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Basic fibroblast growth factor induces primordial follicle development and initiates folliculogenesis

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Abstract

The recruitment of primordial follicles to initiate folliculogenesis determines the population of developing follicles available for ovulation and directly regulates female reproductive efficiency. In the current study, a floating organ culture system was used to examine the progression of primordial (stage 0) follicles to developing (stages 1–4) follicles in 4-day-old pre-pubertal rat ovaries. Basic fibroblast growth factor (bFGF) was found to induce primordial follicle development similar to what has been demonstrated for kit ligand/stem cell factor (KL). The bFGF-treated ovaries contained 85% developing follicles compared with 50% developing follicles for control untreated organ cultures. Correspondingly, the number of primordial follicles in bFGF-treated ovaries decreased to 15% of the total compared with 45% for controls. A bFGF neutralizing antibody was found to decrease the small amount of spontaneous follicle development that occurs during the organ culture. Basic FGF was localized to primordial and early developing follicles by immunocytochemistry and was primarily observed in the oocytes. Treatment of bovine ovarian theca cells and stroma cells with bFGF was found to promote cell growth. Basic FGF produced by the oocyte in early stage follicles appears to act on adjacent somatic cells to promote cell growth and development. Basic FGF, like KL, appears to be a primordial follicle-inducing factor. In summary, bFGF can regulate primordial follicle development that directly influences female reproductive efficiency.

Keywords: Basic fibroblast growth factor; Kit ligand; Primordial; Follicle; Ovary; Development; Mesenchymal–epithelial cell–cell interactions

1. Introduction

Two crucial aspects of ovarian biology about which little is known are the factors that control initiation of primordial follicle development and selection of the dominant follicle. Females are born with a pool of oocytes that organize into primordial follicles. This pool of primordial follicles represents the complete supply of oocytes that have the potential to ovulate. During each menstrual or estrous cycle, primordial follicles initiate follicular development. After primordial follicle development is initiated, the follicles are destined to ovulate or degenerate through atresia. In a mono-ovulator species, one follicle is selected as the dominant follicle and eventually ovulates. When the supply of oocytes (i.e. primordial follicles) is diminished, the menstrual cyclicity ends and humans enter menopause. Therefore, the factors that control initiation of primordial follicle development ultimately determine reproductive fitness and the age of menopausal transition in humans.

Basic fibroblast growth factor (bFGF) has been localized to the oocytes of primordial and primary follicles of many species, but not to putative granulosa cells of human primordial follicles (van Wezel et al., 1995; Yamamoto et al., 1997). The bFGF is also localized to granulosa cells of pre-antral and antral follicles. Theca cells of developing follicles also stain positive for bFGF (van Wezel et al., 1995; Yamamoto et al., 1997), although Wordinger et al. (1993) observed no theca staining in antral mouse follicles. Receptors for bFGF have been reported in rat (Shikone et al., 1992) and bovine (Wandji et al., 1992) granulosa cells. Basic FGF is important in regulating a wide range of ovarian

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functions including granulosa cell mitosis (Gospodarowicz et al., 1989; Lavranos et al., 1994; Rodgers et al., 1996; Roberts and Ellis, 1999), steroidogenesis (Vernon and Spicer, 1994), differentiation (Anderson and Lee, 1993) and apoptosis (Tilly et al., 1992). In addition, bovine granulosa cells have been shown to produce bFGF in the pre-antral and antral follicle stages of development (Neufeld et al., 1987). Whether bFGF is important in initiating follicular development is investigated in the current study.

Kit ligand (KL) was recently shown to have the capacity to initiate primordial follicle development in rat ovaries in culture (Parrott and Skinner, 1999b) and antibodies to KL were shown to inhibit this development (Parrott and Skinner, 1999b). Many germ line mutations of KL and c-kit exist in mice. Null mutations of KL (steel locus, Sl) and c-kit (white spotting locus, W) cause sterility due to defects in oocyte migration (Bennett, 1956; Mintz and Russell, 1957; McCoshen and McCallion, 1975). Other mutations such as steel panda (Sl^{pan}), steel contras, and steel t (Sl^{t}) (McCoshen and McCallion, 1975; Kuroda et al., 1988) do not inhibit oocyte migration, but cause follicular arrest at early stages of ovarian follicular development. The progression of early follicular development in mice deficient for the bFGF gene has not been closely examined. Mice null for bFGF have impaired migration and differentiation of neuronal cells in the cerebral cortex (Dono et al., 1998; Vaccarino et al., 1999), and impaired tissue remodeling associated with wound healing (Ortega et al., 1998). The ability of some of these mice to be fertile suggests that compensatory factors may exist for bFGF to allow this critical biological process to occur.

