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Insulin but not insulin-like growth factor-1 promotes the primordial to primary follicle transition

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Abstract

A critical step in ovarian biology is the transition of the developmentally arrested primordial follicle to the growing primary follicle. The current study utilizes a rat ovarian organ culture system to investigate the role of insulin and insulin-like growth factor-1 (IGF-1) in this process. Four-day-old rat ovaries were cultured and the degree of primordial to primary follicle transition measured. Insulin increased the primordial to primary follicle transition 30% over control with a half maximal effective concentration (EC50) between 2.5 and 5 ng/ml. IGF-1 did not cause an increase in the primordial to primary follicle transition at concentrations up to 100 ng/ml. Ovaries were also treated with epidermal growth factor (EGF) and hepatocyte growth factor (HGF) and neither had an effect on the primordial to primary follicle transition. Ovaries were treated with insulin in conjunction with other factors known to promote the primordial to primary follicle transition in order to discern any potential synergistic effects. Previous experiments have shown that kit ligand (KL), basic fibroblast growth factor (bFGF) and leukemia inhibitory factor (LIF) promote the primordial to primary follicle transition. Insulin was shown to have an additive effect with KL and LIF, but not bFGF. The fact that insulin can influence the primordial to primary follicle transition at low concentrations (i.e. 5 ng/ml) and that IGF-1 has no effect suggests that insulin is acting at the insulin receptor, not the IGF-1 receptor. The observation that insulin has an additive effect with KL and LIF, but not bFGF, suggests the insulin's site of action is likely the oocyte. In summary, observations suggest that insulin acts as an endocrine type factor to help coordinate primordial to primary follicle transition at the level of the oocyte. The significance of the observations in relation to diabetes and female infertility is discussed.

Keywords: Folliculogenesis; Insulin; IGF-1; Ovary; Primordial follicle; Diabetes

1. Introduction

The transition of the primordial follicle to the primary follicle is a poorly understood phenomenon critical for female reproduction. The primordial follicle consists of an oocyte arrested at the diplotene stage of prophase one and surrounded by squamous epithelial or pre-granulosa cells. These follicles remain developmentally arrested until individual follicles leave the resting pool and develop into primary follicles and initiate folliculogenesis. This is termed the primordial to primary follicle transition (Hirshfield, 1991). Primordial follicles do not proliferate after birth and are the sole source of gametes for a female's reproductive life. When follicle numbers are exhausted, a series of physiological changes associated with menopause begins in humans (Richardson et al., 1987). Therefore, the proper coordination of the primordial to primary follicle transition is critical to maintain female fertility. Inappropriate activation of primordial follicle development appears to be a cause of certain reproductive diseases. The most notable of these is a group of conditions known as premature ovarian failure (Santoro, 2001). The investigation of factors that coordinate the primordial to primary follicle transition is the focus of the current study.

Several factors have recently been demonstrated using organ culture studies to directly influence the primordial to primary follicle transition including kit ligand (KL), basic fibroblast growth factor (bFGF) and leukemia inhibitory factor (LIF) (Parrott and Skinner, 1999; Nilsson and Skinner, 2001a; Nilsson et al., 2002). Another factor that may influence primordial follicle

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development is insulin. Insulin receptors have been localized in the ovary (Poretsky et al., 1985; El-Roeiy et al., 1994) to the stromal cells (Poretsky et al., 1984), granulosa and theca cells of developing follicles (Hernandez et al., 1992; El-Roeiy et al., 1993). Studies that specifically examined primordial follicles demonstrated that insulin receptors are primarily localized to the oocyte and are not detected in the granulosa cells (Samoto et al., 1993). Some factors, such as KL and LIF, promote the primordial to primary follicle transition to a greater degree in the presence of insulin (Parrott and Skinner, 1999; Nilsson et al., 2002). Direct ovarian organ culture studies have demonstrated that high concentrations of insulin stimulate primordial follicle development in the hamster (Yu and Roy, 1999). Whether insulin actions are at the IGF-1 receptor and what the site of action of insulin is remains to be elucidated and is investigated in the current study.

