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# Leukemia inhibitory factor (LIF) promotes the primordial to primary follicle transition in rat ovaries

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**Abstract**

In a sexually mature female, primordial follicles continuously leave the arrested pool and undergo the primordial to primary follicle transition. The oocytes increase in size and the surrounding squamous pre-granulosa cells become cuboidal and proliferate to form a layer of cuboidal cells around the growing oocyte. This development of the primordial follicle commits the follicle to undergo the process of folliculogenesis. When the available pool of primordial follicles is depleted reproductive function ceases and humans enter menopause. The current study examines whether leukemia inhibitory factor (LIF) promotes the primordial to primary follicle transition that initiates follicular development. Ovaries from 4 day-old rats were cultured in the absence or presence of LIF or neutralizing antibody to LIF. LIF treatment increased the proportion of follicles that initiated the primordial to primary follicle transition to 59%, compared to 45% in untreated cultured ovaries. The ability of LIF to induce primordial follicle development was enhanced to greater than 75% by the presence of insulin in the culture medium. Anti-LIF neutralizing antibody reduced the proportion of spontaneous developing primordial follicles. Immunocytochemical studies demonstrated higher levels of LIF protein in the granulosa and surrounding somatic cells of primordial and primary follicles compared to the oocyte. In contrast, later pre-antral and antral stage follicles showed LIF expression primarily in the oocyte. In granulosa and theca cell cultures LIF had no effect on cell proliferation. However, LIF treatment did increase expression of Kit ligand (KL) mRNA in cultured granulosa cells. KL has been shown to promote ovarian cell growth and induce primordial follicle development. LIF induction of KL expression may be involved in the actions of LIF to promote primordial to primary follicle transition. In summary, LIF treatment increased the primordial to primary follicle transition in cultured ovaries and LIF may interact with KL to promote primordial follicle development. 

*Keywords:* Ovary; Leukemia inhibitory factor (LIF); Primordial follicle; Folliculogenesis; Growth factors

## 1. Introduction

Primordial follicles are assembled in the first 2–3 days after birth in the rat ovary. A primordial follicle is composed of an oocyte arrested in the diplotene stage of prophase one of meiosis and surrounded by a single layer of squamous pre-granulosa cells. In a sexually mature female primordial follicles continuously leave the arrested pool and undergo the primordial to primary follicle transition. The oocytes increase in size and the surrounding flattened pre-granulosa cells become cuboidal and proliferate to form a single layer around the growing oocyte. Folliculogenesis continues until each growing follicle either ovulates or undergoes atresia and apoptosis. During each reproductive cycle some primordial follicles will undergo the transition to primary follicles while others remain and will be available for future cycles of recruitment (Hirshfield, 1991; Rajah et al., 1992; Peters et al., 1975; Cran and Moor, 1980). When the available pool of primordial follicles is depleted reproductive function ceases and primates, including humans, enter menopause (Faddy et al., 1992; Faddy and Gosden, 1996; Richardson et al., 1987; Gosden et al., 1983). Women who prematurely

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deplete their pool of primordial follicles exhibit an infertile condition called premature ovarian failure (Parrott et al., 2001; Berga, 2001). An improved understanding of the control of the primordial to primary follicle transition and subsequent folliculogenesis might lead to improved therapies for such conditions.

Both Kit ligand (KL) and basic fibroblast growth factor (bFGF) have been shown to promote the primordial to primary follicle transition in the rat (Parrott and Skinner, 2000a; Nilsson et al., 2001). KL is produced by the granulosa cells of developing ovaries (Manova et al., 1993; Motro and Bernstein, 1993) and KL receptors (c-kit) are present in oocytes and theca cells (Manova et al., 1993; Motro and Bernstein, 1993; Manova et al., 1990; Horie et al., 1991). Theca cells will proliferate in culture in response to KL treatment (Parrott and Skinner, 1997) and KL may act to recruit theca cells from surrounding ovarian stroma during folliculogenesis (Parrott and Skinner, 2000b). Similarly, bFGF is present in the oocytes of primordial follicles and the granulosa cells of developing follicles (Nilsson et al., 2001; van Wezel et al., 1995; Yamamoto et al., 1997). Receptors for bFGF are found in granulosa cells (Shikone et al., 1992; Wandji et al., 1992). Granulosa, theca and ovarian stromal cells will proliferate in response to bFGF (Nilsson et al., 2001; Lavranos et al., 1994; Rodgers et al., 1996; Roberts and Ellis, 1999). Therefore, both KL and bFGF are proposed to signal follicular cells to promote the primordial to primary follicle transition (Parrott and Skinner, 2000a,b; Nilsson et al., 2001).