In the current study, the hypothesis is tested that bFGF promotes primordial follicle development and transition to primary follicles. Evidence is provided that bFGF, like KL, is sufficient to induce primordial follicles to initiate development. In addition, the localization of bFGF in the rat ovary and the actions of bFGF on ovarian stromal cells and theca cells are examined.

2. Methods

2.1. Histology and organ cultures

Postnatal 4-day-old rat ovaries were isolated and either immediately fixed or cultured for 14 days and then fixed. Animal use and procedures were approved by the University Animal Care Committee. Ovaries were fixed in histochoice tissue fixative (Amresco, Solon, OH), embedded in paraffin, sectioned (7 μM), stained with Hematoxylin and eosin, and photographed using a Kodak DCS420 digital camera. Whole ovaries were cultured on floating filters (0.4 μm Millicell-CM; Millipore, Bedford, MD) in 0.5 ml Dulbecco's Modified Eagle's Medium–Ham's F-12 medium (1:1, vol/vol) containing 0.1% bovine serum albumin (Sigma, St. Louis, MO), 0.1% albumax (Gibco BRL, Gaithersburg, MD), 5 × ITS-X (5 mg/ml insulin, 2.75 mg/ml transferrin, 3.35 sodium selenite, 1 mg/ml ethanolamine; Gibco BRL), and 0.05 mg/ml L-ascorbic acid (Sigma, St. Louis MO) in a four-well culture plate (Nunc plate; Applied Scientific, South San Francisco, CA) as previously described (Parrott and Skinner, 1999b). Treatments during organ culture included KL and bFGF obtained from R&D Systems (Minneapolis, MN). The dose of KL (25 ng/ml) and bFGF (40 ng/ml) used has been shown to be the optimal dose growth effects on monocultures of ovarian somatic cell cultures (Parrott and Skinner, 1999b). A half-optimal dose was shown to have similar effects, confirming the dose used. Neutralizing bFGF antibody (R&D systems, Minneapolis, MN) immunoglobulin (IgG) was used at a 20 ng/ml concentration and compared with non-immune IgG at the same concentration as a control. Medium was supplemented with penicillin, streptomycin and gentamycin to prevent bacterial contamination. After culture, ovaries were fixed and sectioned as already described.

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. Using 4-day-old rat ovary cultures, these largest cross-sections have been shown to provide representative follicle populations when compared with serial sections through the entire ovary (Parrott and Skinner, 1999b). A minimum of three different experiments each involving six ovaries (i.e. three rats) for each treatment were utilized. Follicles were classified as either primordial (stage 0), or one of the developing pre-antral stages (stages 1–4), as previously described (Parrott and Skinner, 1999b). Briefly, primordial follicles consist of an oocyte partially or completely encapsulated by flattened squamous pre-granulosa cells. Developing (stage 1–4) follicles contain successively more cuboidal granulosa cells in layers around the oocyte (Fig. 1).

2.2. Cell culture and growth assays

Theca cells and stromal–interstitial cells were isolated from freshly obtained bovine ovaries as previously described (Parrott and Skinner, 1998, 1999a). Cells were cultured at subconfluent densities (growth permissive) in the absence of growth factor for 24 h, then cells were treated as indicated for 20 h followed by a 4 h incubation with [^{3}H]thymidine. Counts per min (cpm) of [^{3}H]thymidine incorporated into DNA were determined and normalized to the total DNA per well (Parrott and Skinner, 1999b). Values were generally greater than 1×10^4 cpm/μg DNA. Epidermal growth

factor (EGF) (Sigma, St. Louis MO) was used as a positive control growth stimulator.

