preantral (stage 4) follicles per section. This increase in preantral follicle development was blocked by the c-kit antibody ACK-2. Observations suggest that KL in part mediates the actions of gonadotropins in preantral follicles. Laitinen *et al.* showed that gonadotropins can regulate KL expresssion in human granulosa cells collected from large size follicles for *in vitro* fertilization (52). The current results suggest that KL may also mediate gonadotropin actions in preantral follicles.

KL (also termed stem cell factor, mast cell factor or steel factor) has a wide range of activities on germ cells, melanocytes, mast cells, and primitive hematopoietic cells of the myeloid, erythroid, and lymphoid cell lineages (53, 54). Many of these multipotent stem cells alter their developmental program and differentiate in response to KL. Theca cells are recruited to differentiate from ovarian stromal stem cells during initiation of primordial follicle development. The authors have developed the hypothesis that KL may have a role in the recruitment of stromal stem cells to differentiate into theca cells. In organ culture KL had no effect on ovarian androstenedione production that is a marker of follicular phase theca cell differentiation. Differentiation of theca cells in early primary (stage 1) and primary follicles (stage 2) may not involve production of androstenedione. Elucidation of the ability of KL to recruit stromal stem cells to differentiate into theca cells will require identification of early theca cell markers of differentiation.

Gonadotropins dramatically stimulated androstenedione production by ovary organ cultures. The increased androstenedione production was likely derived from differenti-

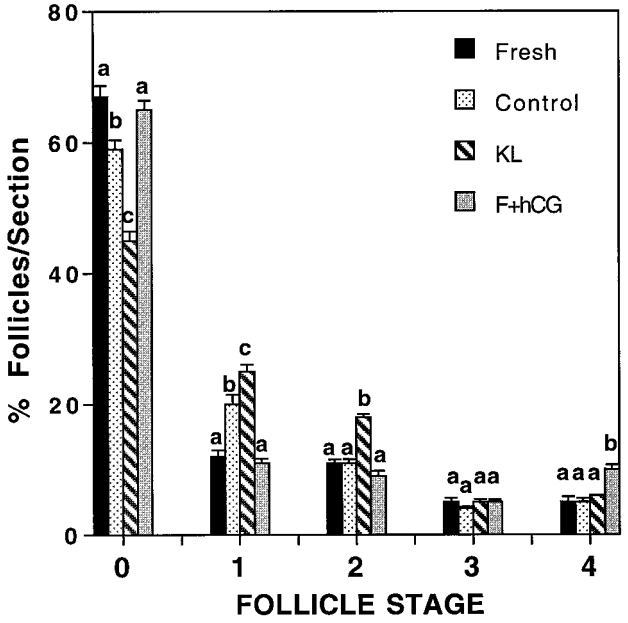

FIG. 5. Primordial follicle development in ovary organ cultures. Ovaries were prepared fresh or cultured for 5 days. Follicles were classified as described in *Materials and Methods*. Data were expressed as percent follicles per cross-section for each classification stage. Follicle classifications were counted and data presented as mean ± SE (percent follicles/section) from four different experiments performed in duplicate. *Bars with different superscript letters* are significantly different within each classification stage as determined by the Tukey-Kramer HSD test. Follicles are classified as primordial (stage 0), early primary (stage 1), primary (stage 2), transitional (stage 3), and preantral (stage 4). Primordial follicle development is represented by a decrease in stage 0 follicles and an increase in stage 1–4 follicles within each treatment. Fresh, Fresh 4-day-old ovaries; C, control; KL, kit ligand; F + hCG, FSH + human CG.