Leukemia inhibitory factor (LIF) is a cytokine identified for its ability to affect growth of myeloid leukemic cells (Gearing et al., 1987; Moreau et al., 1988). LIF is present in human follicular fluid and its levels are regulated according to the stage of antral follicle development. LIF levels in follicular fluid are also responsive to human chorionic gonadotropin (hCG) (Coskun et al., 1998; Arici et al., 1997). Granulosa cells in culture can produce LIF (Coskun et al., 1998; Arici et al., 1997). Prior to the formation of primordial follicles LIF and KL in combination have been shown to be important in primordial germ cell survival in embryonic mice (Yutaka et al., 1999). The LIF receptor complex is a heterodimer composed of LIF receptor beta (LIFR beta) and gp130 (Baumann et al., 1994). LIF receptors are known to be present in the human in the oocytes of antral stage follicles and in early stage embryos (van Eijk et al., 1996). There is a low affinity LIF receptor which is important in multiple organ systems, but apparently not reproductive tissues (Ware et al., 1995). The expression of LIF and its receptors during the early stages of follicle development is unknown and is investigated in the current study.

The present investigation examines whether LIF promotes the primordial to primary follicle transition that initiates follicular development. The cellular localization of LIF in early stage follicles is determined by immunocytochemistry. The actions of LIF on follicular cell growth and gene expression are investigated to provide insight into the mechanisms by which LIF may act on primordial follicles.

## 2. Methods

### 2.1. Histology and organ cultures

Postnatal day 4 rat ovaries were prepared fresh or cultured for 14 days. Fresh ovaries were fixed in Bouin's solution for 1–2 h, embedded in paraffin, sectioned (3–5 μm), and serial sections placed on slides. The sections were then stained with Hematoxylin and eosin. Whole ovaries were cultured as previously described (Parrott and Skinner, 2000a) on floating filters (0.4 μm Millicell-CM, Millipore, Bedford, MD) in 0.5 ml Dulbecco's Modified Eagle's Medium (DMEM)-Ham's F-12 medium (1:1, vol/vol) containing 0.1% bovine serum albumin (BSA, Sigma, St. Louis, MO), 0.1% albumax (Gibco BRL, Gaithersburg, MD), 27.5 μg/ml transferrin, and 0.05 mg/ml L-ascorbic acid (Sigma) in a four-well culture plate (Nunc plate, Applied Scientific, South San Francisco, CA). Some organ culture experiments additionally contained 1 μg/ml insulin (human recombinant, Sigma). Ovaries were randomly assigned to treatment groups with 1–3 ovaries per floating filter. Ovaries (4–7) were examined per treatment group. Treatments during organ culture included anti-LIF neutralizing Ab (1 μg/ml) obtained from R&D Systems, (Minneapolis, MN) and LIF (50 ng/ml) obtained from Chimicon International, Inc. (Temecula, CA). Medium was supplemented with penicillin and streptomycin to prevent bacterial contamination. After culture, ovaries were fixed and sectioned as described above for fresh uncultured ovaries.

The number of follicles at each developmental stage was counted in two serial sections and averaged from the largest cross-section through the center of the ovary. The oocyte nucleus had to be visible in a follicle in order to be counted. Normally 150–200 follicles were present in a cross-section. Follicles were classified as either primordial (stage 0), or as one of the developing preantral stages (stages 1–4) as described previously (Parrott and Skinner, 2000a). Briefly, primordial follicles consist of an oocyte partially or completely encapsulated by flattened squamous pregranulosa cells. Developing (stage 1–4) follicles contain successively more cuboidal granulosa cells in layers around the oocyte (Parrott and Skinner, 2000a; Nilsson et al., 2001).

### 2.2. Cell culture

Theca cells and granulosa cells were isolated from freshly obtained bovine ovaries as previously described (Parrott and Skinner, 1998). Cells were plated and cultured either at subconfluent densities for use in growth assays (in DMEM plus 0.1% BSA plus 0.1% calf serum), or confluent densities for use in assays of mRNA expression (in Ham's F-12 plus 0.1% BSA) (Parrott and Skinner, 1998; Parrott et al., 2000).

### 2.3. Cell growth assays

Cells were cultured at subconfluent densities (growth permissive) in DMEM plus 0.1% BSA plus 0.1% calf serum for 24 h, then cells were treated for 20 h with LIF (50 ng/ml) or 10% calf serum. This was followed by a 6-h incubation with $^{3}$H-thymidine (10 μCi/ml). Counts per min (CPM) of $^{3}$H-thymidine incorporated into DNA were determined as described previously (Nilsson et al., 2001) and normalized to the total DNA per well. DNA levels were assayed by incorporation of SYBR-green® dye (Molecular Probes Inc., Eugene, OR) procedure. Samples were excited at 300 nm and measured at 520 nm. Fluorescence was compared to that of a standard curve of known calf thymus DNA.