2.3. Immunocytochemistry

Freshly isolated ovary sections from 4-day-old rats were stained for the presence of bFGF with bFGF antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:200, using protocols previously described (Levine et al., 2000). Briefly, 7 μm sections were de-parafinized, rehydrated, and quenched in 20% MeOH/3% hydrogen peroxide, and blocked in 5% serum for several hours prior to incubation with primary antibody. Biotinylated secondary antibody was detected using the Vectastain kit (Vector) and diaminobenzadine. Negative control experiments were performed involving the use of no primary antibody, non-immune antibody and bFGF peptide pre-absorption/competition as previously described (Parrott and Skinner, 1999a; Levine et al., 2000).

2.4. Statistics

Treatment groups are compared with controls with an analysis of variance (ANOVA) using Dunnet's test (Hsu, 1996). Pair comparisons were performed using Tukey–Kramer's HSD. All statistics were calculated with the help of JMP version 3.1 software (SAS Institute, Inc., Carey, NC).

3. Results

Prepubertal 4-day-old rat ovaries contain a large number of primordial follicles that are capable of initiating development. These ovaries are small (i.e. 1–2 mm) and can be cultured as whole organs. The ovarian organ cultures were treated with (40 ng/ml) bFGF to examine effects on primordial follicle development. Some ovaries were treated with (25 ng/ml) KL as a positive control for initiating primordial follicle development. Ovaries cultured and treated for 14 days had varying degrees of follicle development, but the total number of follicles per section did not change and the size of the ovary did not change (data not shown) (Parrott and Skinner, 1999a). Therefore, the observations made were reflected in a change in follicle development and not due to alterations in follicle number or atresia. After culture, ovaries were sectioned and follicles were classified as primordial (stage 0) or developing

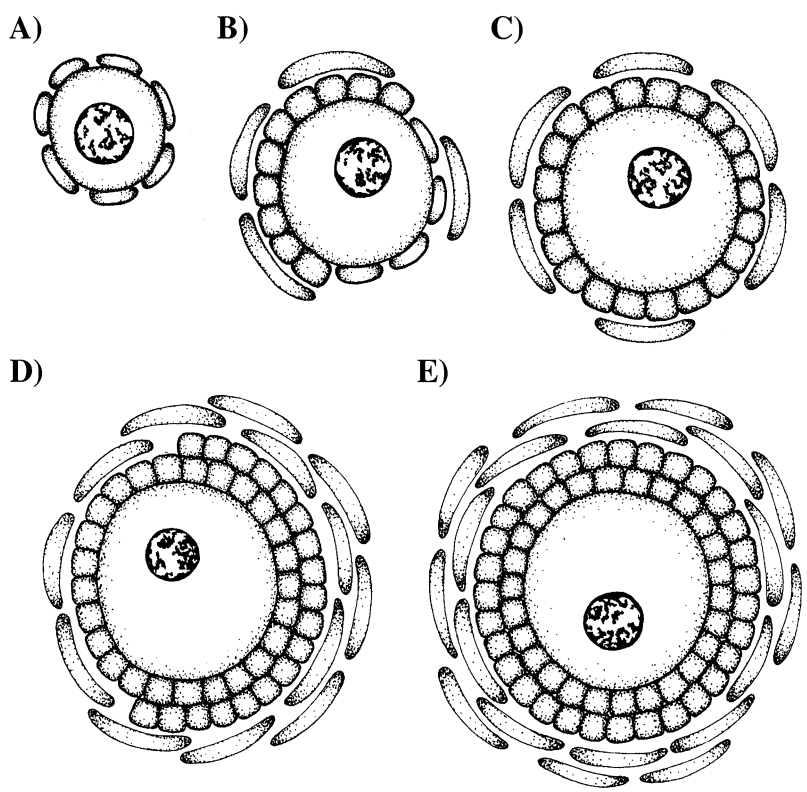

Fig. 1. Schematic representation of the morphology associated with stage 0–4 follicles. Stages 1–4 are combined and referred to as developing follicles, in contrast to the stage 0 primordial follicles. (A) Primordial follicles (stage 0) consist of an oocyte partially or completely encapuslated by squamous pregranulosa cells. (B) Stage 1 (early primary) follicles have some cuboidal granulosa cells. (C) Stage 2 (primary) follicles have a single layer of cuboidal granulosa cells surrounding the oocyte. (D) Stage 3 (transitional) follicles have one to two layers of cuboidal granulosa. (E) Stage 4 (pre-antral) follicles have two or more layers of cuboidal granulosa cells.

