Platelet-derived growth factor modulates the primordial to primary follicle transition

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Abstract

Primordial follicles steadily leave the arrested pool and undergo a primordial to primary follicle transition during the female reproductive lifespan. When the available pool of primordial follicles is depleted reproduction ceases and humans enter menopause. The present study was designed to investigate the actions of several growth factors previously identified as candidate regulatory factors for the primordial to primary follicle transition with a microarray analysis. Ovaries from 4-day-old rats were placed into culture and treated for 2 weeks with platelet-derived growth factor (PDGF), anti-PDGF neutralizing antibody, vascular endothelial growth factor (VEGF), neuregulin (NRG), or kit ligand (KITL) as a positive control. PDGF-treatment resulted in a significant decrease in the percentage of primordial follicles and a concomitant increase in the percentage of developing primary follicles compared to controls. In contrast, ovaries treated with an anti-PDGF neutralizing antibody had a significant increase in the percentage of primordial follicles demonstrating an inhibition of endogenous follicle development. Ovaries incubated in the presence of VEGF or NRG had no change in follicle development. Observations indicate that PDGF, but not VEGF or NRG, promotes the primordial to primary follicle transition. Immunohistochemical localization indicated that the PDGF protein was present in the oocytes of both primordial and developing follicles. PDGF-treatment of cultured ovaries resulted in an increase in KITL mRNA expression. KITL has been previously shown to promote the primordial to primary follicle transition. KITL-treatment of ovaries had no effect on expression of *Pdgf* or any PDGF homologs or receptors. Therefore, PDGF appears to be produced by the oocyte and acts as one of several extracellular signaling factors that regulate the primordial to primary follicle transition. These observations provide insight into the cell–cell interactions involved in the regulation of primordial follicle development and can be used in the future development of therapies for some forms of infertility.
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Introduction

In mammals most of the oocytes in an ovary are arrested in the first meiotic division and contained in primordial follicles. A primordial follicle is composed of an oocyte arrested in the diplotene stage of prophase I of meiosis and surrounded by a single layer of squamous pre-granulosa cells. The pool of available primordial follicles is formed in the neonatal period and has been considered to be finite in number (Hirshfield 1991, Kezele *et al.* 2002*a*). Recent evidence raises the possibility that new follicles with oocytes may form in adulthood (Johnson *et al.* 2004, 2005). Over the course of a female reproductive lifespan, primordial follicles steadily leave the arrested pool and undergo a primordial to primary follicle transition in which the surrounding pre-granulosa cells become cuboidal and begin to proliferate (Peters *et al.* 1975, Cran & Moor 1980,

Hirshfield 1991, Rajah *et al.* 1992). These developing follicles gain successive layers of granulosa cells and theca cells, while the oocyte increases in size. Eventually, a fluid-filled antrum is formed. While many developing follicles undergo atresia and apoptosis, some continue to develop until they ovulate. When the available pool of primordial follicles is depleted, reproduction ceases and humans enter menopause (Gosden *et al.* 1983, Richardson *et al.* 1987, Faddy *et al.* 1992, Faddy 2000).

Several paracrine growth factors have been shown to act locally within the ovary to regulate the primordial to primary follicle transition. Bone morphogenetic protein 15 (BMP15), basic fibroblast growth factor (bFGF/FGF2), the neurotrophins NT4 and bone-derived neurotropic factor (BDNF), the c-kit receptor for kit ligand (KITL) and the Trk-B receptor for NT4 and BDNF are all present in the oocytes of primordial follicles. All these growth factors

and receptors have been implicated in promoting the primordial to primary follicle transition (Manova *et al.* 1990, Horie *et al.* 1991, van Wezel *et al.* 1995, Dube *et al.* 1998, Laitinen *et al.* 1998, Parrott & Skinner 1999, Nilsson *et al.* 2001, Paredes *et al.* 2004). Several growth factors found to be present in the pre-granulosa cells surrounding primordial follicles or in granulosa cells were also found to promote the primordial to primary follicle transition including KITL and leukemia inhibitory factor (LIF) (Manova *et al.* 1993, Motro & Bernstein 1993, van Wezel *et al.* 1995, Yamamoto *et al.* 1997, Parrott & Skinner 1999, Nilsson *et al.* 2001). The thecal/interstitial cells surrounding follicles express bone morphogenetic protein 4 (BMP4) and BMP7, both of which promote the primordial to primary follicle transition. BMP4 has also been shown to be important for follicle survival (Lee *et al.* 2001, Nilsson & Skinner 2003). Anti-Müllerian hormone/Müllerian inhibitory substance (AMH/MIS) is a growth factor produced by the granulosa cells of developing pre-antral and antral follicles which inhibits the primordial to primary follicle transition (Baarends *et al.* 1995, Durlinger *et al.* 1999, 2002).