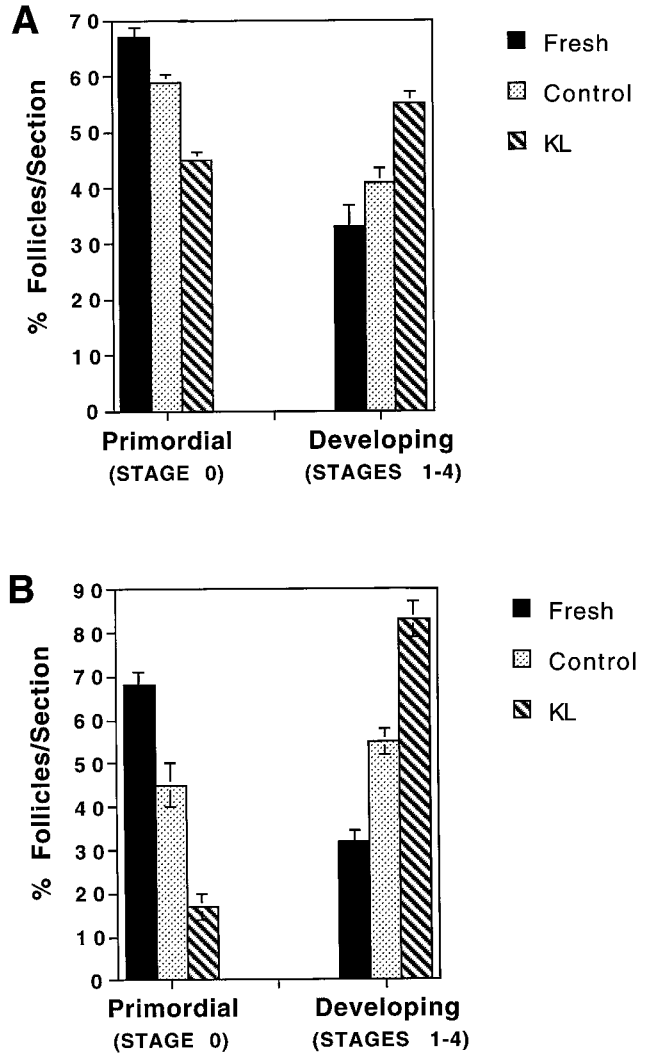

FIG. 6. Primordial follicle development in short-term and long-term ovary organ cultures. Ovaries were prepared fresh or cultured for 5 (A) or 14 (B) days. Follicles were classified as described in *Materials and Methods*. Data were expressed as percent follicles per section for each classification stage. Follicle classifications were counted and data presented as mean ± SE (percent follicles/section) from four different experiments performed in duplicate. *Bars with different superscript letters* are significantly different within each classification as determined by the Tukey Kramer HSD test. Follicles are classified as primordial (stage 0), early primary (stage1), primary (stage 2), transitional (stage 3), and preantral (stage 4). Primordial follicle development is represented by a decrease in stage 0 follicles and an increase in stage 1–4 follicles within each treatment. (Fresh) fresh 4-day-old ovaries; Control; (KL) kit ligand.

ated theca cells in preantral follicles (stage 4). Gonadotropins directly stimulate theca cell steroid production in developing follicles (55). Interestingly, the ACK-2 c-kit antibody enhanced the ability of gonadotropins to stimulate androstenedione production by cultured ovaries. Previous experiments have shown that theca cells from later stage follicles (*i.e.* preantral and antral stages) can proliferate in response to KL *in vitro* (28). Therefore, KL may indirectly decrease theca cell differentiated functions by promoting entry of the cells into the cell cycle. An important role of KL in preantral follicles (*i.e.* stage 4) may be to stimulate theca cell proliferation to establish several theca cell layers. Inhibition of KL actions with ACK-2 may indirectly increase theca cell androstenedione production in gonadotropin-stimulated follicles. As a result, observable structural development of preantral follicles may be distinct from actual androstenedione production. Stimulation of a androstenedione production by ACK-2 was likely due to effects on existing preantral follicles. A similar effect of ACK-2 has been reported on Leydig cell testosterone production (38). Serum testosterone levels were elevated 24 h after injection with ACK-2.

Recently, a role for c-kit has also been suggested for the development of preantral follicles from primary follicles (6). Some of the effects observed are likely through the primordial follicles, but this was not directly examined. Administration of ACK-2 to mice appeared to block development of follicles. It was not clear if these effects were direct on follicles or indirect due to the use of whole mice as a model system. The current study suggests a role for KL in inducing the primordial follicles to develop to the primary follicle stage. Therefore, this previous study (6) compliments the current observations and suggests KL has a role throughout follicle development.