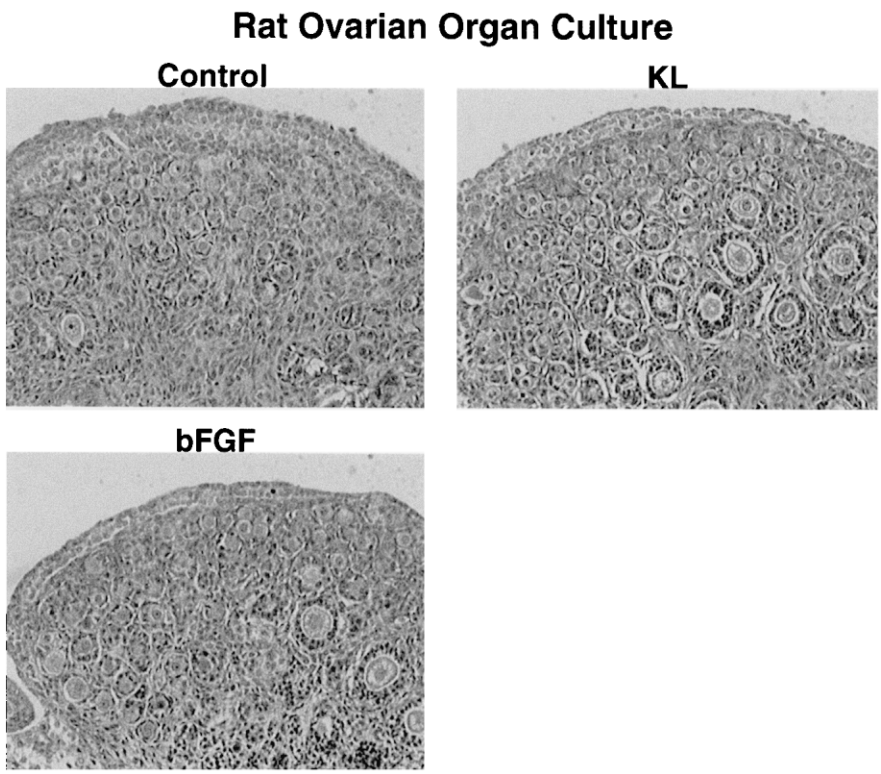

Fig. 2. Postnatal 4-day-old rat ovaries were cultured, fixed, paraffin embedded, sectioned and stained with Hematoxilin and eosin. Representative sections are shown here for untreated (Control) ovaries and those treated with 25 ng/ml KL (KL) or 40 ng/ml bFGF (bFGF) for 14 days in culture. The number of developing (stage 1–4) follicles is increased in ovaries cultured with KL or bFGF. Representative sections at a magnification of $100\times$.

(stage 1–4) as previously described (Parrott and Skinner, 1999a) and shown in Fig. 1.

Follicles were counted in duplicate ovaries from a minimum of three independent experiments (i.e. a minimum of 7000 total follicles counted) in order to quantify the effect of bFGF on initiation of primordial follicle development. Data were expressed as percent follicles per section for each classification stage. Freshly isolated 4-day-old ovaries contain approximately $70 \pm 2\%$ primordial follicles (stage 0) (Figs. 2 and 3). Untreated (i.e. control) 14-day-cultured ovaries contain approximately $40 \pm 3\%$ primordial follicles and $60 \pm 2\%$ developing follicles, suggesting some spontaneous primordial follicle development occurs (Figs. 2 and 3). This is compared with $30 \pm 2\%$ developing follicles in freshly isolated ovaries. Ovaries cultured with bFGF showed a significant ($P \leq 0.05$) decrease in the number of primordial follicles to $15 \pm 2\%$ and a corresponding increase to $85 \pm 3\%$ in developing stage 1–4 follicles compared with controls (Fig. 3). Similar results were obtained with KL treatment as previously described (Fig. 3) (Parrott and Skinner, 1999a). The combined treatment of bFGF and KL gave the same results as either factor alone with a decrease in primordial follicles to 15% and increase to 85% developing follicles ($P < 0.05$). The distribution of developing follicles were 45% stage 1, 36% stage 2, 9% stage 3, 9% stage 4 and negligible secondary or antral stages (data not shown) (Parrott and Skinner, 1999a). The data obtained with the 14-day cultures (Fig. 3) were similar to the 5-day cultures (data not shown) except the percentage of primordial follicles developing was reduced to 40% for control cultures and 60% for treated cultures (data not shown), as compared with the 55% control cultures and 85% treated cultures shown in Fig. 3 (Parrott and Skinner, 1999a).

