

**Glial Derived Neurotrophic Factor Promotes Ovarian
Primordial Follicle Development and Cell-Cell Interactions
During Folliculogenesis**

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TO THE UNIVERSITY HONORS COLLEGE:

As thesis advisor for GRETCHEN DOLE,

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PRÉCIS

Mammalian egg cells are contained in ovaries in structures called follicles. Female fertility is limited by the rate of early ovarian follicle development. One of the steps necessary for follicles to develop and become candidates for ovulation is the primordial to primary follicle transition. Primordial follicles are formed during fetal development, and are composed of an egg (oocyte) surrounded by supporting cells (granulosa cells). Once follicles are formed the eggs contained within permanently cease to divide, and therefore the original population of primordial follicles composes the entire complement of follicles a female will have for her entire lifetime. Primordial follicles are arrested in development, and have the potential to remain in a quiescent state throughout adult life. During the primordial to primary follicle transition, primordial follicles initiate development. They will either develop fully to maturation and ovulation, or undergo normal follicle death. Once all of the primordial follicles have left their arrested state and met one of these two fates, menopause occurs. Therefore, it is the rate at which primordial follicles leave their arrested state and initiate development that determines a female's fertility, and also at what age menopause will occur.

A network of extracellular signaling factors regulates the primordial to primary follicle transition. These secreted proteins allow communication between the major ovarian cell types – oocytes (germ cells) and granulosa cells (somatic cells). The known protein network includes six stimulatory and two inhibitory factors, however these represent only part of the hypothesized signaling system. Elucidation of the processes regulating primordial follicle development is essential in designing therapies for reproductive diseases as well as for the manipulation of menopause.

For many women, the years leading up to menopause can include hot flashes and irregular periods, and therefore can become very uncomfortable. Once a woman enters this pre-menopausal phase, it is possible that physicians could medically speed up the development of her remaining follicles in order to shorten this pre-menopausal phase.

Similar technology may also be of use to women in which development is proceeding too quickly. The normal onset of menopause is after age 50, however approximately 1 in 100 women enter menopause before they are 40 and even at an age as early as 20. These women have “premature ovarian failure”, and have reduced fertility and increased risk of developing osteoporosis as a result. For women who are at risk for premature ovarian failure, follicle development could be slowed with therapies in order to preserve their fertility and bones for a longer period of time.

Finally, manipulation of early follicle development could be used for women who are at risk for developing breast and ovarian cancer. Women who carry mutant BRCA1 and BRCA2 genes have significantly higher risks of developing breast and ovarian cancer, and their risk increases with each reproductive cycle. For this reason, many BRCA carriers choose to have their ovaries removed surgically in order to induce menopause. The ability to medically induce menopause in these women would achieve the same end while avoiding the risks of invasive surgery.

Because of these potential applications, the goal of the current study was to further characterize the signaling pathways that regulate normal follicle development. The compound under investigation is glial derived neurotrophic factor (GDNF). GDNF is primarily involved in neuron survival and repair, however, GDNF and its receptors are also expressed in the ovary. The current research was designed to determine whether glial derived neurotrophic factor

(GDNF) participates in the network of cell-cell signaling interactions that regulates primordial follicle development.

To characterize the role of GDNF in the developing ovary, a variety of methods were utilized. Organ culture studies demonstrated the direct impact of GDNF treatment on follicle development. Immunohistochemistry and reverse transcription – polymerase chain reaction (RT-PCR) were used to determine which ovarian cell types - oocyte or granulosa - express GDNF and its receptor at all stages of development. Also, real-time PCR and microarray analysis were utilized to establish how GDNF affects the expression levels of other proteins in the signaling network regulating follicle development.

Ovaries treated with GDNF contained a significant increase in the proportion of developing follicles. Immunohistochemical studies demonstrated that GDNF is localized to oocytes (germ cells) in follicles of all developmental stages, as well as to granulosa cells (somatic cells) in late-stage follicles. GDNF receptor alpha 1 ($GFR\alpha 1$) staining was localized to oocytes of primordial and primary follicles, and at reduced levels in oocytes of late-stage follicles. $GFR\alpha 1$ was also present in many somatic cell types in the adult ovary. The localization studies were confirmed with RT-PCR. Microarray analysis was used to identify changes in gene expression following GDNF treatment and further explore the signaling network regulating early follicle development.

Observations indicate that GDNF promotes primordial follicle development and mediates cell-cell interactions required during follicle development. Further research involves *in vivo* studies to determine if manipulation of follicle development in living animals is possible.

TABLE OF CONTENTS

List of Figures and Tables	2
Introduction	3
Research Question	5
Materials and Methods	
Organ Culture.....	6
Morphological Analysis.....	7
Immunohistochemistry.....	8
Antral Follicle Cell Preparation.....	9
Reverse Transcription-PCR.....	10
Real-time PCR.....	11
Microarray and Bioinformatics.....	12
Statistics.....	14
Results	15
Discussion	25
Conclusion	31
Acknowledgements	32
References	33

LIST OF FIGURES AND TABLES

Figures

Figure 1: Histology of Cultured Neonatal Rat Ovaries.....	15
Figure 2: Follicle Distribution in Control and GDNF Treated Rat Ovaries.....	16
Figure 3: Follicle Pool Size in Treated and Untreated Rat Ovaries.....	17
Figure 4: GDNF and GFR α 1 Immunohistochemical Localization in Cultured Postnatal day-4 Rat Ovaries.....	18
Figure 5: GDNF and GFR α 1 Immunohistochemical Localization in Adult Rat Ovaries.....	19
Figure 6: GDNF Immunohistochemistry in a Large Antral Follicle.....	20
Figure 7: RT-PCR Showing Expression of GDNF and GFR α 1 mRNA in Oocytes and Granulosa Cells from Antral Follicles.....	22
Figure 8: Kit Ligand (KL) Expression in GDNF Treated Ovaries.....	22
Figure 9: Microarray of Control and GDNF Treated Postnatal day-4 Rat Ovaries after 2 days of Culture.....	23

Tables

Table 1: Ovarian Localization of GDNF and GFR α 1 Expression.....	21
Table 2: Microarray of Control and GDNF treated postnatal day-4 rat ovaries after 2 days of Culture.....	24