The current study suggests that KL can initiate follicle growth *in vitro*. Mutations in Steel such as Steel Panda (SI^{pan}), Steel + (SI^{+}), and Steel Contrast (SI^{con}) cause ovarian follicles to arrest at early stages of development after initiation has

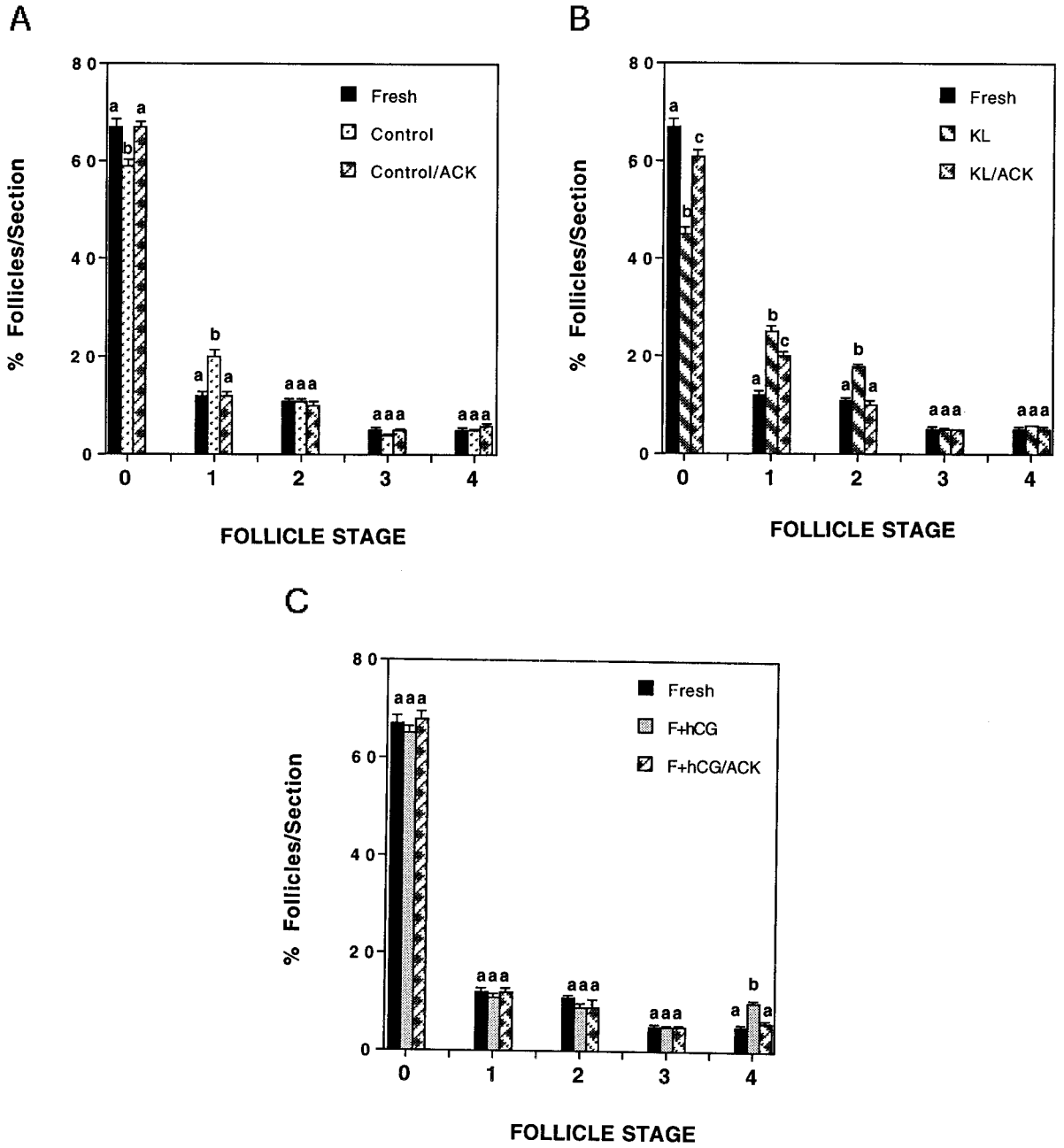

FIG. 7. ckit antibody ACK-2 actions on primordial follicle development. Ovaries were prepared fresh or cultured for 5 days. A, Ovaries were cultured in the presence of normal rat IgG or ACK-2 antibody. B, Ovaries were cultured in the presence of normal rat IgG or ACK-2 and treated with KL. C, Ovaries were cultured in the presence of normal rat IgG or ACK-2 and treated with F + hCG. Fresh, Fresh 4-day-old ovaries; control, untreated cultured ovaries; KL, kit ligand; F + hCG, FSH and human CG; ACK-2, function-blocking c-kit antibody. Follicles are classified as described in *Materials and Methods*. Follicles are classified and data presented as mean ± SE (percent follicles/section for each stage) from four different experiments performed in duplicate. Follicles were classified as primordial (stage 0), early primary (stage1), primary (stage 2), transitional (stage 3), and preantral (stage 4). Primordial follicle development is represented by a decrease in stage 0 follicles and an increase in stage 1–4 follicles within each treatment. *Bars with different superscript letters* are significantly different within each classification stage as determined by the Tukey-Kramer HSD test.

occurred. It is possible that there are additional factors *in vivo* that play a role in follicle development that are absent from the *in vitro* experiments. In addition, Steel mutant mice may have developed other unidentified compensatory mechanisms that affect follicle development. Experiments designed to examine the potential effects of KL on follicle development *in vivo* are planned.