The bFGF neutralizing antibody was used to determine if the spontaneous primordial follicle development observed could be inhibited. As shown in Fig. 3A, the untreated ovary cultures contained approximately $40 \pm 3\%$ primordial follicles and $60 \pm 2\%$ developing follicles. The presence of the bFGF IgG antibody maintained primordial follicle development at approximately $50 \pm 5\%$ in comparison with the non-immune control IgG that had the reduction to 40% primordial follicles (Fig. 3B). Therefore, some, but not all, spontaneous follicle development could be inhibited with the bFGF antibody.

Immunocytochemistry was utilized to localize bFGF during follicle development. Basic FGF is present at elevated levels in the oocytes of primordial and early developing follicles in rat ovaries (Fig. 4). Although

some staining occurs in all cell types, the highest levels are seen in the oocytes of these early stage follicles. Reduced oocyte bFGF staining was observed in pre-antral follicles with a corresponding increase in granulosa cell staining (Fig. 4). The negative non-immune antibody control (Fig. 4) and bFGF peptide pre-absorption control (data not shown) demonstrated that the predominant oocyte staining was specific.

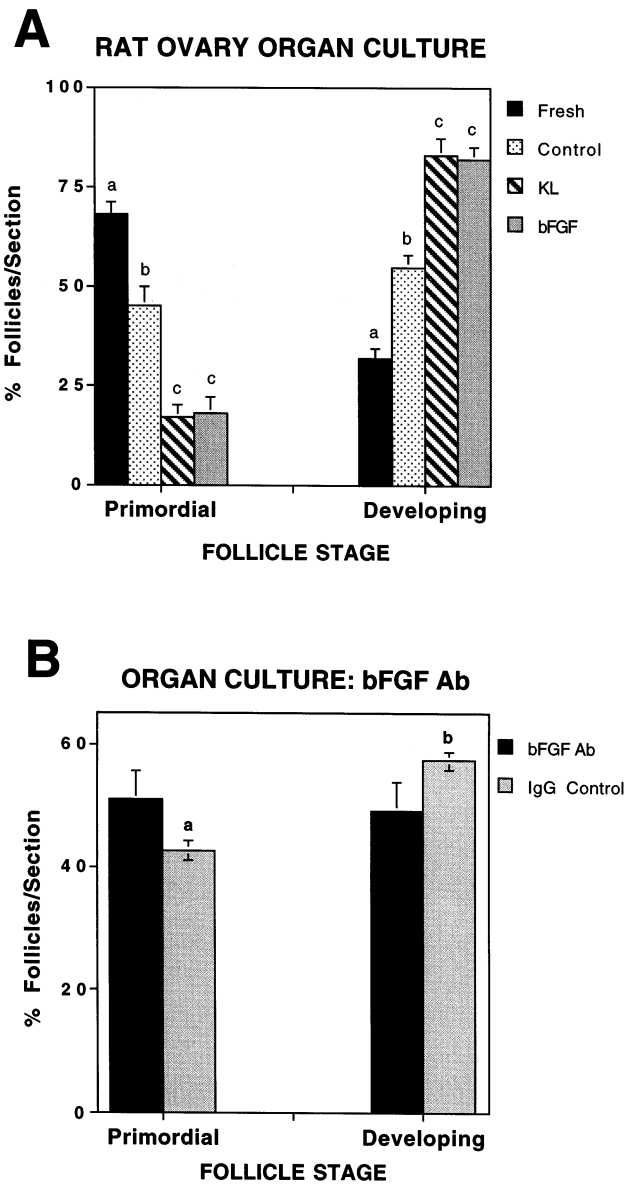

Fig. 3. Postnatal 4-day-old rat ovaries were analyzed (A) when either freshly isolated (Fresh) or cultured for 2 weeks in the absence (Control) or presence of 25 ng/ml KL (KL) or 40 ng/ml bFGF (bFGF): or (B) ovaries were cultured in the presence of bFGF antibody IgG or non-immune IgG, 20 ng/ml. The ovarian follicles in the histologic sections of these ovaries were counted and categorized as either primordial (stage 0) or developing follicles (stages 1–4). All the follicles were counted in serial cross-sections at the widest portion of the ovary and the contralateral ovary was used in the control cultures for comparison. These data were expressed as the percentage of follicles that fell into each category per section. Treatment groups are significantly ($P \leq 0.05$) different from each other if columns have different superscript letters as determined by ANOVA and Tukey–Kramer HSD. Data are presented as the mean ± S.E. of the mean from a minimum of three different experiments performed in duplicate (i.e. six different rats and ovaries per treatment).