Insulin is known to have various effects on ovarian cell types. Insulin has been shown to stimulate androgen production by cultured theca cells (Barbieri et al., 1983; Bergh et al., 1993; McGee et al., 1996), as well as estrogen and progesterone production by cultured granulosa cells (Willis et al., 1996). Whether the actions of insulin are mediated through the insulin or IGF-1 receptor remains to be elucidated. IGF-1 and its receptor belong to the insulin super-family. Both insulin and IGF-1 can stimulate each other's receptor with low affinity binding (Jones and Clemmons, 1995). In the rat, IGF-1 is produced in the granulosa cells of mature follicles (Yoshimura, 1998). IGF-1 synergizes with FSH to increase aromatase, LH receptor and progesterone production in the granulosa cells (Yoshimura, 1998). In the theca cells, IGF-1 synergizes with LH to increase steroidogenic enzymes and androgen production (Bergh et al., 1993; McGee et al., 1996; Willis et al., 1996). In mice with a null mutation in the IGF-1 gene, follicle development is normal until the late pre-antral stage where development arrests (Baker et al., 1996). Therefore, IGF-1 appears to be important in the later stages of follicular development, but not in the initial stages of follicle development associated with the primordial to primary follicle transition (Baker et al., 1996).

The current study investigates whether the previously reported effect of insulin on primordial follicle development (Yu and Roy, 1999; Nilsson et al., 2002) is due to the high concentrations of insulin used in the organ culture acting on the IGF-1 receptor or whether this is a direct effect of insulin on the insulin receptor. The site of action of insulin is also investigated. The hypothesis tested is that insulin promotes the primordial to primary follicle transition through the insulin receptor and not the IGF-1 receptor. Novel observations are presented regarding the low concentration of insulin required to promote primordial follicle development, a lack of IGF-1 action and synergism with other factors suggesting the oocyte is the site of insulin action. The significance of the observations presented in relation to diabetes and female infertility is discussed.

2. Methods

2.1. Organ cultures and histology

Postnatal 4-day-old rat ovaries were cultured for 14 days. Whole ovaries were cultured as previously described (Parrott and Skinner, 1999) on floating filters (0.4 um 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), 2.75 μg/ml transferrin and 0.05 mg/ml L-ascorbic acid (Sigma) in a 4-well culture plate (Nunc plate, Applied Scientific, South San Francisco, CA). Ovaries were randomly assigned to treatment groups with one to three ovaries per floating filter. From three to eight ovaries were examined per treatment group. Ovaries were treated with: insulin (1.25 ng–5 μg/ml) (bovine, Sigma), LIF (100 ng/ml) (R&D Systems, Minneapolis, MN), IGF-1 (R&D Systems), KL (100 ng/ml) (R&D Systems), bFGF (100 ng/ml) (R&D Systems), HGF (100 ng/ml) (R&D Systems) or EGF (100 ng/ml) (R&D Systems). Medium was supplemented with penicillin, streptomycin and gentamycin to prevent bacterial contamination. After culture, ovaries were fixed in Bouin's fixative (Sigma) for 2 h. Ovaries were then embedded in paraffin, sectioned at 4 μm and stained with hematoxylin and eosin. The follicles at each developmental stage were counted in two serial sections through the center of the ovary and averaged. Normally, in each experiment two ovaries were in each treatment group. Experiments were repeated three times. Therefore, $N = 6$ for most treatment groups. Normally 150–200 follicles were present in a cross-section and treatments did not effect total follicle numbers. For example, control ovaries had 198 ± 28 follicles per section, while 5 μg/ml insulin-treated ovaries had 173.5 ± 16. This has been true for previous studies using this system (Parrott and Skinner, 1999; Nilsson and Skinner, 2001b; Nilsson et al., 2002). Therefore, follicle apoptosis or survival is not associated with the effects observed, only changes in follicle composition or stage.