For more than 70 yr, whole ovaries have been maintained in organ culture to study follicular development (56–60). Early studies also examined the development of primoridal follicles in freshly isolated ovarian tissue (61–63). Despite this extensive investigation, the factors that control initiation of primordial follicle development remain to the elucidated. In the current study, 4-day-old rat ovaries were maintained

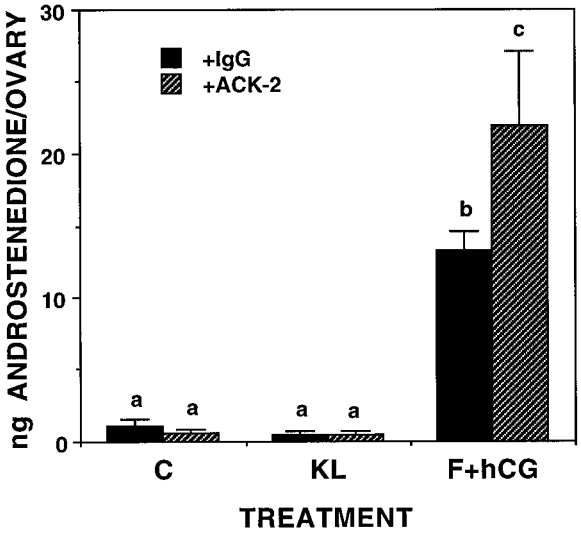

FIG. 8. Androstenedione production by cultured 4-day-old ovaries. Ovaries were cultured in the presence of normal rat IgG or ACK-2 c-kit antibody. Androstenedione accumulation in the conditioned media was determined. Data are normalized per ovary (ng androstenedione/ovary) and presented as mean ± SE from four different experiments performed in duplicate. *Bars with different superscript letters* are significantly different as determined by the Tukey-Kramer HSD test. C, Untreated cultures; KL, kit-ligand; F + hCG, FSH and human CG; IgG, normal rat IgG; ACK-2, function-blocking c-kit antibody.

in a simple floating-filter organ culture system to study the factors that control primordial follicle development. KL dramatically induced development of primordial follicles in these ovaries. The c-kit antibody ACK-2 blocked initiation of primordial follicle development. Because both oocytes and theca/stromal cells express the c-kit receptor, KL may promote early follicular development by acting on both germ cells (*i.e.* oocytes) and somatic cells (*i.e.* theca and stromal cells). These experiments suggest that KL may be both necessary and sufficient to induce primordial follicle development in ovary organ cultures. The speculation is made that KL may be a "theca cell organizer" (20) that is involved in initiation of primordial follicle development. An understanding of the factors that control primordial follicle development will be useful in understanding in part the mechanisms that underlie many phenomena such as female sterility, premature ovarian failure, polycystic ovary disease, precocious or delayed puberty, early or delayed onset of menopause, and ovarian cancer.

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