Although effects of bFGF on granulosa cells have been reported, actions of bFGF on other ovarian somatic cells has not been investigated. Experiments were performed to better understand the potential sites of bFGF action in the ovary. Bovine theca cells and stroma cells were cultured in the presence of bFGF and other growth factors to evaluate the actions of these factors on cell growth, as measured by [^{3}H]thymidine incorporation into DNA. Basic FGF was found to stimulate theca cell proliferation (Fig. 5A). Epidermal growth factor (EGF) and 10% calf serum were used as positive controls and have previously been shown to stimulate theca cell growth. Similarly, bFGF was found to stimulate ovarian stroma cell growth to the same level as seen with EGF treatment (Fig. 5B). Therefore, bFGF can stimulate both theca cell and ovarian stroma cell growth (Fig. 5).

4. Discussion

Basic FGF treatment of rat ovaries results in a decrease in the number of primordial follicles and a concomitant increase in developing follicles. This demonstrates that bFGF, like KL, induces (i.e. increases) primordial follicle development (i.e. primordial transition to primary follicle stage) to initiate folliculogenesis. Kit ligand and bFGF are the first factors shown to directly promote primordial follicle development, in contrast to the large number of factors shown to promote later stage follicle development. How KL and bFGF may interact in this process of primordial follicle development remains to be determined. The inability of the combined treatment of KL and bFGF to provide an additive effect suggests that one factor may precede the other and/or that the in vitro follicle development observed is optimal and cannot be increased. The bFGF neutralizing antibody decreased the rate of spontaneous follicle development in the ovary culture (Fig. 3B), but did not have the dramatic effect of the KL neutralizing antibody previously shown (Parrott and Skinner, 1999a). This observation supports a role for bFGF in primordial follicle development, but that both KL and bFGF are probably required. Other factors have been shown to be present at the early follicle stages that also may be involved in the induction process. Growth differentiation factor-9 (GDF-9) is expressed in early stage primary follicle oocytes and appears to influence follicle development to the pre-antral and antral stages (Dong et al., 1996). The GDF-9 knockout mice have a block in follicle development at