Platelet-derived growth factor (PDGF), neuregulin (NRG) and vascular endothelial growth factor (VEGF) were all identified as candidate signaling factors to regulate the primordial to primary follicle transition with a microarray analysis previously performed (Kezele *et al.* 2005*b*). These factors change mRNA expression levels during primordial to primary follicle transition, suggesting that they may regulate early follicle development (Kezele *et al.* 2005*b*). The present study was designed to investigate the potential regulatory roles of PDGF, VEGF and NRG in primordial to primary follicle transition.

The most prevalent isoforms of PDGF are the PDGF-AA, PDGF-BB homodimers, and PDGF-AB heterodimers. PDGF-AA, PDGF-AB and PDGF-BB bind to the PDGF receptor-alpha (PDGFRα), while the PDGFRβ binds primarily PDGF-BB. After binding and activation of the monomeric α and β receptors, these receptors dimerize and activate various intracellular signaling kinase cascades (Fredriksson *et al.* 2004, Tallquist & Kazlauskas 2004). PDGF receptors have been shown to be present in the theca and stroma compartments of porcine ovaries (Taylor 2000). PDGF has been demonstrated to affect the proliferation or function of both theca cells (Duleba *et al.* 1999, Shores & Hunter 2000, Taylor 2000) and granulosa cells (Hammond *et al.* 1985, Anderson & Lee 1993, Lafrance *et al.* 1993), as well as ovarian surface epithelium (Dabrow *et al.* 1998). No studies have been reported that examine any effect PDGF may have on the early stages of follicle development.

NRGs are the protein products of a family of related genes. The NRG1 gene is the one for which the most biological functions are known. The NRG1 gene produces several alternatively spliced proteins, some of which are transmembrane proteins with an extracellular

domain. This extracellular domain can be cleaved off to release paracrine signaling molecules (Falls 2003). NRGs bind to the Erbβ receptors, with NRG1 binding to Erbβ2, Erbβ3 and Erbβ4 (Falls 2003). NRG1-β is involved in colonization of the genital ridge with primordial germ cells (Kierszenbaum & Tres 2001), while NRG1-α and NRG1-β are both implicated in growth of ovarian cancer cells.

VEGF is a well-characterized growth factor that binds to receptors VEGFR1 and VEGFR2. VEGF is best known as a stimulator of angiogenesis. It is in this role that VEGF has been shown to act in the ovary by stimulating vascular development in the theca layer of pre-antral and antral follicles and stimulating follicle growth (Zimmermann *et al.* 2003, Hunter *et al.* 2004, Iijima *et al.* 2005).

The objective of the present study was to investigate what role PDGF, NRG and VEGF may play in the primordial to primary follicle transition. The effect of these growth factors on the primordial follicle transition was examined using a rat ovary organ culture system. The ability of the growth factors to regulate expression of KITL, a known stimulator of primordial follicle development (Parrott & Skinner 1999), was also examined. A better understanding of the factors that regulate the primordial to primary follicle transition can lead to treatments for some infertilities such as premature ovarian failure.

Materials and Methods

Organ culture and treatments

Postnatal 4-day-old rat ovaries were dissected from freshly euthanized rat pups. All animal protocols were approved by the Washington State University Animal Care and Use Committee. Whole ovaries were cultured as previously described (Nilsson *et al.* 2001) on floating filters (0.4 μm Millicell-CM, Millipore, Bedford, MD, USA) in 0.5 ml Dulbecco's Modified Eagle's Medium-Ham's F-12 medium (1:1, vol/vol) containing 0.1% BSA (Sigma), 0.1% Albumax (Gibco BRL), 27.5 μg/ml transferrin, 200 ng/ml insulin (human recombinant, Sigma), and 0.05 mg/ml ʟ-ascorbic acid (Sigma) in a 4-well culture plate (Nunc Plate, Applied Scientific; South San Francisco, CA, USA). A culture experiment consisted of several wells in a culture plate with each well receiving a separate treatment. Two or three ovaries from different rats were placed on floating filters into each well and cultured. Since each ovary receiving a particular treatment is genetically unique and can respond independently to that treatment, then each ovary is considered an experimental unit ($n=1$). Treatments during organ culture included recombinant rat PDGF-AB heterodimer (R&D Systems, Inc.; Minneapolis, MN, USA) at 50 ng/ml, rat KITL (Amgen; Thousand Oaks, CA, USA) at 50 ng/ml, anti-PDGF neutralizing antibody (R&D Systems) at 20 μg/ml,

human NRG1-β1 extracellular domain (R&D Systems) at 50 ng/ml, human NRG1-β1 EGF domain (R&D Systems) at 50 ng/ml, or rat VEGF (R&D Systems) at 50 ng/ml. Preliminary experiments testing the effects of NRG1-β1 EGF domain and VEGF at 250 ng/ml in culture demonstrated no difference than experiments performed at 50 ng/ml (data not shown). This suggest that 50 ng/ml is at or above the maximum effective dose for these growth factors. Medium was supplemented with penicillin and streptomycin to prevent bacterial contamination. After culture, ovaries were fixed, sectioned and stained with hematoxylin and eosin (H&E) for use in morphological analysis. Alternatively, if mRNA levels were to be measured from cultured ovaries, after culture ovaries from one treatment group were pooled and homogenized in 1 ml Trizol (Gibco BRL) and stored at −20 °C until RNA isolation.