INTRODUCTION

Female fertility of most mammals is determined by the primordial follicle pool size in the neonatal period and the rate at which primordial follicles leave the arrested pool to begin development. A resting pool of primordial follicles is formed late in embryogenesis or in the first days after birth (Hirshfield 1991; Skinner 2005). A primordial follicle consists of an oocyte arrested in prophase I of meiosis and surrounded by flattened pre-granulosa cells (Parrott, *et al.* 1999). Once a primordial follicle begins to develop it will either continue to develop fully into an ovulatory follicle or will undergo atresia via cellular apoptosis at some stage of folliculogenesis. The development process is initiated by the primordial to primary follicle transition, during which the flattened pre-granulosa cells surrounding the oocyte become cuboidal granulosa cells indicative of the primary follicle (Kezele, *et al.* 2002; Hirshfield 1991). The rate at which follicles leave the primordial pool and transition into developing primary follicles determines a female's future fertility. Elucidation of the processes regulating primordial follicle development is essential in designing therapies for reproductive diseases such as premature ovarian failure in which follicles undergo primordial to primary transition at an abnormally fast rate (Richardson, *et al.* 1987; Santoro 2001). Potential therapies could also regulate the transition into menopause or induce menopause for women at risk for developing breast and ovarian cancer. The network of known extracellular signaling factors regulating primordial to primary follicle transition includes kit ligand (KL) (Nilsson, *et al.* 2004; Parrott, *et al.* 1999), leukemia inhibitory factor (LIF) (Nilsson, *et al.* 2002), bone morphogenesis protein 4 (BMP4) (Nilsson, *et al.* 2003), bone morphogenesis protein 7 (BMP7) (Lee, *et al.* 2004), platelet-derived growth factor (PDGF) (Nilsson, *et al.* 2006), and basic fibroblast growth factor (bFGF) (Nilsson, *et al.* 2004), all of which promote primordial follicle development (Skinner

2005). Anti-Müllerian Hormone/Müllerian inhibitory substance (AMH/MIS) and stromal-derived factor 1 (SDF1/CXCL12) inhibit primordial to primary follicle transition (Holt, *et al.* 2006; Ikeda, *et al.* 2002; Nilsson, *et al.* 2007). The current research was designed to determine whether glial derived neurotrophic factor (GDNF) is a part of this network of cell-cell interactions that regulates primordial follicle development.

Glial cell-line derived neurotrophic factor (GDNF) has previously been shown to exhibit neuro-restorative and neuro-protective actions for dopaminergic neurons in the nigrostriatal pathway of the brain (Wissel, *et al.* 2006; Gash, *et al.* 1998; Kordower, *et al.* 2000; Kirik, *et al.* 2004). It also mediates tube formation in mammary glands via the MAP kinase pathway (Karihaloo, *et al.* 2005). GDNF signaling occurs via a protein complex. The receptor GDNF family-receptor alpha 1 (GFR α 1) preferentially binds GDNF, and the ligand-receptor complex activates the ubiquitous tyrosine kinase receptor RET (Carmillo, *et al.* 2005; Amoresano, *et al.* 2005; Pozas, *et al.* 2005; Vargas-Leal, *et al.* 2005). Activation of RET via this complex leads to activation of intracellular signaling pathways involved in cell proliferation and differentiation (Naughton, *et al.* 2006).

Testicular GDNF is expressed in the Sertoli cells while its receptors GFR α 1 and RET are expressed in the spermatogonial cells (Dettin, *et al.* 2003). Signaling from the GDNF/GFR α 1/RET complex is necessary for spermatogonial stem cell self-renewal and proliferation in neonatal mice (Naughton, *et al.* 2006; Widenfalk, *et al.* 2000; Wu, *et al.* 2005). GDNF expression in the testis decreases as males reach maturity. In contrast, GDNF expression in the ovary increases with maturity especially in follicles close to ovulation (Golden, *et al.* 1999). GDNF mRNA is expressed at lower levels in developing follicles (Golden, *et al.* 1999; Widenfalk, *et al.* 2000) and localized to oocytes (Aravindakshan, *et al.* 2006). Microarray

experiments by the current investigators suggested that RET receptor expression in ovaries changed with AMH treatment (Nilsson, *et al.* 2007). This observation, coupled with the results of the previous studies on GDNF, led to the hypothesis that GDNF signaling may regulate the primordial to primary follicle transition.

RESEARCH QUESTION

The current study investigates the role of GDNF in promoting primordial to primary follicle transition and characterizes GDNF and GFR α 1 expression in neonatal and adult ovaries.

MATERIALS AND METHODS

Organ Culture:

Sprague-Dawley rats were obtained from a Washington State University breeding colony. All animal procedures were approved by the WSU Animal Care and Use Committee. Ovaries dissected from four day-old female rat pups were maintained in a whole organ culture system on floating filters (0.4 μm Millicell-CM; Millipore Corp., Billerica, MA) in 0.5 ml DMEM-Ham's F-12 medium (1:1, vol/vol; Life Technologies, Inc., Rockville, Maryland) containing 0.1% BSA (Sigma, St. Louis, MO), 0.1% albumax (Life Technologies, Inc., Rockville, Maryland), 200 ng/ml insulin (rh Insulin; Sigma, St. Louis, MO), 0.05 mg/ml L-ascorbic acid (Sigma, St. Louis, MO) and 27.5 $\mu\text{g/ml}$ transferrin (Sigma, St. Louis, MO) in a four-well culture plate (Nunc plate; Applied Scientific, South San Francisco, CA). Medium was supplemented with final concentration 5 $\mu\text{g/ml}$ gentamicin, 3.25 $\mu\text{g/ml}$ streptomycin, and 3.25 units/ml penicillin to prevent bacterial contamination. Ovaries were treated with no factor (control), glial cell-line derived neurotrophic factor (R-METHU GDNF, 50 ng/ml; Amgen, Thousand Oaks, CA), kit ligand/stem cell factor (rm SCF, 50ng/ml; R&D Systems, Minneapolis, MN), or GDNF and KL combined. The 50ng/ml GDNF dose was found to be optimal since an increased dose (500ng/ml) stimulated the same biological response. Two to three ovaries were placed on each filter, and no two ovaries from the same animal were placed into the same treatment group. Culture medium and treatments were replaced every two days. After ten days, ovaries were fixed in Bouin's fixative (Sigma, St. Louis, MO) for one hour followed by immersion in 70% ethanol. Tissues were paraffin embedded, sectioned at 3 μm , and hemotoxylin/eosin stained. The experiments were repeated so that each treatment group contained five to seven different ovaries.