2.2. Statistics

Treatment groups in each experiment are compared to each other using a Student's t-test analysis of variance (ANOVA) (Hsu, 1996). Groups were considered significantly different if $P \leq 0.05$. Specific comparisons are outlined in Section 3 and the figures. All statistics were

calculated with the help of JMP v3.1 software (SAS Institute, Inc., Carey, NC).

3. Results

The experiments classify follicles as either primordial or as developing pre-antral follicles, as described by Parrott and Skinner (1999). Briefly, primordial follicles consist of an oocyte, partially or completely encapsulated by flattened squamous pre-granulosa cells (Fig. 1A). Follicles that have made the primordial to primary follicle transition consist of an oocyte accompanied by one or more cuboidal granulosa cells (Fig. 1B). As previously demonstrated, the total number of follicles in a section did not change with any treatment in the organ culture, only the follicle composition or stages.

The initial experiment examined insulin's effect on the primordial to primary follicle transition. Four-day-old rat ovaries were treated with different concentrations of insulin, ranging from 1.25 ng/ml to 5 μg/ml (Fig. 2). Basic-FGF has been shown to stimulate primordial follicle development and was used as a positive control. Insulin doses at or above 5 ng/ml promoted the primordial to primary follicle transition ≈30% above untreated controls. This stimulus is comparable to the positive control of bFGF treatment. Insulin doses of 2.5 ng/ml and less had negligible effects on primordial to primary follicle transition (Fig. 2). Insulin appears to stimulate the primordial to primary follicle transition at a half-maximal concentration between 2.5 and 5 ng/ml (≈3.75 ng/ml) (Fig. 2).

Previous studies have shown that high doses of insulin (1 μg/ml) have an additive effect with LIF to promote the primordial to primary follicle transition (Nilsson et al., 2002). The next experiment was designed to determine if low doses of insulin also produce this effect. Ovaries were treated with LIF in conjunction with 5 ng/ml of insulin. The 5 ng/ml dose was the lowest concentration of insulin found to affect the primordial to primary follicle transition (Fig. 2). Insulin and LIF alone each caused a 25–30% increase in the primordial to primary follicle transition. The combined treatment produced a nearly additive increase of ≈50% in the primordial to primary follicle transition (Fig. 3). In the culture system utilized, insulin appears to have an additive effect with LIF at concentrations as low as 5 ng/ml.

Due to the ability of insulin to cross-react with the IGF-1 receptor, the next experiment examined if IGF-1

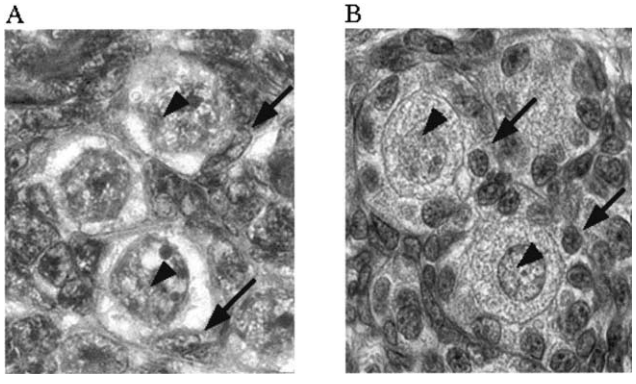

Fig. 1. (A) Histology of primordial follicles with an oocyte (arrowhead) partially or completely encapsulated by flattened (arrow) squamous pre-granulosa cells, 200× magnification. (B) Histology of primary follicles with an oocyte (arrowhead) accompanied by one or more cuboidal (arrow) granulosa cells, 200× magnification.