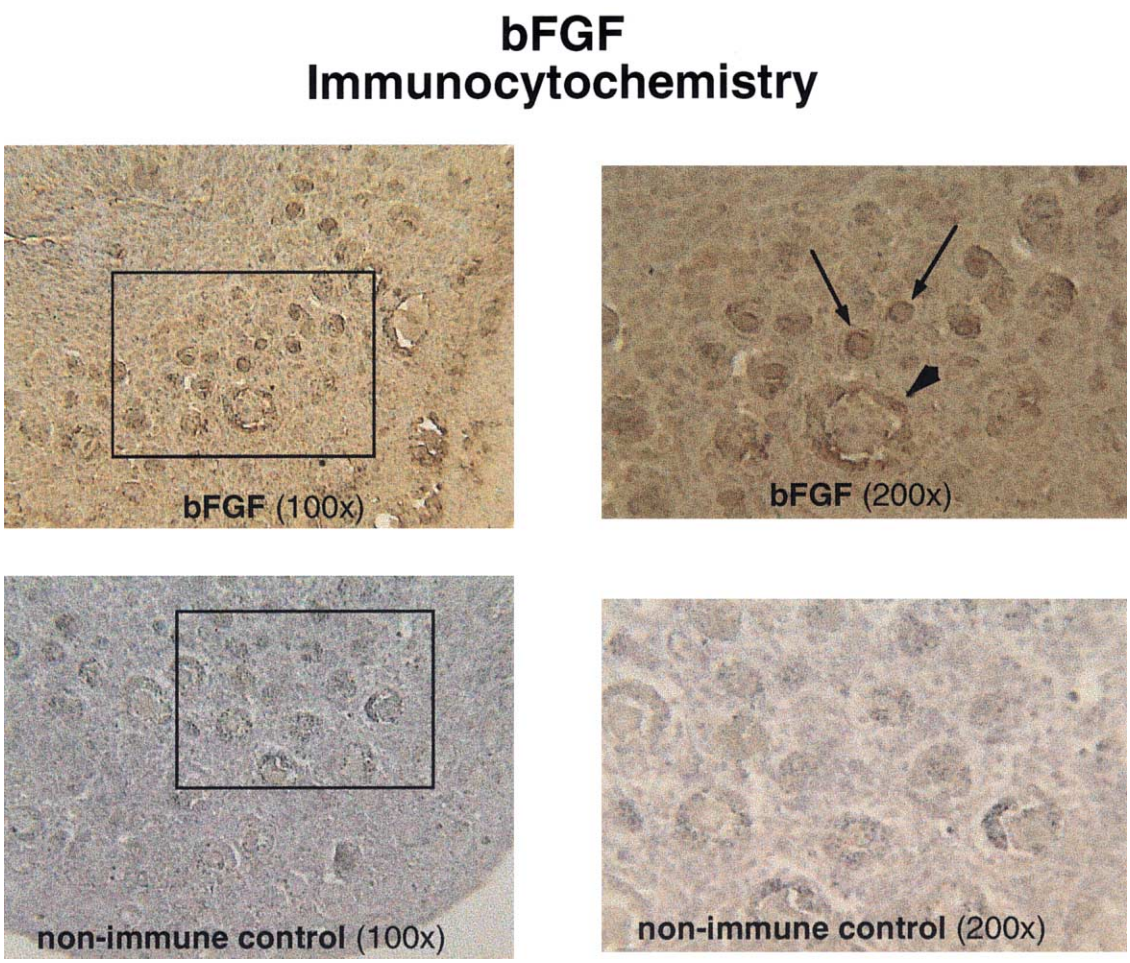

Fig. 4. Immunocytochemical localization of bFGF in 4-day-old rat ovaries. Dark-brown staining indicates a higher concentration of bFGF in oocytes of early stage ovarian follicles. The non-immune control was subjected to the same procedure without the addition of primary antibody. The panels to the right are higher magnification (200 ×) of the boxes inset in the panels to the left (100 ×). The arrows indicate primordial and early primary follicles with oocyte staining and the arrowhead indicates a pre-antral follicle with granulosa staining.

the primary stage, not the primordial stage (Dong et al., 1996). GDF-9 is not expressed in the primordial follicle (Dong et al., 1996) and the knockout phenotype suggests GDF-9 may not have a role in primordial follicle development. The role GDF-9 may have in primordial follicle development and/or influence KL or bFGF expression remains to be examined. It will be of interest to determine the cascade of events involved in primordial follicle development.