Morphological Analysis:

The number of follicles at each developmental stage was counted and averaged in two serial sections from the largest cross-section through the center of the ovary and averaged. Previously, the data obtained from this analysis of two mid-diameter cross-sections have been shown to provide similar results as analysis of compiled data from all serial sections (data not shown) (Parrott, *et al.* 1999; Nilsson and Skinner 2003). In addition, total follicle number per section does not change between treatment groups as shown in previous studies (Kezele *et al.* 2005a; Nilsson *et al.* 2001, 2006, 2007). Morphological analysis of the primordial to primary follicle transition is only valid for factors that do not affect follicle viability. If the total follicle numbers per cross section vary greatly between treatment groups, percentages of primordial and developing follicles may be skewed. However, if follicle viability is not affected, only the percentage of follicles at each developmental stage changes with treatment (Nilsson, *et al.* 2001; Nilsson, *et al.* 2002). After determining that follicle viability was not affected by treatment, follicles in ovarian cross sections were classified as primordial (stage 0), or developing (stages 1-4: early primary, primary, transitional and preantral) as previously described (Oktay, *et al.* 1995). Primordial follicles consist of an oocyte arrested in prophase I of meiosis that is partially or completely encapsulated by flattened squamous pregranulosa cells. Early primary follicles have initiated development (ie, undergone primordial to primary follicle transition) and contain at least two cuboidal granulosa cells. Primary, transitional, and preantral follicles exhibit one or more complete layers of cuboidal granulosa cells. Four-day old ovaries contain predominately primordial follicles (Parrott, *et al.* 1999). Hemotoxylin/eosin stained ovarian sections were analyzed at 400X magnification using light microscopy.

Immunohistochemistry:

Ovary sections from freshly isolated adult or cultured postnatal day-4 (P4) ovaries (cultured for 10 days) were immunostained as previously described (Nilsson, *et al.* 2002) for the presence of GDNF using anti-GDNF primary antibody (Anti-GDNF rabbit IgG, 5 µg/ml; Santa Cruz Biotechnology, Santa Cruz, CA). Briefly, 3 µm sections were deparaffinized, rehydrated through a graded ethanol series, boiled in 10 mM Sodium Citrate buffer, quenched in 3% hydrogen peroxide/20% Methanol and 0.1% Triton-X solution, and then blocked with 10% goat serum (normal goat serum; Vector Laboratories, Inc., Burlingame, CA) for twenty minutes prior to incubation with 5 µg/ml primary antibody for twelve hours. Sections were then washed in PBS and incubated with 1:300 diluted biotinylated secondary antibody for 45 minutes (goat anti-rabbit IgG; Vector Laboratories, Inc., Burlingame, CA), washed again, and incubated with streptavidin peroxidase (Zymed, San Francisco, CA) prior to color development with a DAB peroxidase substrate kit (Vector Laboratories, Inc., Burlingame, CA). Following development, sections were dehydrated, coverslips mounted with zylene-based medium (Cytoseal-XYL; Richard Allan Scientific, Kalamazoo, MI), and analyzed at 200X, 400X, and 1000X magnification using light microscopy. Negative control experiments were performed using a non-specific primary antibody at 5 µg/ml (Rabbit IgG; Sigma, St. Louis, MO). Non-cultured adult rat ovaries were also stained for the presence of GDNF with the same antibody (Anti-GDNF rabbit IgG, 5-10 µg/ml; Santa Cruz Biotechnology, Santa Cruz, CA) using the protocol described below for GFR α 1 localization.

Neonatal cultured and freshly isolated adult rat ovaries were stained for GFR α 1 localization using anti-GFR α 1 primary antibody (Anti-GFR α 1 rabbit IgG, 5-10 µg/ml; Santa Cruz Biotechnology, Santa Cruz, CA). The protocol for GFR α 1 immunohistochemistry

excluded the boiling step and introduced a second wash in 0.1% Triton-X solution following incubation with primary antibody. Negative control experiments were performed using a non-specific primary antibody at 5-10 µg/ml (Rabbit IgG; Sigma, St. Louis, MO). The protocol was otherwise identical to that used for GDNF staining.

Antral Follicle Cell Preparation:

Female 24 day-old rats were injected with 10 IU PMSG subcutaneously to induce follicle development. 48 hours post-injection ovaries were extracted, bursae and fat trimmed away, and ovaries placed in plates of F12/DMEM media (1:1, vol/vol; Life Technologies, Inc., Rockville, Maryland) supplemented with final concentration 5 µg/ml gentamicin, 3.25 µg/ml streptomycin, and 3.25 units/ml penicillin to prevent bacterial contamination. Ovaries were then incubated at 37 °C for 30 minutes in EGTA (6 mM; Sigma, St. Louis, MO), transferred to a sucrose solution (0.5 M; Life Technologies, Inc., Gaithersburg, Maryland) and incubated for another 30 minutes (both solutions made in media above). Following incubation, ovaries were again placed in F12/DMEM + antibiotics medium, where large follicles were lanced open to extrude granulosa cells and oocytes. Oocytes were aspirated into a separate tube creating two cell preparations, each enriched for either oocytes or granulosa cells from antral follicles. The oocyte preparation was further subjected to gentle aspiration through a 100 µl pipette tip and the resulting supernatant, containing dislodged granulosa cells, was discarded. This created oocyte preparations in which some granulosa cells would still adhere to many oocytes, and so a small amount of granulosa cell contamination is expected. All other cell types remain associated with the ovarian connective tissue. The isolated cell populations were placed in Trizol reagent at -75° (Sigma) for storage until RNA isolation.

Reverse Transcription-PCR:

Granulosa cells and oocytes were extracted from antral rat follicles or microdissected as described above. Cells were immersed in Trizol reagent (Sigma, St. Louis, MO) and RNA extracted according to manufacturer's protocols. RNA samples were DNase treated with the TURBO DNA-free kit (Ambion, Austin, TX) prior to reverse-transcription of RNA to cDNA according to a standard oligo-dT RT protocol in a reaction volume of 25 μ l. Negative controls for each sample were created at this step by omitting MMLV enzyme in the RT reaction. All PCR primer sets span intron/exon boundaries. PCR used the following primers for GDNF (NCBI: NM_019139), forward: 5'-CTGGAAGATTCCCCGTATGA-3' and reverse: 5'-TCTTCGGGCATATTGGAGTC-3'. A second PCR for GFR1 used 2 μ l of the first PCR product as template, and the previous reverse primer was used in conjunction with a nested forward primer: 5'-CTGTCTGCCTGGTGTGCT-3'. GDNF Thermocycling conditions were as follows: 95°C 4 min, 35 cycles of 95°C 30s, 60°C 60s, 72°C 30s, followed by 72°C 5 min. Second GDNF PCR required 20 cycles. Primers for GFR α 1 (NCBI: NM_012959.1) were forward: 5'-GGCAGTCCCGTTCATATCAG -3' and reverse: 5'-AGCAGAAGAGCATCCCGTAG- 3'. Second PCR for GDNF used 1 μ l of the first PCR product as template and identical primers. The protocol for GFR α 1 amplification was as follows: 95°C 4 min, 35 cycles of 95°C 30s, 60°C 60s, 72°C 30s, followed by 72°C 5 min. Second GFR α 1 PCR required 20 cycles. GDNF and GFR α 1 PCR products were electrophoretically analyzed and sequenced to confirm identity. Amplification of the ribosomal protein S2 was used as a reference standard. The S2 reference gene primers (NCBI: NM_031838) were rS2-F, 5'CTGCTCCTGTGCCCAA-GAAG3' and rS2-R,

5'AAGGTGGCCTTGGCAAAGTT3'. Ribosomal S2 mRNA expression does not change in ovarian cells regardless of treatment (Kezele, *et al.* 2005a).