### 2.4. Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

To assay for KL mRNA expression the granulosa cells were plated at confluent density in Ham's F-12 plus 0.1% BSA for 24 h and then cells were treated for 2 days with LIF (50 ng/ml). At the end of treatment total RNA was prepared from cultured cells using Trizol reagent (Gibco BRL, Rockville, MD). RNA was stored at −70 °C until use. As described previously (Parrott et al., 2000) the RNA samples were reverse transcribed to cDNA by adding 1 μg RNA to 1 μl each of 1 μM 3′-KL and cyclophilin primers. Primer sequences are detailed below. Before reverse transcription tubes containing total RNA and 3′-primers were heated to 65 °C for 10 min and cooled to room temperature. Total RNA was reverse transcribed for 1 h at 37 °C using the following conditions: 1 μg total RNA, 1 μM specific 3′-primers, 0.1 mM dNTP's, 10 mM DTT, 40 Units RNAse inhibitor (Promega, Madison, WI), and 200 Units M-MLV reverse transcriptase (Gibco BRL, Gaithersburg, MD) in 40 μl RT buffer (50 mM Tris–HCl pH 8.3, 75 mM KC1, 3 mM $MgCl_2$). After 1 h samples were heated to 95 °C for 5 min to inactivate the reverse transcriptase enzyme. Steady state levels of KL and cyclophilin (i.e. 1B15) mRNA's were analyzed using a specific quantitative reverse transcription-polymerase chain reaction (RT-PCR) assay for each gene (Parrott et al., 2000). Known quantities of cDNA for portions of the KL or cyclophillin gene were assayed in parallel with the unknown samples to act as a standard curve. These quantitative RT-PCR assays have previously been described in detail (Parrott and Skinner, 1997). The primers used were: KL, 5′-GGA CAA GTT TTC GAA TAT TTC TGA AGG CTT GAG TAA TTA TTG-3′ (5′-primer; 42-mer) and 5′-AGG CCC CAA AAG CAA ACC CGA TCA CAA GAG-3′ (3′-primer; 30-mer), which generated a specific 452-bp KL PCR product, and 1B15, 5′-ACA CGC CAT AAT GGC ACT GGT GGC AAG TCC ATC-3′ (5′ primer, 33-mer) and 5′-ATT TGC CAT GGA CAA GAT GCC AGG ACC TGT ATG-3′ (3′ primer, 33-mer) which generated a specific 105-bp product. Amplification was performed using the following conditions: 0.4 μM each primer, 16 μM dNTP's, and 1.25 Units AmpliTaq polymerase in 50 μl GeneAmp PCR buffer (containing 1.5 mM $MgCl_2$, Perkin–Elmer). Each PCR amplification consisted of an initial denaturing reaction (5 min, 95 °C); 26–30 cycles of denaturing (30 s, 95 °C), annealing (1 min, 60 °C), and elongation (2 min, 72 °C) reactions; and a final elongation reaction (10 min, 72 °C). At least 0.25 μCi of $^{32}$P-labeled dCTP (Redivue, Amersham Life Sciences, Arlington Heights, IL) was included in each sample during amplification for detection purposes. Specific PCR products were quantified by electrophoreses of all samples on 4–5% polyacrylamide gels, exposing the gels to a phosphor screen for 8–24 h, followed by quantifying the specific bands on a Phosphor Imager (Molecular Dynamics, Sunnyvale, CA). Each gene was assayed in separate PCR reactions from the same RT. All KL data were normalized for 1B15. The optimal number of cycles for amplification was determined for each assay in order to achieve maximum sensitivity while maintaining linearity (i.e. logarithmic phase of PCR reactions). KL PCR products were amplified for 30 cycles, and 1B15 PCR products were amplified for 26 cycles. The sensitivity of each quantitative PCR assay was below 1 fg, which corresponds to less than 125 fg target mRNA/μg total RNA.