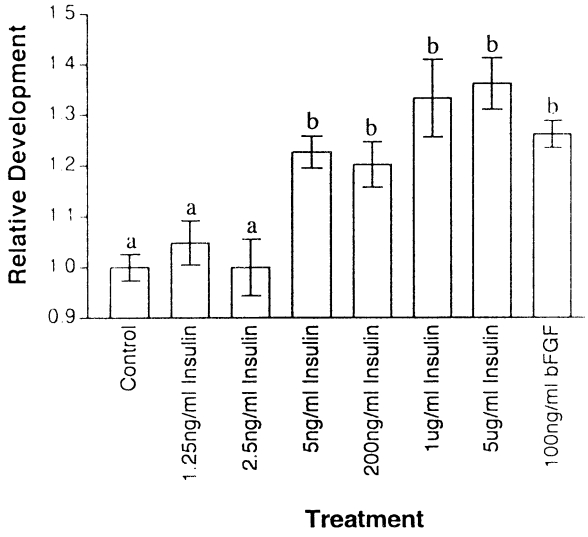

Fig. 2. Primordial to primary follicle transition in 4-day-old rat ovaries cultured in varying doses of insulin. Data displayed as relative primordial to primary follicle transition compared to control untreated cultures. Different superscript letters represent values significantly different from each other, $P < 0.001$. Data is presented as the mean ± S.E.M. from three different experiments performed in replicate.

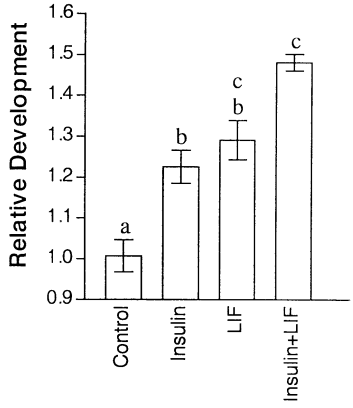

Fig. 3. Primordial to primary follicle transition in 4-day-old rat ovaries cultured with insulin (5 ng/ml) and/or LIF (100 ng/ml). Data displayed as relative primordial to primary follicle transition compared to control cultures. Different superscript letters represent values significantly different from each other, $P < 0.05$. Data is presented as the mean ± S.E.M. from three different experiments performed in replicate.

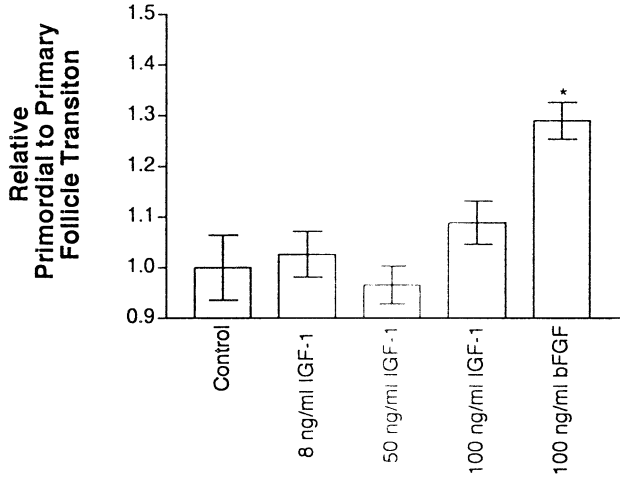

Fig. 4. Primordial to primary follicle transition of 4-day-old rat ovaries cultured in IGF-1. Basic-FGF (100 ng/ml) used as a positive control. Data displayed as relative primordial to primary follicle transition compared to control cultures. The asterisks represent a significant difference from Control, $P < 0.005$. Data is presented as the mean ± S.E.M. from three different experiments performed in replicate.

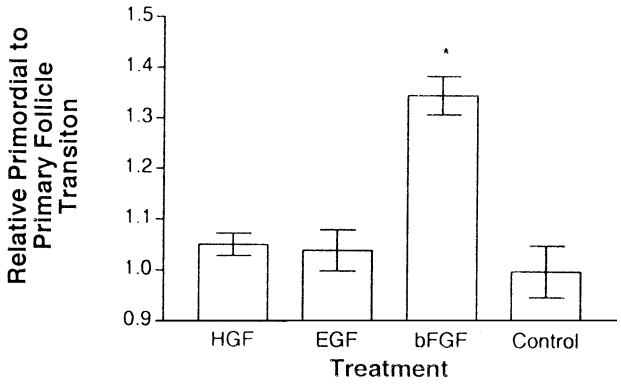

Fig. 5. Primordial to primary follicle transition of 4-day-old rat ovaries culture in the absence or presence of 100 ng/ml HGF or EGF. Basic-FGF (100 ng/ml) was used as a positive control. Data displayed as relative primordial to primary follicle transition compared to control cultures. No concentration of HGF or EGF produced a level of primordial to primary follicle transition different than control. The asterisks represent a significant difference from Control, $P < 0.002$. Data is presented as the mean ± S.E.M. from three different experiments performed in replicate.