Real-time PCR:

Ovaries from four day-old female rat pups were maintained in the organ culture system described above for two days with GDNF treatment (R-METHU GDNF, 50 ng/ml; Amgen, Thousand Oaks, CA), or were left untreated as controls. Two ovaries from each culture well were combined to make each RNA sample. After 2 days of culture, ovaries were homogenized in Trizol reagent (Sigma, St. Louis, MO) and RNA extracted according to manufacturer's protocols. RNA samples were DNase treated with TURBO DNA-free kit (Ambion, Austin, TX) prior to reverse-transcription of RNA to cDNA according to a standard oligo-dT RT protocol in a reaction volume of 25 μ l. Negative controls for each sample were created at this step by omitting MMLV enzyme in the RT reaction. 5 μ l of each 1:10 diluted cDNA sample, as well as each 1:10 diluted negative control sample, were 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 manufacturer's instructions for detection of Kit Ligand (*Kitl*). The *Kitl* primers (NCBI: NM_021843) were *rKitl-720*: 5'ATTTATGTTACCCCCTGTTGCAGCC3' and *rKitl-859*: 5'CAATTACAAGCGAAATGAGAGCCG3'. SYBR Green was also used to detect the ribosomal protein gene *S2*, which was used as a reference standard for real-time PCR. Real-time PCR was performed on an ABI-7000 real-time machine with the following protocol: 50 °C 2 min , 95 °C 2 min, then 40 cycles of 95 °C 15 s and 66 °C 30 s. Fluorescent detection data were analyzed for KL mRNA levels and normalized to *S2* mRNA levels, and then GDNF-treated sample KL levels were normalized to untreated control KL mRNA levels.

Microarray and Bioinformatics:

Postnatal day-4 rat ovaries were cultured for two days in the presence or absence of GDNF (R-METHU GDNF, 50 ng/ml; Amgen, Thousand Oaks, CA). Culture conditions were identical to those described for 10-day organ culture experiments. Each sample contained 6-10 pooled ovaries, and no two ovaries from the same animal were placed into the same treatment group. For each control and treated sample, two biological replicates were produced using different sets of ovaries. After culture ovaries were placed into Trizol reagent for RNA extraction as per manufacturer's protocols.

RNA was hybridized to the Affymetrix (Santa Clara, CA) Rat 230 2.0 gene chip. One chip was used for each biological replicate (i.e. four chips total: two for control and two for treated). The number of chips required for specific experiments has been previously reviewed (Chen, *et al.* 2004). 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 treated cultured ovaries were reverse transcribed into cDNA, which was transcribed into biotin-labeled RNA. Biotin-labeled RNA was then hybridized to the Affymetrix Rat 230 2.0 gene chips. Biotinylated RNA was then visualized by labeling with phycoerythrin-coupled avidin. The microarray chip was scanned on an Affymetrix Gene Chip Scanner 3000 (Affymetrix). The microarray image data were converted to numerical data with GeneChip Operating Software (GCOS version 1.2; Affymetrix) using a probe set target signal of 125. An analysis was performed with GCOS to assess the relative abundance of the transcripts based on signal and detection calls (present, absent, or marginal). The 11 perfect match and 11 mismatch oligonucleotides for a specific gene were used to statistically determine present/absent calls using a one-sided Wilcoxon's signed rank test.

In GCOS, excel files were generated with expression signals and absent/present calls for each probe set. Using the excel files, R^2 for control or treated sample replicates were calculated (>0.98), indicating negligible total variability between chips, experiments and samples. Excel files from GCOS were imported into Gene-spring software (Silicon Genetics, Redwood City, CA) and normalized using the recommended defaults. This includes setting signal values below 0.01 to a value of 0.01, total chip normalization to the 50th percentile, and per gene normalization to the median. Unless otherwise indicated, in order for a transcript to be considered present, it had to be both tagged as present in the GCOS present/absent call, and have an expression level greater than 75. In order for a transcript to be considered changed between treatment groups, it had to exhibit at least a 1.5-fold change between the means of the treatments and have a Student's *t*-test *P* value of <0.05 between control and treatment samples. Therefore, the data presented are for genes that were determined to be statistically present and found to be statistically different from control with a given treatment.

Previous studies have demonstrated that microarray data are validated with quantitative PCR data (Kezele, *et al.* 2005b; Shima, *et al.* 2004). Due to the presence of 11 different oligonucleotide sets for each specific gene being used on the microarray versus only a single primer set for a gene in a quantitative PCR, the microarray is more effective at eliminating false positive or negative data and provides a more robust quantitation of changes in gene expression. However, validation of microarray data was performed with the KL gene using a real-time PCR procedure, as described in Methods. As presented in Results, similar data were obtained with the real-time PCR analysis as with the microarray analysis.

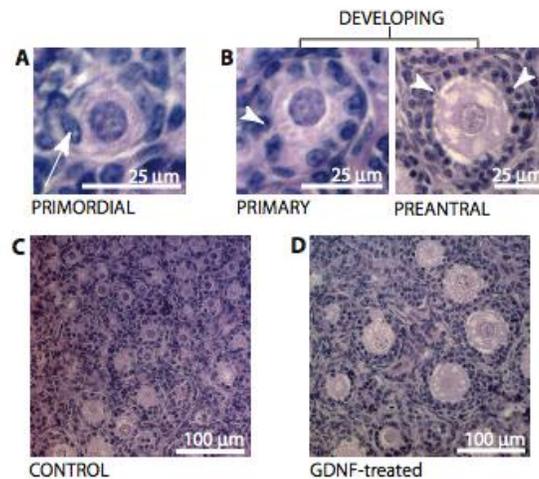
Statistics:

Organ culture treatment groups were compared using an analysis of variance (one-way ANOVA). Following a significant result with ANOVA, treated groups were compared with the control using Dunnet's Multiple Comparison Post-hoc test. Real-time PCR treatment groups were compared using a one-sample T-test. All statistics were calculated with the help of GraphPad Prism version 4.0b software (GraphPad Software, Inc., San Diego, CA).