### 2.5. Immunocytochemistry

Localization of LIF protein was determined by immunocytochemical analysis. Four-day-old rat ovaries were cultured for 2 weeks and then fixed in Bouin's solution (0.9% picric acid, 9% formaldehyde, 5% acetic acid) for 1–2 h. Ovaries were paraffin embedded and sectioned at 3–5 μm. Ovaries were de-parafinized in xylenes and hydrated through an ethanol series of 100, 90 and 70%. Antigens were exposed by boiling sections for 5 min in 0.01 M sodium citrate buffer, pH 6.0. Ten percent rabbit serum in phosphate buffered saline (PBS) was used as a blocking agent prior to incubating sections with primary antibody overnight at 4 °C. Anti-LIF antibody (Santa Cruz Biotechnology Inc., Santa Cruz,

CA; made in goat) was used at 20 or 4 μg/ml. Negative controls were incubated in the presence of non-immune goat IgG as a primary antibody or with anti-LIF antibody and 100 μg/ml blocking LIF peptide. Secondary antibody was biotinylated anti-goat made in rabbit. Strep-avidin peroxidase (Zymed Laboratories Inc, South San Francisco, CA) localized the enzymatic color reaction to the sites of primary antibody binding. The color reaction was performed using the color reaction kit from Vector Laboratories, Inc., Burlingame, CA. Slides were then dehydrated through a xylenes/ethanol series and coverslipped. The presence of LIF protein was indicated by a brown color on the sections.

### 2.6. Statistics

Treatment groups are compared to controls (Hsu, 1989) and pairs comparisons are performed using Student's $t$-test. Multiple comparison tests are performed using Tukey–Kramer HSD. Groups were considered significantly different if $P \leq 0.05$. All statistics were calculated with the help of JMP v3.1 software (SAS Institute, Inc., Carey, NC).

## 3. Results

In order to determine if LIF promotes development of primordial follicles 4 day-old rat ovaries were cultured in the absence or presence of 50 ng/ml LIF or 1 μg/ml anti-LIF neutralizing antibody (Fig. 1). The ovaries were fixed, sectioned and stained for morphological analysis either fresh (i.e. at the time of dissection prior to culture) or after culture and treatment. Data were from three separate experiments. A total of approximately 4000 follicles were counted. Total follicle numbers per ovary did not change between treatment groups (data not shown), as previously demonstrated (Parrott and Skinner, 2000a). Fresh uncultured ovaries have 68% primordial and 32% developing follicles. Untreated cultured ovaries reflect the spontaneous primordial to primary follicle transition and development that occurs in culture and have 45% developing follicles. LIF treatment increased the percentage of developing follicles to 59% (Fig. 1). Treatment with neutralizing antibody to LIF reduces slightly the spontaneous primordial follicle development that occurs in culture to 40% developing follicles. Therefore, LIF can promote the primordial to primary follicle transition (Fig. 1).

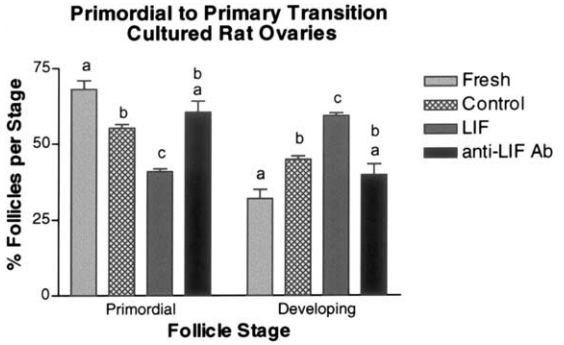
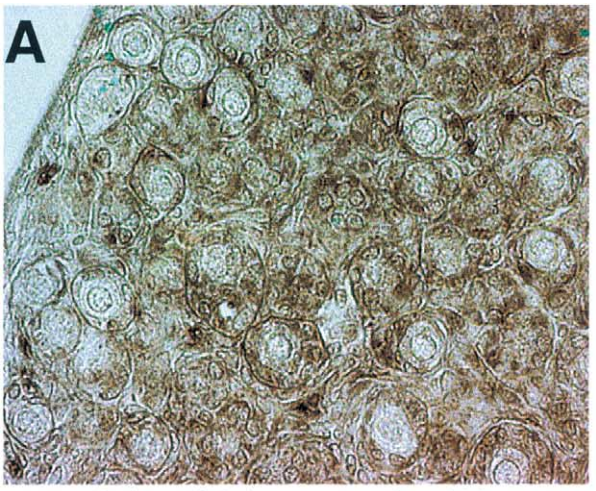



Fig. 1. LIF promotes the primordial to primary follicle transition in cultured rat ovaries. Ovaries from 4-day-old rats were either immediately fixed and stained (Fresh) or were placed into culture for 14 days. Cultured ovaries were treated with no factors (Control), LIF (LIF) or neutralizing antibody to LIF (anti-LIF Ab). After culture all ovaries were fixed, stained and subjected to morphological analysis. The follicles per ovary cross-section were categorized as being either primordial or developing (which includes all follicles having undergone the primordial to primary transition). Bar height indicates the proportion of follicles that are of either primordial or developing stages for each treatment group. Data are from three separate experiments with two replicates (ovaries) per experiment with the mean ± SEM are presented. Bars with different superscript letters indicate treatment groups are significantly different ($P < 0.05$).