can stimulate the primordial to primary follicle transition. Ovaries were treated with concentrations of IGF-1 ranging from 8 to 100 ng/ml (Fig. 4). Basic-FGF was used as a positive control and was found to stimulate primordial follicle development. Each dose of IGF-1 had a level of primordial to primary follicle transition that was not significantly different from the untreated control (Fig. 4). IGF-1 apparently does not affect the primordial to primary follicle transition under the experimental conditions utilized.

Epidermal growth factor (EGF) and hepatocyte growth factor (HGF) are both important in developing antral follicles. Their role in later stages of folliculogenesis make them good candidates for factors that might stimulate the primordial to primary follicle transition. Four-day-old rat ovaries were cultured with EGF and HGF. EGF and HGF had no effect on the primordial to primary follicle transition compared to control untreated organs (Fig. 5).

The final experiment was designed to determine if insulin may synergize with other factors known to stimulate the primordial to primary follicle transition. Ovaries were treated with KL or bFGF in the absence or presence of insulin (Fig. 6). Both KL and bFGF produced a 25–30% increase in the level of primordial to primary follicle transition. Insulin produced the expected 25–30% increase in the level of primordial to primary follicle transition. Ovaries treated with insulin and KL produced a >40% increase in the degree of primordial to primary follicle transition (Fig. 6). Ovaries treated with both insulin and bFGF only had the 25–30% increase in primordial to primary follicle transition, similar to that seen with either factor alone (Fig. 6). It appears that insulin can provide an additive response with KL and LIF, but not with bFGF on primordial follicle development.

4. Discussion

Culture of ovaries and ovarian fragments containing primordial follicles has provided insight into the primordial to primary follicle transition (Nilsson and Skinner, 2001b). Culture of newborn mouse ovaries in serum-containing media shows that the transition can occur spontaneously in vitro (Eppig and O'Brien, 1996). Bovine follicle fragments (Fortune et al., 2000) and rat ovaries (Parrott and Skinner, 1999; Nilsson and Skinner, 2001a) cultured in serum free media also show some spontaneous primordial to primary transition (Hirshfield, 1991; McGee et al., 1996; Nilsson et al., 2002). These studies suggest the factors that coordinate the primordial to primary transition are present in or around the primordial follicle. The primordial to primary follicle transition appears to be influenced by autocrine and/or paracrine factors. Using an organ culture system kit ligand (KL), basic fibroblast growth factor (bFGF) and leukemia inhibitory factor (LIF) have previously been shown to promote the primordial to primary follicle transition (Parrott and Skinner, 1999; Nilsson and Skinner, 2001a; Nilsson et al., 2002).

The current study demonstrates that insulin can help coordinate the primordial to primary follicle transition at a very low concentration with an EC_{50} between 2.5 and 5 ng/ml (i.e. ≈3.75 ng/ml). Insulin was found to have an additive effect with LIF and KL on primordial follicle development at these low doses. Interestingly,