RESULTS

Ovaries from four day-old female rat pups were maintained in organ culture for ten days in the absence (control), or presence of 50 ng/ml glial cell-line derived neurotrophic factor (GDNF), 50 ng/ml kit ligand/stem cell factor (KL), or GDNF and KL combined. Following culture follicles in ovarian cross sections were classified as primordial or developing. Follicles containing an oocyte partially or fully encapsulated by flattened pre-granulosa cells were designated primordial (Figure 1A), whereas follicles containing an oocyte accompanied by two or more cuboidal granulosa cells were designated developing (Figure 1B). Developing follicles can be further classified into separate stages (transitional, primary, secondary, preantral), however for the purpose of analyzing primordial to primary follicle transition a comparison of the percentage of primordial follicles to the percentage of total developing follicles per section was made. At 200X magnification, untreated control ovaries (Figure 1C) contain notably fewer large developing follicles than ovaries treated with GDNF (Figure 1D).

Figure 1: Histology of Cultured Neonatal Rat Ovaries. A) Histology of a primordial follicle with an oocyte surrounded partially or completely by flattened (squamous) pre-granulosa cells (arrow). B) Histology of primary and preantral secondary follicles (both classified as developing) with an oocyte surrounded by one or more cuboidal granulosa cell layers (arrowhead). C) Postnatal day-4 rat ovary cultured for ten days with no treatment. D) Postnatal day-4 rat ovary cultured for ten days with GDNF treatment.



Cultured control (untreated) ovary cross-sections contained 41.3 ± 3.4 % (Mean \pm SEM) developing follicles (stage 1-4) due to spontaneous primordial to primary follicle transition (Figure 2). In contrast, ovary sections treated with GDNF contained an increased proportion of developing follicles ($p < 0.01$) at 54.3 ± 1.9 %. Ovarian sections treated with both GDNF and KL also had an increased percentage of developing follicles ($p < 0.01$; 56.9 ± 2.6 %) compared to controls. As a positive control, ovary sections treated with KL contained 49.7 ± 0.21 % developing follicles ($p < 0.05$). Total follicle pool size did not significantly change with varying treatments (Figure 3). Although no statistically significant change was observed, a slight reduction in number was found after treatment. The decrease observed could no account for the degree of stimulation after treatment. The higher percentage of developing follicles in GDNF-treated ovaries indicates that more follicles have undergone primordial to primary follicle transition. Therefore, GDNF promotes primordial follicle development in rat ovaries.

Figure 2: Follicle Distribution in Control and GDNF Treated Rat Ovaries. Postnatal day-4 rat ovaries were maintained for ten days in organ culture with no treatment (control), GDNF treatment, KL treatment, or GDNF and KL combined. KL is a known promoter of primordial to primary follicle transition. The percent follicle category (control or developing) is presented (mean \pm SEM) from a minimum of three experiments in replicate with (**) indicating $p < 0.01$ compared with control, and (*) indicating $p < 0.05$ compared with control.

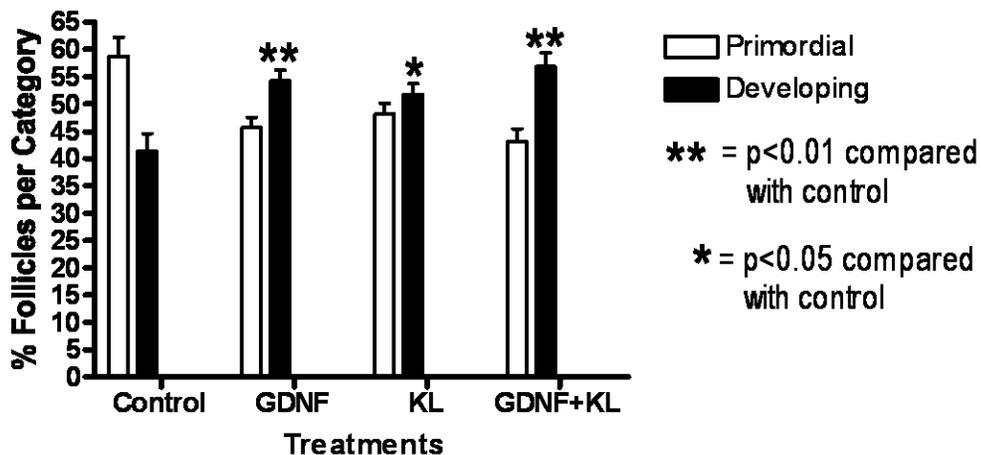
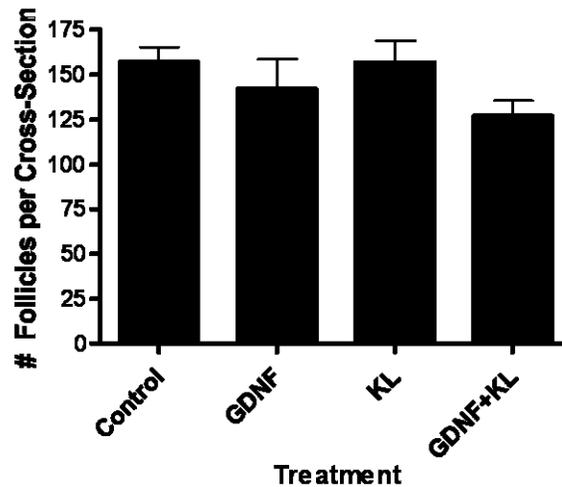
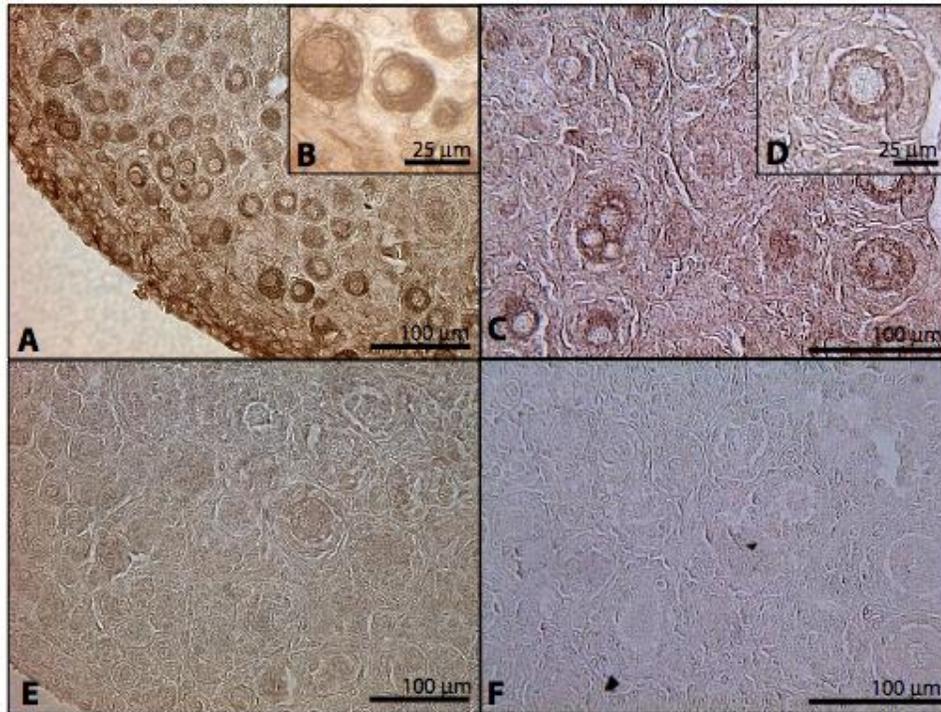