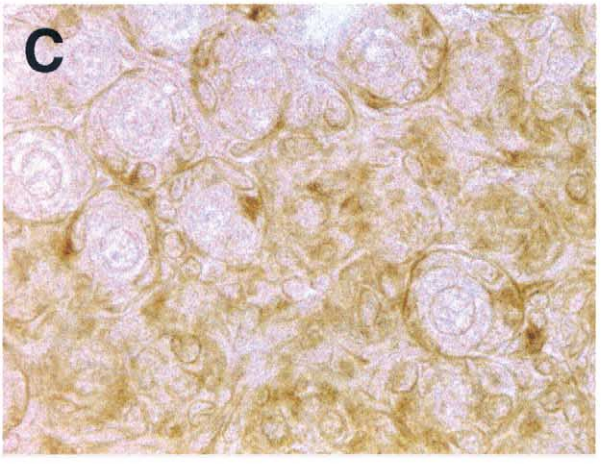
Immunocytochemistry was performed to localize LIF protein expression in 4 day-old rat ovaries after 14 days of culture and pubertal 25 day-old rat ovaries (Fig. 2). LIF protein is present in the granulosa cells of primordial and primary follicles, but is absent or at very low levels in the oocytes of these stages of follicle development. This is in contrast to the high level of LIF localization seen in the oocytes of pre-antral and antral follicles of 25 day-old rats (Fig. 2). Granulosa cell expression of LIF continues through all stages. The negative control with blocking LIF peptide demonstrated the staining observed was specific (Fig. 2). These studies indicate that LIF is present in the granulosa cells of primordial follicles and so is available as a paracrine signaling agent to influence the primordial to primary follicle transition.

Previous studies demonstrated that KL has a marked effect on promoting the primordial to primary follicle transition (Parrott and Skinner, 2000a). Ovaries cultured in the presence of KL had the proportion of developing follicles increased to nearly 80%. In those studies all culture experiments were performed with insulin in the culture medium. In the current study, organ cultures were performed without insulin in the medium. The ability of KL to promote primordial follicle transition appeared decreased in the absence of insulin (data not shown). Ovaries were cultured with 1 μg/ml insulin, 50 ng/ml LIF or the combination of insulin and LIF in order to test whether insulin enhances the development of primordial follicles in response to LIF treatment (Fig. 3). Control untreated ovaries showed 54% developing follicles. Insulin alone moderately increased the proportion of developing follicles to 60%, but was not statistically significantly different. The proportion of developing follicles in ovaries treated with LIF alone was significantly different from controls at 63%. The combination of insulin and LIF markedly increased the proportion of developing follicles to 77%

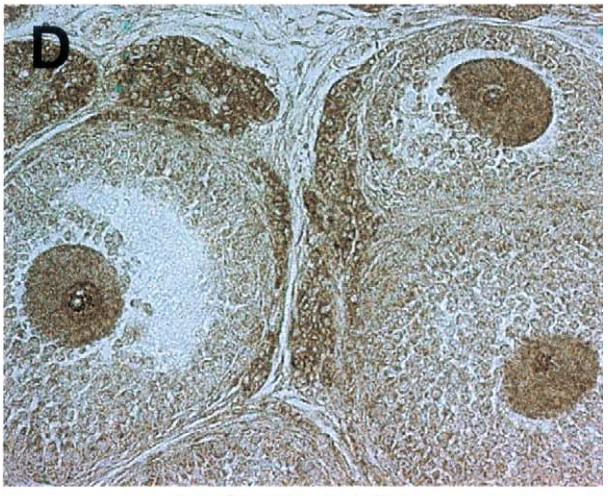



Cultured 4-day rat ovaries 400x

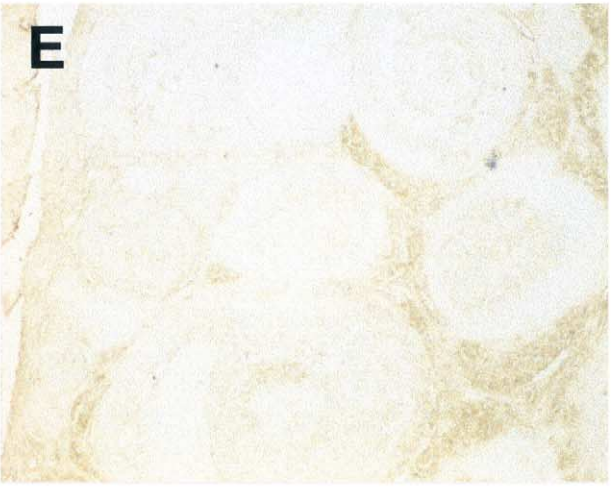

Cultured 4-day rat ovaries: blocking peptide




Cultured 4-day rat ovaries 1000x




25-day rat ovaries




25-day rat ovaries: blocking peptide

Fig. 2 (*Continued*)