Morphological evaluation

Four-day-old rat ovaries were fixed fresh or 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-paraffinized in xylenes and hydrated through an ethanol series. Sections were stained with hematoxylin and eosin using standard protocols. The number of follicles at each developmental stage was counted in two serial sections and averaged from the largest cross-sections through the center of the ovary. The oocyte nucleus had to be visible in a follicle in order for the follicle to be counted. Normally, 100–200 follicles were present in a cross-section. Previously, it has been demonstrated that the total follicle number per section does not change after 2 weeks of culture compared to freshly isolated 4-day-old ovaries (Parrott & Skinner 1999). Follicles were classified as either primordial (stage 0), or as one of the developing preantral stages (stages 1–4) as described previously (Parrott & Skinner 1999). Briefly, primordial follicles consist of an oocyte partially or completely encapsulated by squamous pre-granulosa cells. Developing (stages 1–4) follicles contain successively more cuboidal granulosa cells in layers around the oocyte (Parrott & Skinner 1999, Nilsson *et al.* 2001). The results of follicle counting are presented as percentages (%primordial vs %developing) to account for differences between individual mice in total number of oocytes per section.

Immunohistochemistry

Localization of PDGF protein was determined by immunohistochemical analysis. Rat ovaries were fixed and prepared for immunostaining as for H&E staining as described above. Antigens were exposed by boiling sections for 5 min in 0.01 M sodium citrate buffer at pH 6.0. A solution of 10% goat serum in PBS was used as a blocking agent prior to incubating sections with primary antibody overnight at 4 °C. Slides were incubated with polyclonal rabbit anti-human PDGF antibody (Santa Cruz Biotechnology, Inc.; Santa Cruz, CA) at 10 μg/ml overnight at 4 °C. Secondary antibody (biotinylated goat anti-rabbit IgG, Vector, Burlingame, CA, USA) was detected by using the Vectastain kit (Vector) and diaminobenzadine (Vector). Negative controls were incubated in the presence of non-immune rabbit IgG as a primary antibody at 10 μg/ml.

Real-time PCR

Ovaries were isolated from 4-day-old rats and placed into culture as described above. Cultured ovaries were treated for 2 days with recombinant rat PDGF-AB heterodimer (R&D Systems) at 50 ng/ml, or rat KITL (Amgen) at 50 ng/ml, or were left untreated as controls. Three ovaries from the same culture well were pooled to make each RNA sample. RNA was extracted using the Trizol reagent (Sigma). RNA samples were DNAse treated using the TURBO-DNA-free kit (Ambion, Austin, TX). Two micrograms total RNA from each sample was reverse transcribed to cDNA using a standard oligo-dT RT protocol in a reaction volume of 25 μl. cDNA samples were diluted 1:10 and 2 μl of diluted sample/well was used as template for real-time PCR analysis. Each sample was run in triplicate. The Platinum SYBR Green qPCR Supermix kit (Invitrogen) was used according to manufacturers instructions. The KITL primers were rKL-720, 5′ATTTATGTTACCCCCTGTT-GCAGCC3′ and rKL-859, 5′CAATTACAAGCGAAATGA-GAGCCG3′. The ribosomal protein gene S2 was used as a references standard for real-time PCR. The S2 reference gene primers were rS2-F, 5′CTGCTCCTGTGCCCAA-GAAG3′ and rS2-R, 5′AAGGTGGCCTTGGCAA-AGTT3′. Ribosomal S2 mRNA expression does not change in ovarian cells treated with hormones (Kezele *et al.* 2005*a*). Real-time PCR was performed on an ABI-7000 real-time machine with the following protocol: 60 °C 2 min, 95 °C 10 min, then 40 cycles of 95 °C 20 s and 68 °C 90 s. Fluorescent detection data were analyzed such that KITL product levels were normalized to S2 gene levels, and then PDGF- and KITL-treated sample KITL levels were normalized to untreated control KITL levels.