Figure 3: Follicle Pool Size in Treated and Untreated Rat Ovaries. Postnatal day-4 rat ovaries were maintained for ten days in organ culture with no treatment (control), GDNF treatment, KL treatment, or GDNF and KL combined. Two cross sections from the center of each ovary were analyzed and the total follicle numbers per cross-section averaged. The mean \pm SEM from a minimum of three different experiments in replicate are presented with no statistical different ($p>0.05$) between treatments.



Immunohistochemistry was used to localize the proteins for GDNF and its receptor GFR α 1 in postnatal day-4 (P4) rat ovaries after ten days of organ culture. Dark-colored GDNF-specific staining was localized to oocyte cytoplasm in primordial and primary follicles of P4 sections (Figure 4 A, B). GFR α 1 protein in neonatal ovaries was also localized to the oocyte cytoplasm of primordial and primary follicles (Figure 4 C, D). Non-specific staining throughout the tissue is determined by comparison with sections stained with a non-specific primary antibody (Figure 4 E, F).

Figure 4: GDNF and GFR α 1 Immunohistochemical Localization in Cultured Postnatal day-4 Rat Ovaries. A-B) GDNF; C-D) GFR α 1; E) non-specific IgG developed according to GDNF protocol; F) non-specific IgG developed according to GFR α 1 protocol. Representative micrograph of a minimum of three different experiments in replicate.



Immunohistochemistry was also used to localize GDNF and GFR α 1 in freshly isolated adult female ovaries. In primordial and primary follicles, GDNF and GFR α 1 are both present in the oocyte (Figure 5 A, C). This confirms the results seen in cultured ovaries. In pre-antral follicles, GDNF localization expands include oocytes, nearby granulosa cells, and theca (Figure 5E). GFR α 1 localization in pre-antral follicles remains at low levels in the oocyte, and expands to the granulosa cells and theca (Figure 5G). GDNF localization in antral follicles remains in oocytes and nearby cumulus granulosa cells (Figure 5I). GDNF is also present in the theca throughout adult sections (Figure 5 M, N). In antral follicles GFR α 1 is present at low levels in oocytes and mural granulosa cells (Figure 5K). In some antral follicle oocytes GFR α 1 is detectable at levels no higher than those of the background staining seen in the negative controls (not shown),

suggesting that GFR α 1 protein may not be present in these oocytes. GFR α 1 is also present in the theca and ovarian surface epithelium of adult rat ovaries (Figure 5 O, P). These findings are summarized in Table 1. Corresponding negative control sections developed according to matched GDNF and GFR α 1 protocols represent non-specific background staining at each follicular stage (Figure 5 B, D, F, H, J, L).

Figure 5: GDNF and GFR α 1 Immunohistochemical Localization in Adult Rat Ovaries. (A-D) Localization in primordial and primary follicles: A) GDNF; C) GFR α 1. E-H) Localization in pre-antral follicles: E) GDNF; G) GFR α 1. I-L) Localization in antral follicles: I) GDNF; K) GFR α 1. M-P) Localization in theca and granulosa cells: M, N) GDNF; O, P) GFR α 1. Sections stained with non-specific IgG corresponding to protocol-matched GDNF and GFR α 1 stained sections (B, D, F, H, J, L). Arrowheads point to oocyte cytoplasm, arrows point to thecal cells, (*) indicates granulosa cells, and OSE indicates ovarian surface epithelium. Representative micrographs from a minimum of three different experiments in replicates.