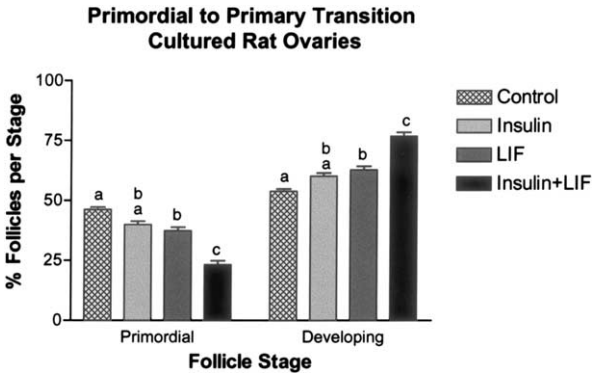



Fig. 3. Insulin enhances the ability of LIF to promote the primordial to primary follicle transition. Ovaries from 4-day-old rats were placed into culture for 14 days. Cultured ovaries were treated with no factors (Control), insulin (Insulin), LIF (LIF) or both insulin and LIF (Insulin + LIF). After culture ovaries were fixed, stained and subjected to morphological analysis. The follicles per ovary cross-section were categorized as being either primordial or developing (which includes all follicles having undergone the primordial to primary transition). Bar height indicates the proportion of follicles that are of either primordial or developing stages for each treatment group. Data are from three separate experiments done in replicate with the mean ± SEM presented. Bars with different superscript letters indicate treatment groups are significantly ($P < 0.05$) different by Tukey–Kramer HSD test.

(Fig. 3). These observations suggest insulin acts together with LIF to further increase the proportion of primordial follicles undergoing transition and development.

Bovine theca and granulosa cells were isolated from antral follicles and cultured with or without LIF in order to test whether LIF acts directly on granulosa or theca cells to promote cell proliferation (Fig. 4). Neither granulosa nor theca cell growth was influenced by LIF treatment (50 ng/ml) as measured by $^{3}$H-thymidine incorporation into DNA. Treatment with 10% calf serum as a positive control did result in increased cell proliferation for both cell types. Therefore, LIF was unable to act directly on granulosa or theca cells to promote cell proliferation, which is a part of follicle development.

KL is known to promote growth of granulosa and theca cells as well as stimulate recruitment of theca from the stroma surrounding follicles (Parrott and Skinner, 1997, 2000b). In order to examine possible mechanisms by which LIF acts to promote primordial to primary follicle transition, the hypothesis was tested that LIF induces KL expression in granulosa cells. Following LIF stimulation, KL protein could potentially induce some of the events of the primordial to primary follicle transition. Bovine granulosa cells were isolated from antral follicles and cultured (Fig. 5). The granulosa cells were treated with 50 ng/ml LIF for 3 days and KL mRNA levels was determined by quantitative RT-PCR. LIF treatment increased KL mRNA levels more than twofold compared to untreated controls. Therefore, LIF and KL may interact and/or cooperate to promote primordial follicle development (Fig. 6).

## 4. Discussion

In order to determine if LIF promotes the primordial to primary follicle transition 4-day-old rat ovaries were cultured in the absence or presence of LIF. A significant ($P < 0.05$) increase in the proportion of developing

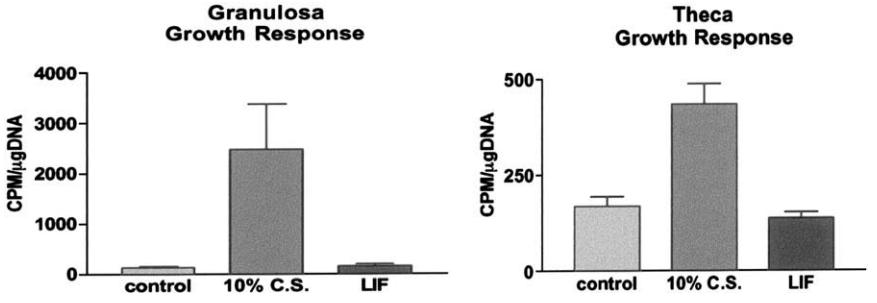



Fig. 4. LIF does not promote proliferation of either theca or granulosa cells in culture. Granulosa (left panel) and theca cells (right panel) were isolated from antral bovine follicles and placed into culture. Cells were treated with 50 ng/ml LIF or 10% calf serum as a positive control, or were untreated. Cell proliferation was measured by $^{3}$H-thymidine incorporation (CPM/µg DNA). Data are from three separate experiments with three