Microarray analysis

RNA was hybridized to the Affymetrix (Affymetrix; Santa Clara, CA) rat 230 2.0 gene chip. The Genomics Core in the Center for Reproductive Biology at Washington State University performed the analysis as previously described (McLean *et al.* 2002, Shima *et al.* 2004). Briefly, RNA from control and KITL-treated (50 ng/ml

for 2 days as described above) whole cultured ovaries was reverse transcribed into cDNA and cDNA was transcribed into biotin labeled RNA. Biotin labeled RNA was then hybridized to the Affymetrix 230 2.3 gene chips. Each gene set is composed of 16 pairs of 24-mer oligonucleotides, with one sense strand specific for the gene and one anti-sense strand with single point mutations for use as comparative negative control. The oligonucleotides span the gene so 5′ and 3′ regions are contributing to the final signal obtained. Biotinylated RNA was then visualized by labeling with phycoerythrin-coupled avidin. The microarray was scanned on a Hewlett-Packard Gene Array Scanner (Hewlett-Packard Co.; Palo Alto, CA, USA). Two microarray chips from two different RNA samples were analyzed for each of the control and KITL-treated ovary preparations.

Bioinformatics

Microarray output was examined visually for excessive background noise and physical anomalies. GCOS software (Affymetrix) was used for analyses. An absolute analysis using GCOS was performed to assess the relative abundance of the transcripts based on signal and detection (present, absent, or marginal) for the 16 different oligonucleotides per gene and comparison for analysis. The absolute analysis from GCOS was imported into GeneSpring 7.0 software (Silicon Genetics; Redwood City, CA). The data were normalized within GeneSpring using the default/recommended normalization methods. These include setting of signal values below 0.01–0.01, total chip normalization to the 50th percentile, and normalization of each gene to the median. These normalizations allowed for the visualization of data based on relative abundance at any given time point rather than compared to a specific control value. Means of the raw signal values for control or KITL-treated replicate gene arrays were used to determine expression or change in expression of mRNA for selected genes. Previous studies have shown that microarray data correlate well with real-time quantitative PCR and Northern analysis (McLean *et al.* 2002, Sadate-Ngatchou *et al.* 2004).

Statistics

Pair comparisons were performed using Student's *t*-test. Multiple comparison tests were performed using Dunnet's analysis after a significant difference had been found with ANOVA. Dunnet's test compares each treatment group to the designated control group. In the case of real-time PCR data, in which all values are normalized so that controls are equal to 1, a one-sample *t*-test was performed to test if the means differ significantly from 1.0. Groups were considered significantly different if $P \leq 0.05$. All statistics were

calculated with the help of GraphPad Prism version 3.0a for Macintosh, GraphPad Software (San Diego, CA, USA).

Results

Ovaries of 4-day-old rats contain predominately primordial follicles. In order to determine the effects of growth factors on the primordial to primary follicle transition, ovaries from 4-day-old rats were placed into culture and treated with PDGF (50 ng/ml), anti-PDGF neutralizing antibody (20 µg/ml), KITL (50 ng/ml), NRG1-β1 (50 ng/ml), VEGF (ng/ml) or were left untreated as negative controls. After 2 weeks, the ovaries were fixed, sectioned, and stained for morphological evaluation.

The total number of combined primordial and developing follicles per ovary cross-section was 151 ± 10 for controls, 116 ± 9 for PDGF-treated ovaries, 130 ± 13 for anti-PDGF treated ovaries, and 114 ± 2 for KITL-treated ovaries. Approximately, 1500 follicles per treatment were counted for this study. These total ovary counts are not significantly different (using an ANOVA analysis) between treatment groups, indicating that no treatments result in a loss or gain in follicles compared to controls. These results are similar to those found by Parrott & Skinner (1999). Treatments can result in a change in the relative proportion of primordial and developing follicles. PDGF-treatment resulted in a significant ($P < 0.05$) increase in the percentage of developing follicles compared to the percentage of developing follicles in controls (Fig. 1). This is necessarily accompanied by a concomitant decrease in the percentage of primordial follicles compared to that of controls. Observations indicate an increase in the rate of primordial to primary follicle transition. Conversely, ovaries treated with anti-PDGF neutralizing antibody were found to have a significant ($P < 0.05$) decrease in primary follicles indicating a decrease in primordial to primary follicle transition compared to controls. KITL is known to stimulate primordial to primary follicle transition (Parrott & Skinner 1999) and was used as a positive control for the organ culture system. KITL-treatment resulted in a significant ($P < 0.05$) increase in the proportion of primary follicles indicating an increase in primordial to primary follicle transition (Fig. 1).

The actions of NRG and VEGF were also evaluated using the ovarian organ culture procedure. Ovaries from 4-day-old rats were placed into culture and treated with NRG1-β1 (50 ng/ml), VEGF (50 ng/ml) or were left untreated as controls. Neither NRG1-β1 nor VEGF were shown to promote the primordial to primary follicle transition (Fig. 2).