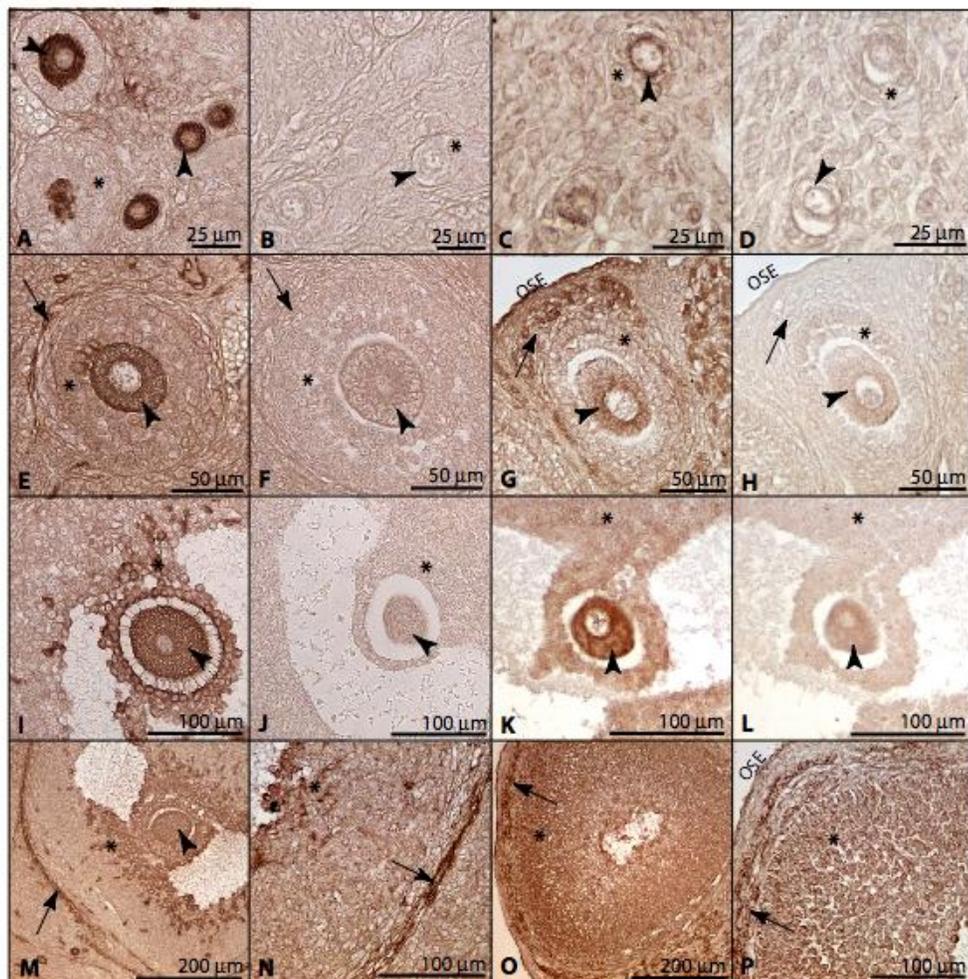
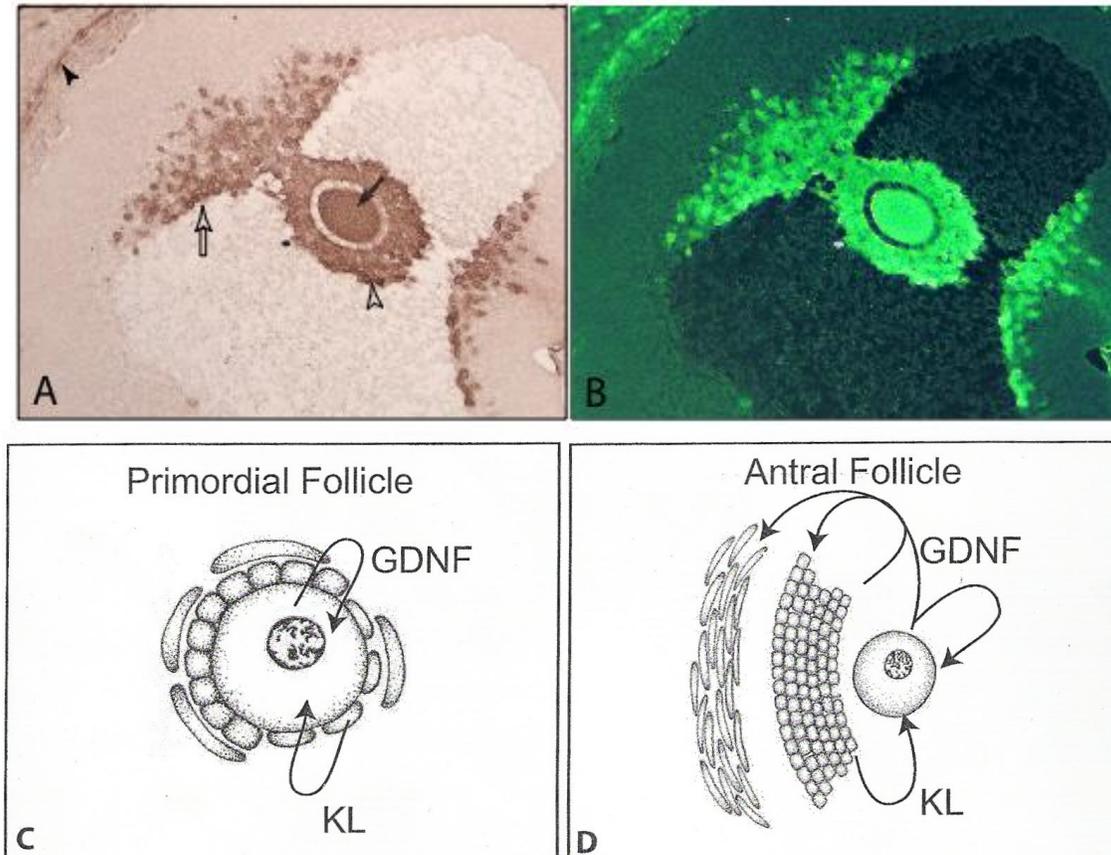


Figure 6: GDNF Immunohistochemistry in a Large Antral Follicle (A, B). (A) The arrow indicates the oocyte, the open arrow head the cumulus granulosa, the open arrow the oocyte proximal granulosa, and the closed arrowhead indicates the interna-theca cells. (B) panel is artificial color contrast of panel (A). Representative micrograph from three different experiments in replicate. (C) Schematic of primordial follicle expression and action of GDNF and KL and (D) schematic of large antral follicle expression and action of GDNF and KL.



Observations suggest GDNF has an autocrine role in the primordial follicle since it is produced by the oocyte and has receptors on the oocyte (Figure 4, Figure 5 and Figure 6). In the preantral follicle GDNF continues to have an autocrine role, but the receptor is also present on developing theca cells (Figure 5). In contrast, antral follicle GDNF is expressed by the oocyte and adjacent cumulus granulosa cells (Figure 5I, M & Figure 6), but the receptor $GFR\alpha 1$ is expressed by the oocyte and mural granulosa (Figure 5K, O). GDNF expression in the antral

follicle is in the oocyte and in cells most proximal to the oocyte (Figure 6 A, B). GDNF expression is also observed in the theca cells (Figure 5M, N & Figure 6). Therefore, GDNF transitions from an autocrine factor in the primordial follicle to a factor with autocrine and paracrine abilities in the antral follicle (Figure 6 C, D), (Table 1).

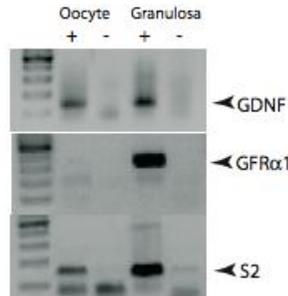
Table 1: Ovarian Localization of GDNF and GFR α 1 Expression.