Fig. 2. LIF immunohistochemistry. Ovaries from 4-day old rats after 14 days of culture without LIF or neutralizing antibody treatments (A, B and C), or from 25-day pubertal rats (D and E) were immunostained using anti-LIF antibody to show where LIF protein is localized. Brown stain indicates the presence of LIF protein. (A) Several primordial and primary follicles are present, where the large oocyte is surrounded by a single layer of flattened pre-granulosa cells and/or cuboidal granulosa cells. Note that the oocytes have low levels of LIF staining compared to surrounding cells. Magnification 400 ×. (B) Anti-LIF Ab was pre-incubated with a fivefold excess of LIF blocking peptide before applying to cultured rat ovary sections as a negative control. Magnification 400 ×. (C) Higher magnification (1000 ×) of cultured immunostained ovaries. (D) Later stage follicles with several layers of granulosa cells and large oocytes. High levels of staining for LIF protein are seen in the oocytes. Magnification 200 ×. (E) Anti-LIF Ab was pre-incubated with a fivefold excess of LIF immune peptide before applying to 25-day rat ovary sections as a negative control. Magnification 200 ×. Data are representative of a minimum of three different experiments.

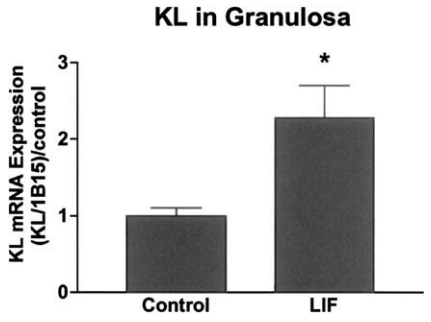



Fig. 5. LIF induces KL mRNA expression in cultured granulosa cells. Granulosa cells were isolated from antral bovine follicles and placed into culture. Cells were treated with 50 ng/ml LIF or were untreated for 3 days. RT-QPCR was performed to determine levels of KL mRNA expression. Data are expressed as KL mRNA/1B15 mRNA (pg/well) normalized to untreated control values. Data are from three separate experiments with two replicates per experiment with the mean ± SEM presented. Asterisk indicates a significant difference ($P < 0.05$) between treatments by Student's $t$-test.

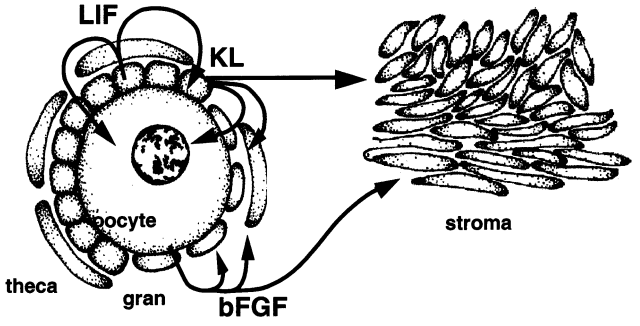



Fig. 6. Model of potential cellular signaling interactions during primordial to primary follicle transition. Interactions between theca, granulosa (gran), flattened pre-granulosa cells and the oocyte involving leukemia inhibitory factor (LIF), Kit ligand (KL) and basic fibroblast growth factor (bFGF) are indicated. The entire follicle is surrounded by stromal/interstitial cells that also require paracrine interactions. Arrows indicate proposed signaling interactions.

follicles was observed with LIF treatment. This indicates that LIF can promote the primordial follicle transition. LIF appears to act in a similar manner as KL and bFGF that have also been shown to promote primordial to primary follicle transition (Parrott and Skinner, 2000a; Nilsson et al., 2001). Rat ovaries were also cultured in the presence of neutralizing antibody to LIF. LIF antibody resulted in significantly ($P < 0.05$) fewer developing follicles than seen in LIF treated ovaries. Observations from the cultured ovary experiments suggest that locally expressed LIF can regulate the primordial to primary follicle transition that occurs over the animals' reproductive lifetime.