An immunohistochemical procedure was performed to localize PDGF protein in ovaries. Cultured 4-day-old ovaries have primordial follicles as well as a variety of developing pre-antral follicles. Anti-PDGF antibody (Santa Cruz Biotechnology, Inc.) was used to localize PDGF in both freshly isolated 4-day-old rat ovaries
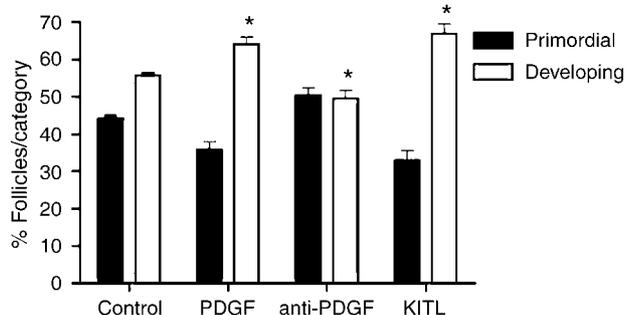
Figure 1 Effect of PDGF-treatment on primordial to primary follicle transition in cultured ovaries. Ovaries from 4-day-old rats were placed into culture for 14 days. Cultured ovaries were treated with PDGF, anti-PDGF neutralizing antibody, KITL or were left untreated as controls. After culture all ovaries 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). Data are presented as the mean percentage ± s.e.m. with data pooled from four separate experiments ($n=6–12$ ovaries per treatment group). One-way ANOVA showed a significant ($P<0.0001$) effect of treatment. Asterisks indicate that the percentage of developing follicles for a treatment is significantly ($P<0.05$) different than that of the control by post-hoc Dunnet's test.

and 4-day-old rat ovaries after culture for 14 days. Immunohistochemical localization indicated that PDGF protein was present in the oocytes of both primordial and developing follicles (Fig. 3). From examination of staining intensity, it is possible that PDGF protein expression is higher in the oocytes of developing rather than primordial follicles. However, one cannot exclude the possibility that differences in fixation or handling of ovaries resulted in these staining differences. There was negligible staining in the surrounding somatic cells (Fig. 3).
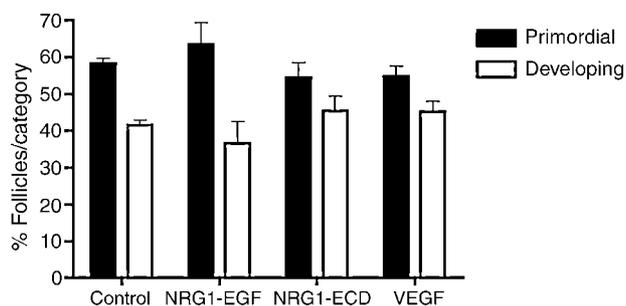

Figure 2 Lack of effect of NRG1 or VEGF-treatment on primordial to primary follicle transition in cultured ovaries. Ovaries from 4-day-old rats were placed into culture for 14 days. Cultured ovaries were treated with NRG1-EGF, NRG1-ECD, VEGF or were left untreated as controls. After culture all ovaries 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. Data are presented as the mean percentage ± s.e.m. with data pooled from three separate experiments ($n=3–5$ ovaries per treatment group). One-way ANOVA showed no significant ($P>0.05$) effect of treatment.

Four-day-old rat ovaries were cultured for 2, 5, 10 or 14 days in the presence or absence of KITL (50 ng/ml) in order to determine the appropriate length of culture prior to collecting tissue for morphological analysis vs collecting RNA for gene expression analysis. After 2, 5, 10 and 14 days KITL-treated and control ovaries were fixed, stained and subjected to morphological analysis. Results indicated that no statistically significant morphological difference in follicle transition was apparent between treated and control ovaries at 2 or 5 days, but that at 10 and 14 days of culture KITL-treated ovaries had an increased percentage of developing follicles compared to controls (Fig. 4). These results suggest that mRNA collected from ovaries after 2 days of treatment with growth factors would reflect changes in gene expression due to the treatment, without reflecting changes in gene expression due to a different population of follicles and differentiated cells.