A number of factors have been shown to indirectly influence primordial follicle development and/or recruitment of follicles. These factors may influence the apparent interactions between developing follicles and primordial follicles. An example of such a factor is anti-Müllerian hormone. Null mice with a knockout of the anti-Müllerian hormone gene deplete their primordial follicles by 13 months of age and show increased numbers of developing follicles at 4 months of age (Durninger et al., 1999). Studies on the development of fetal hamster ovaries show that exogenous insulin is required for follicles to continue developing normally past the primordial and primary stages (Yu and Roy, 1999). Therefore, factors that decrease or increase pre-antral or antral follicle development may influence primordial follicle development or recruitment due to the interactions between these follicles. In the current 4-day-old rat ovarian cultures, the advanced pre-antral and antral follicles are rare. Interactions with these advanced follicle stages is probably not a factor in the observations presented in the current study.

Basic FGF may influence primordial follicle development by acting on oocytes, theca cells, stromal interstitial cells or granulosa cells (Shikone et al., 1992; Wandji et al., 1992; Horie et al., 1993; Manova et al., 1993, 1990; Motro and Bernstein, 1993) The potential ability of bFGF to directly regulate theca cell or ovarian stromal cell function has not been thoroughly investigated. The large size of bovine ovaries (i.e. similar to human ovaries) allows pure populations of theca cells and ovarian stromal cells to be isolated and cultured

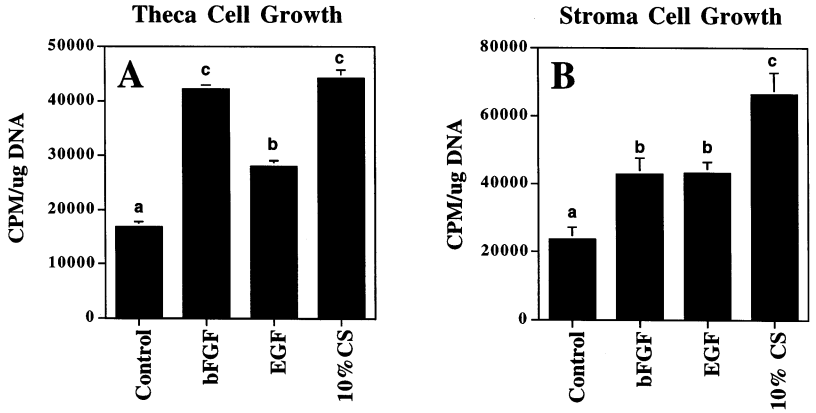

Fig. 5. [^{3}H]Thymidine incorporation into cultured (A) theca or (B) ovarian stroma. Treatments (24 h) are 10% calf serum (10% CS), 50 ng/ml epidermal growth factor (EGF), 50 ng/ml basic FGF (bFGF) or no treatment (Control). Treatment groups are significantly ($P \leq 0.05$) different from each other with different superscript letters, as determined by ANOVA and Tukey–Kramer HSD. Data are the mean ± S.E.M. from three different experiments.

(Parrott and Skinner, 1998, 1999b). In the current study, bovine ovaries were used to investigate the direct actions of bFGF on theca cell and ovarian stromal cell growth. Basic FGF was found to stimulate growth of both theca and stroma cells. Previous research has demonstrated that bFGF also stimulates granulosa cell growth (Gospodarowicz et al., 1989; Lavranos et al., 1994; Rodgers et al., 1996; Roberts and Ellis, 1999). Therefore, all the somatic cell types surrounding an oocyte can potentially respond to bFGF. Localization of bFGF demonstrated high levels of bFGF in the oocytes of early stage (i.e. primordial) follicles. These results suggest that oocyte derived bFGF may promote ovarian granulosa, stromal and theca cell growth during primordial and early follicular development. bFGF appears to in part induce primordial follicle development by directly acting on adjacent ovarian cells to promote somatic cell growth.

Factors that control the initiation of primordial follicle development are essential for female reproduction. Initiation of primordial follicle development determines the number of follicles available for dominant follicle selection and ovulation. Although bFGF appears to be important for primordial and early follicular development, other factors that remain to be elucidated are also likely involved. Interactions between various factors such as bFGF, KL, anti-Müllerian hormone and GDF-9 will provide a potential sequence of events involved in this initial process of folliculogenesis. Identification of the actions of bFGF provides a potential therapeutic target to control follicular development in humans. The ability to control the number of primordial follicles that initiate development over time may be useful for regulating the onset of menopause and treating pathological conditions such as premature ovarian failure.

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