Immunocytochemical analysis confirmed that LIF is expressed in early stage ovarian follicles. LIF was seen to be present in the granulosa cells of primordial and later stage follicles, but was absent from the oocytes of primordial and primary follicles. In contrast, LIF was present in the oocytes of later pre-antral and antral stage follicles (Eckert and Niemann, 1998). LIF may signal in a paracrine manner from the pre-granulosa cells of primordial follicles to either the arrested oocyte or to surrounding stromal tissue. Such signaling, alone or in combination with other factors, could promote the primordial to primary follicle transition. In later stage antral follicles LIF expression has shifted to become higher in the oocyte. LIF may also have a different signaling role in these later follicles than in primordial follicles.

Insulin was shown to have an effect in promoting the primordial follicle transition in cultured ovaries. Insulin in combination with LIF acted synergistically to cause a marked increase in the proportion of developing follicles after treatment. Insulin has previously been shown to act synergistically with FSH in granulosa cells to increase steroidogenic activity (Davoren and Hsueh, 1984; Garzo and Dorrington, 1984). Insulin has also been a component of several oocyte or ovary in vitro culture systems (Yu and Roy, 1999; Eppig and O'Brien, 1996; Roy and Treacy, 1993; Roy and Greenwald, 1989). Insulin may be a co-regulator in the signaling pathway controlling primordial follicle transition. Alternatively, systemic insulin may provide trophic support to the cells of the ovary and allow them to respond maximally to LIF signaling.

Several possible mechanisms by which LIF may affect primordial follicle transition and development were examined. KL and bFGF have been shown to directly affect the proliferation of granulosa, theca and ovarian stromal cells, as well as promote primordial follicle transition (Nilsson et al., 2001; Parrott and Skinner, 1997, 2000b; Lavranos et al., 1994; Rodgers et al., 1996; Roberts and Ellis, 1999). However, neither granulosa nor theca cells were shown to proliferate in response to LIF treatment. Both KL and bFGF promote cell proliferation within 24 h of treatment (Parrott and Skinner, 1997; Nilsson et al., 2001). Therefore, LIF may act indirectly to promote the proliferation of granulosa and theca that occurs as a primordial follicle begins to develop. One possibility is that LIF may act to increase expression of other growth factors that in turn affect granulosa or theca cell proliferation. To test this possibility granulosa cells from antral follicles were cultured in the presence of LIF and then KL mRNA levels were determined. LIF treatment increased KL mRNA expression. In the growth assays bovine granulosa cells from antral follicles were cultured and treated with LIF for only 1 day. It is likely that this was insufficient time to allow KL transcription and translation to affect DNA synthesis. The assay for KL mRNA expression was performed after treating granulosa cells for 3 days with LIF. Therefore, LIF may act to up-regulate KL expression that in turn promotes granulosa cell proliferation. Alternatively, it is possible that the rat

granulosa cells react differently to LIF than do bovine cells from antral follicles. Further study is needed to confirm the mechanisms by which LIF acts on primordial follicle cells.

LIF is not essential for follicular development since LIF knock-out mice develop antral follicles and ovulate fertilizable oocytes (Stewart et al., 1992). However, LIF expression in the uterus is required for successful blastocyst implantation and pregnancy. Basic FGF knock-out mice are also able to ovulate viable oocytes (Ortega et al., 1998). This would suggest that several parallel signaling systems may exist to promote the primordial to primary follicle transition and initiate subsequent folliculogenesis. It is not surprising that compensatory factors may exist to control a process so important to reproductive function. Also, an interacting network of signaling factors may provide more precise, responsive control of primordial follicle transition and development. Fig. 6 shows a model by which LIF, bFGF and KL paracrine signals may act on the oocyte, granulosa and surrounding theca/interstitial cells during a primordial or primary follicle transition.

In summary, LIF was shown to promote the primordial to primary follicle transition in cultured ovaries. One mechanism by which LIF may act is to induce the expression of KL from granulosa cells. Therefore, LIF may be an important element of the regulatory pathways controlling recruitment of follicles from the primordial follicle pool. Future studies will further characterize the interactions between LIF, bFGF, KL and other factors to elucidate how they control follicle development. If the control network being worked out in rats is also described and confirmed in humans, then some speculations might be made about human disease associated with follicle development. Elucidation of the control of the primordial to primary follicle transition will help explain the potential pathologies of some presently unexplained forms of premature ovarian failure in which primordial follicles undergo transition and begin developing at an unusually high rate. In these women the oocyte pool is rapidly depleted, resulting not only in loss of fertility but also in early onset of menopausal symptoms (Parrott et al., 2001; Berga, 2001). Further study is needed to determine if imbalances in cell signaling are a root cause of some premature ovarian failures. An understanding of the signaling events and the interactions among growth factors that control primordial follicle transition and follicle growth may help in the development of therapeutic strategies to treat or prevent oocyte depletion.

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