RNA was isolated from ovaries cultured for 2 days with or without PDGF-treatment (50 ng/ml). Real-time PCR analysis of KITL expression revealed that KITL mRNA was modestly, but significantly ($P<0.05$), increased by PDGF-treatment (Fig. 5). RNA was also isolated from ovaries cultured for 2 days with or without KITL-treatment (50 ng/ml) and that RNA used for microarray analysis using the Affymetrix 230 2.0 rat chip. Selected genes related to PDGF and PDGF receptors were analyzed. No change in mRNA levels were seen between control and KITL-treated ovaries for PDGFa, PDGFb, PDGFc, PDGFd, PDGF receptor alpha (PDGFRa), PDGF receptor beta (PDGFRb), or PDGFa-associated protein 1 (Table 1). Therefore, PDGF was found to stimulate expression of KITL mRNA, but KITL had no effect on PDGF or PDGF receptor expression. KITL-treatment had no effect on expression of NRG1, the NRG receptor signaling pathway protein Tob2, VEGFa isoform or VEGF receptor (Table 1). KITL-treatment did stimulate the expression of mRNA for the NRG receptor signaling pathway protein Tob1 and the VEGFc isoform (Table 1). As previously reported, the microarray data have been validated with an alternate procedure involving quantitative PCR analysis (Kezele et al. 2005b).

Discussion

Rat ovaries were cultured in the presence of PDGF or neutralizing antibody to PDGF. Morphological evaluation of the treated ovaries indicates that PDGF induces a moderate but significant increase in primordial to primary follicle transition. Conversely, treatment with an antibody that neutralizes endogenous levels of PDGF in the ovary results in a significant decrease in primordial follicle transition. These data suggest that ovarian expression of PDGF can regulate the rate at which primordial follicles leave the resting pool and begin developing. Immunohistochemical analysis indicates that PDGF protein is present in oocytes of primordial
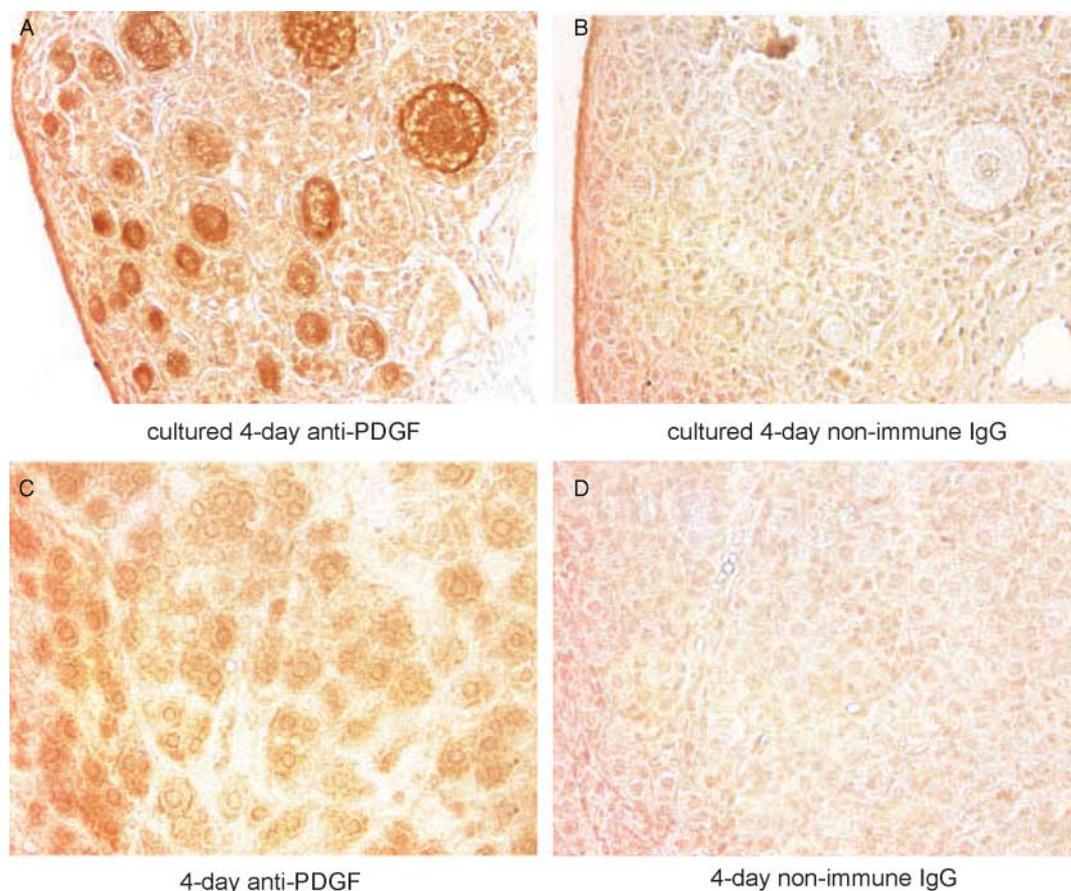
Figure 3 Immunohistochemical localization of PDGF protein in cultured ovaries. The presence of PDGF protein is indicated by a dark brown stain in ovary sections. A) Four-day-old ovaries cultured for 14 days showing PDGF staining at high intensity in oocytes. B) Control cultured ovary section stained using non-immune IgG as a primary antibody. C) Freshly isolated 4-day-old ovaries showing PDGF staining in the oocytes of primordial follicles. D) Control freshly isolated ovary section stained using non-immune IgG as a primary antibody. Images are representative of two different experiments using ovaries from different rats. Microscope magnification=400×. Total microscope magnification at the time of image capture was 400×.