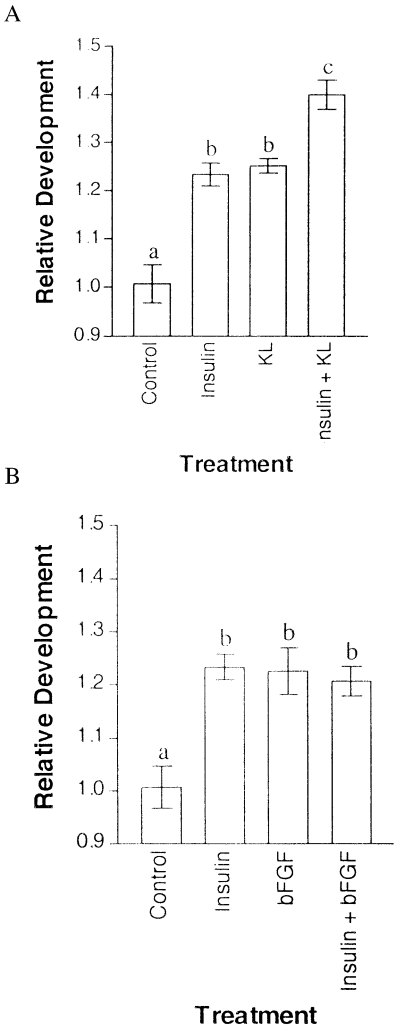

Fig. 6. Primordial to primary follicle transition of 4-day-old rat ovaries cultures with insulin (1 μg/ml) in the presence of absence of (A) KL (100 ng/ml) or (B) bFGF (100 ng/ml). Data displayed as relative primordial to primary follicle transition compared to control cultures. Different superscript letters represent values significantly different from each other, $P < 0.05$. Data is presented as the mean ± S.E.M. from three different experiments performed in replicate.

IGF-1 had no effect on the primordial to primary follicle transition at any dose tested. Due to the low doses of insulin utilized, the IGF-1 receptor does not appear to be involved as a target for insulin. These results suggest that the insulin affect is due to insulin acting on the insulin receptor. Insulin appears to act as an endocrine type hormone factor to influence the primordial to primary follicle transition.

Investigation of the combined actions of insulin and the other growth factors gave insight into insulin's site of action. Previous observations demonstrated that insulin at 1 μg/ml has an additive effect with LIF on the primordial to primary follicle transition (Nilsson et al., 2002). This previous study demonstrated insulin alone did not cause a statistically significant increase in primordial follicle development, but it did cause a trend to increase primordial follicle development. Only high concentrations of insulin were used in this previous study, which tend to have a higher degree of variability possibly due to the pharmacological dose and signal modification. The current study demonstrates that insulin has a similar additive effect with LIF at the low concentration of 5 ng/ml insulin. The current study also demonstrates that insulin has an additive effect with kit ligand. Interestingly, no evidence was found for a synergistic effect between insulin and bFGF. KL and LIF are both expressed by the granulosa cells and affect the other cell types (i.e. oocyte and developing theca cell) in the developing primordial follicle (Nilsson and Skinner, 2001a; Nilsson et al., 2002). Basic-FGF is produced by the oocyte and affects the granulosa and theca cells (Nilsson and Skinner, 2001a). In the primordial follicle, insulin receptors are primarily detectable only in the oocyte, not in the granulosa (Samoto et al., 1993). Insulin displays an additive effect with KL and LIF, but not bFGF. Therefore, the current study supports the previous observation that the insulin receptor and actions of insulin are on the oocyte by demonstrating that insulin influences the actions of KL and LIF at the oocyte, but has no effect on the actions of bFGF on the granulosa and theca cells. These observations suggest that insulin probably acts on the oocyte when it stimulates the primordial to primary follicle transition.

Previous studies have shown that insulin can influence the ovarian follicle. Insulin has been shown to stimulate androgen production in cultured theca cells (Barbieri et al., 1983; Bergh et al., 1993; McGee et al., 1996). Insulin has been shown to stimulate estrogen and progesterone production by cultured granulosa cells (Willis et al., 1996). The high concentration of insulin generally used in these experiments makes it difficult to determine if the effects are caused by insulin actions on the IGF-1 receptor. One study using antibodies to inhibit the IGF-1 receptor demonstrated that insulin's affect on the antral follicle granulosa cells is likely caused by stimulation of the insulin receptor (Willis and Franks, 1995). The current study demonstrates insulin can promote the primordial to primary follicle transition through the insulin receptor on the oocyte. Whether insulin actions are simply to regulate metabolism (e.g. glucose transport) and oocyte viability and/or to influence other oocyte cellular functions remains to be elucidated. However, the fact that insulin displays an additive effect with KL and LIF, but not bFGF, suggests a potentially more specific role for insulin in oocyte development.