and early developing follicles. PDGF receptor localization and action have previously been shown in the somatic granulosa and theca cell populations (Hammond *et al.* 1985, Anderson & Lee 1993, Lafrance *et al.* 1993, Duleba *et al.* 1999, Shores & Hunter 2000, Taylor 2000). PDGF is similar to bFGF/FGF2 in that both paracrine growth factors are expressed by the oocyte and promote the primordial to primary follicle transition (van Wezel *et al.* 1995, Nilsson *et al.* 2001) (Fig. 6). It is not surprising that a process so vital to reproduction is regulated by multiple factors and may feature redundancy.

Treatment of cultured ovaries with PDGF resulted in an increase in KITL expression. This suggests that one mechanism by which PDGF promotes primordial to primary follicle transition is by stimulating KITL mRNA expression, which itself promotes primordial follicle transition (Parrott & Skinner 1999). Previous investigations have also demonstrated that PDGF can increase expression of KITL in mast cells (Hiragun *et al.* 1998). In addition, in cultured rat ovaries it has been shown that bFGF treatment, which is known to promote primordial
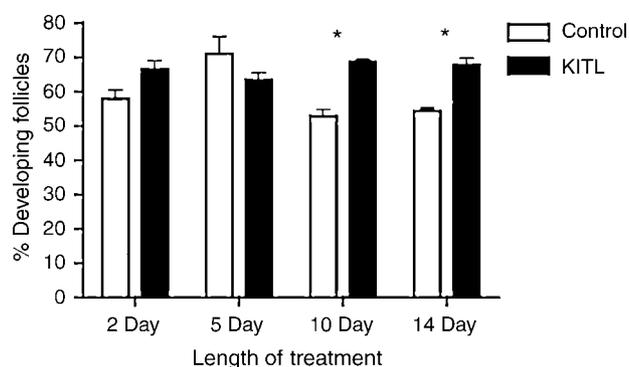

Figure 4 Effect of KITL-treatment over time on primordial to primary follicle transition in cultured ovaries. Ovaries from 4-day-old rats were placed into culture for 2, 5, 10, or 14 days in the absence or presence of KITL. Following culture all ovaries were fixed, stained and subjected to morphological analysis. The follicles per ovary cross-section were categorized as either primordial or developing. Data are presented as the mean percentage±s.e.m. with data pooled from three different experiments (*n*=3–6 ovaries per treatment group). Asterisks indicate that a KITL-treated group is significantly (*P*<0.05) different from its control at that time point by Student's *t*-test.
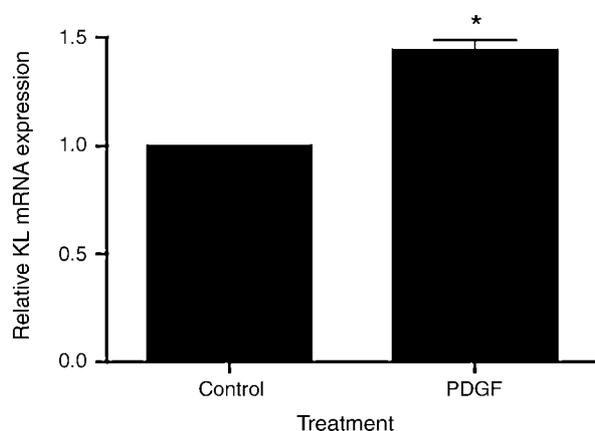
Figure 5 Kit ligand (KITL) mRNA expression in cultured ovaries in response to PDGF-treatment. Ovaries from 4-day-old rats were placed into culture and treated with PDGF or were not treated for 2 days. Real-time PCR was performed on RNA isolated from cultured whole ovaries to determine levels of KITL mRNA expression. Data are expressed as KITL mRNA/S2 mRNA normalized to untreated control values. Data are from three separate experiments with the mean±S.E.M. presented. Asterisk indicates a significant difference ($P<0.05$) from control as determined by a one-sample t-test.

follicle transition, will also increase KITL mRNA expression (Nilsson *et al.* 2001, Nilsson & Skinner 2004). This suggests that one stimulatory growth factor inducing expression of another stimulatory paracrine growth factor may be a common mechanism for regulating cell growth and differentiation in the local follicle environment (Fig. 6).

Neither NRG1 nor VEGF were shown to promote primordial to primary follicle transition, even though mRNA expression of these genes has been shown to change during follicle transition (Kezele *et al.* 2005*b*).