Studies have shown that the IGF-1 knockout mouse has normal follicle development, including the primordial to primary transition, up to the late pre-antral stage (Baker et al., 1996). IGF-1 is intimately involved in ovarian steroidogenesis at the later stages of follicle

development (Bergh et al., 1993; McGee et al., 1996; Yoshimura, 1998). The current study demonstrates that IGF-1 has a negligible effect on the primordial to primary follicle transition.

Currently, there appear to be at least four growth factors known to stimulate the primordial to primary follicle transition (Fig. 7). These are insulin, LIF, KL and bFGF (Parrott and Skinner, 1999; Nilsson and Skinner, 2001a; Nilsson et al., 2002). Combined observations suggest a model (Fig. 7) for the coordination of the primordial to primary follicle transition. KL is produced by the pre-granulosa and acts on the oocyte and adjacent stroma to recruit theca cells (Parrott and Skinner, 1999). LIF is provided by the pre-granulosa cells to act on the oocyte (Nilsson et al., 2002). Basic FGF is produced in the oocyte and acts on the granulosa and adjacent stromal cells (Nilsson and Skinner, 2001a). Insulin is the first endocrine type factor found that stimulates primordial follicle development. It has been found to have an additive effect with KL and LIF, but not bFGF. This differential effect of insulin, along with the localization of insulin receptors to the oocyte (Samoto et al., 1993), suggests that insulin is probably acting upon the oocyte. Whether the actions of insulin influence oocyte viability and metabolism or act in a regulatory manner remains to be further investigated. The characterization of factors that coordinate the primordial to primary follicle transition provides insight into the normal physiology of follicle development. This information may be of use in the design of therapeutic approaches to manipulate and treat pathological conditions arising from inappropriate activation of the primordial to primary follicle transition (e.g. premature ovarian failure) (Santoro, 2001).

In addition to the positive stimulus for primordial follicle development discussed above, negative regulators derived from larger developing follicles also appear to be involved. Alterations in developing follicles appear to influence primordial to primary follicle transition (Hirshfield, 1991). Recently anti-Müllerian hormone (AMH) has been shown to inhibit primordial to primary follicle transition (De Vet et al., 2002; Durlinger et al., 2002). AMH is produced by granulosa cells of developing follicles, but not primordial follicles and appears to act on the primordial follicle to inhibit development. Therefore, a combination of positive stimulators, such as insulin and negative regulators, such as AMH, are required to regulate primordial follicle development.

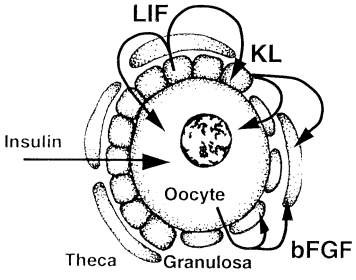

Fig. 7. Proposed cell–cell interactions involved in the primordial to primary follicle transition. Kit ligand (KL) and leukemia inhibitory factor (LIF) are produced by the granulosa and act on the oocyte or theca. Basic-fibroblast growth factor (bFGF) is produced by the oocyte and acts on the somatic cells. Insulin is an endocrine-type hormone that acts on the oocyte.

The observation that insulin acts as an endocrine factor on the insulin receptor to facilitate primordial to primary follicle transition provides new insight into potential mechanisms of female infertility associated with diabetes. Previous studies have demonstrated female infertility associated with diabetes (Selby and Oakley, 1992; Falcone, 2001). The mechanism for this infertility can be due to a central effect on the pituitary–gonadal axis or abnormal antral follicle development, such as polycystic ovarian disease (Griffin et al., 1994). The potential that abnormal insulin levels may impair or alter early follicle development associated with the primordial to primary follicle transition has not been previously considered. Observations presented in the current study suggest that abnormally low insulin levels could inhibit or retard primordial follicle development. Further studies are now required to determine if abnormal primordial follicle development may, in part, be a causal factor in female infertility associated with diabetes.

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