This suggests that these growth factors are either involved in some signaling process other than promoting primordial follicle transition, or that they require as yet unknown cofactors to affect follicle transition. VEGF protein expression has been demonstrated in developing primary, but not primordial follicle oocytes (Celik-Ozenci *et al.* 2003). Further investigations will be needed to determine the role these growth factors play in early follicle development.

The results from the present experiments indicate that PDGF can join the growing list of extracellular signaling factors that regulate the primordial to primary follicle transition. The actions of some of these factors are summarized in Fig. 6. KITL from granulosa cells acts on the oocyte and also upon the surrounding stroma to recruit theca cells (Parrott & Skinner 1999, Parrott & Skinner 2000). Theca/interstitial cells produce BMP4, which acts as a follicle survival factor (Nilsson & Skinner 2003). KGF, also from the theca, acts on granulosa cells to promote KITL expression and follicle development (Kezele *et al.* 2005*a*). Granulosa cells produce LIF that acts on the oocyte as well as on other granulosa cells (Nilsson *et al.* 2002). Insulin acts in an endocrine manner on the oocyte (Kezele *et al.* 2002*b*). AMH/MIS from larger growing follicles acts to inhibit the primordial to primary follicle transition (Baarends *et al.* 1995, Durlinger *et al.* 1999, Durlinger *et al.* 2001). The oocyte produces bFGF that acts upon the granulosa and theca cells (Nilsson *et al.* 2001). Similar to PDGF, bFGF also stimulates KITL expression to facilitate primordial follicle development (Nilsson & Skinner 2004). In this study, it is proposed that the oocyte produces PDGF, which acts upon surrounding granulosa and theca/interstitial cells to promote primordial follicle transition (Fig. 6). In summary, a network of compensatory cell–cell

Table 1 Microarray gene expression data for selected genes from duplicate RNA samples isolated from 4-day-old rat ovaries cultured for 2 days in the absence (control) or presence of 50 ng/ml kit ligand (KITL). Data are expressed as the mean±S.E.M. of the scaled raw signal values, along with the NCBI Genbank accession number for each gene. Asterisk (−) indicates a significant ($P<0.05$) mRNA expression difference between KITL-treated and control samples.

|	Control		KL		
Common name	Mean	±S.E.M.	Mean	±S.E.M.	Genbank no.
PDGFa	99	11	87	10	L06238
PDGFa	128	10	150	14	BE100812
PDGFa	170	19	221	50	AA866419
PDGFRa	1187	74	1135	114	AI232379
PDGFRb	105	20	110	12	AI071374
PDGFRb-like	67	7	81	22	BM384311
PDGFa-associated protein	1078	63	1076	106	NM_022595
Neuregulin 1	282	37	235	25	U02315
Transducer of ERBB2 1 (Tob1) (NRG receptor)	256	20	318*	43	NM_133317
Transducer of ERBB2 2 (Tob2) (NRG receptor)	98	8	112	11	BI275222
VEGFa	44	14	51	7	AF080594
VEGFc	117	10	151*	14	NM_053653
VEGF receptor 2 (KDR)	463	28	482	46	U93307
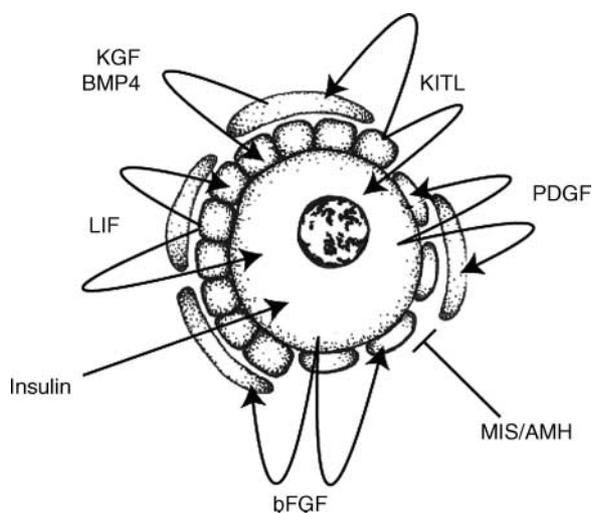
Figure 6 A model of the regulation of the primordial to primary follicle transition by paracrine growth factors, including the actions of platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), leukemia inhibitory factor (LIF), bone morphogenetic protein-4 (BMP4), keratinocyte growth factor (KGF), Anti-Müllerian Hormone/Müllerian inhibitory substance (AMH/MIS), and insulin.

interactions involving multiple growth factors is required to modulate the rate of primordial follicle transition and subsequent follicular growth. A more complete understanding of the molecular and cellular control of primordial follicle development will provide insights into potential new therapeutic targets to treat certain types of infertility or to control the transition to menopause in women.

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