1. Pre-antral follicles have three or more layers of granulosa1 cells.
2. Antral follicles have a fluid-filled antrum.

	Primordial	Primary	Pre-Antral¹	Antral²
GDNF				
Oocyte	++	++	++	++
Granulosa	-	-	+	++
Theca	-	-	+	++
GFRα1				
Oocyte	++	++	+	+/-
Granulosa	-	-	+	+
Theca	-	-	++	++

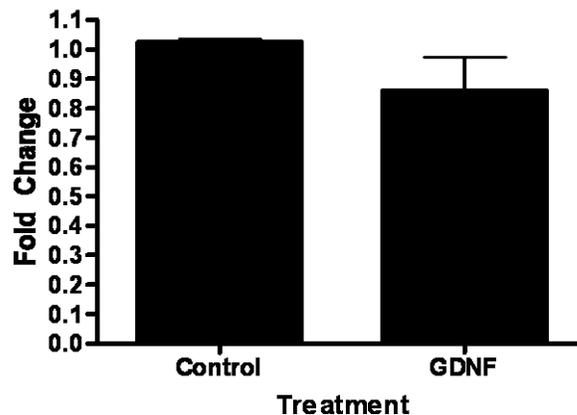
To confirm the immunohistochemical analysis, an RT-PCR was used to determine which ovarian cell types expressed GDNF and GFR α 1 mRNA. RNA was extracted separately from isolated oocytes and granulosa cells of rat antral follicles. Negative control samples (created by omitting MMLV enzyme from the RT reaction) did not produce a significant PCR product (Figure 7). GFR α 1 mRNA was localized to granulosa cells of antral follicles, whereas GDNF mRNA was present in oocytes and granulosa cells of antral follicles (Figure 7). Ribosomal gene S2 was amplified as a constitutively expressed gene reference standard. Observations confirm the expression of GDNF by the oocyte and demonstrated decreased GFR α 1 expression in antral follicle oocytes.

Figure 7: RT-PCR Showing Expression of GDNF and GFR α 1 mRNA in Oocytes and Granulosa Cells from Antral Follicles. GDNF and GFR α 1 bands shown were imaged after two rounds of PCR. Products from reactions with (+) and without (-) MMLV reverse transcriptase enzyme are shown. S2 was amplified as a constitutively expressed reference gene. Data are representative of a minimum of three different experiments.



A real-time PCR procedure was used to measure the potential regulation of KL expression in cultured rat ovaries treated with and without GDNF. The ribosomal gene S2 was amplified as a constitutively expressed gene reference standard and was used to normalize data. There was no significant difference in KL expression between control and GDNF treated ovaries (Figure 8). Therefore, GDNF does not appear to regulate KL expression during primordial follicle development. The level of GDNF and GFR α 1 mRNA expression in the whole ovary was found to be negligible, such that KL regulation of GDNF expression in whole ovary studies were not useful (data not shown).

Figure 8: Kit Ligand (KL) Expression in GDNF Treated Ovaries. Real-time PCR amplification of KL mRNA derived from postnatal day-4 rat ovaries cultured for two days in the presence or absence of GDNF treatment. The mean \pm SEM from three different experiments is presented as relative expression and normalized with S2 mRNA.



The actions of GDNF on the neonatal rat ovary were further investigated by determining the effects of GDNF on the ovarian transcriptome with a microarray analysis. P4 rat ovaries were cultured for 2 days in the absence or presence of GDNF and then RNA was collected for microarray analysis. GDNF was found to alter the expression of 28 genes (above 1.5 fold change cut-off) with 17 stimulated and 11 decreased in expression (Table 2 & Figure 9). A list of specific genes is provided in Table 2 and categories of altered genes are presented. GDNF was found to affect several growth factors and secreted cytokines including anti-Müllerian hormone (AMH), connective tissue growth factor (CTGF), fibroblast growth factor 8 (FGF8), growth differentiation factor 9 (GDF9) and stamioalcin1 (Stc1). Observations demonstrate that GDNF participates with previously described members of the signaling network regulating follicle development. This analysis has also identified candidate genes for further analysis.

Figure 9: Microarray of Control and GDNF Treated Postnatal Day 4 Rat Ovaries after 2 days of Culture. Dendrogram shows transcripts that increased (red) or decreased (green) in GDNF treated versus control ovaries. Gene symbol provided for each altered expression with (-) indicating an EST.

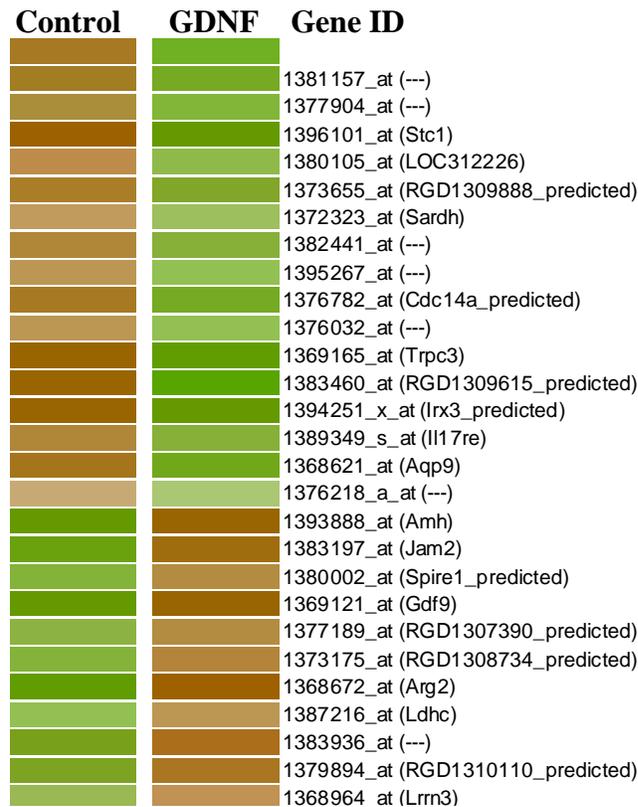


Table 2: Microarray of Control and GDNF Treated Postnatal Day-4 Rat Ovaries after 2 days of Culture. Selected genes with altered gene expression after GDNF treatment. Ratios compare gene expression between control and GDNF treated ovaries.

Gene Symbol & Category	Control Raw	GDNF Raw	GDNF/Cont Ratio	Genbank	Gene Title
Cell Cycle					
Cdc14a_predicted	165	104	0.63	AW915083	CDC14 cell division cycle 14 homolog A
Cytoskeleton					
Jam2	126	204	1.62	AA849471	junction adhesion molecule 2
Spire1_predicted	80	125	1.58	H31747	spire homolog 1 (Drosophila) (predicted)
Growth Factors					
Amh	146	219	1.50	AI059285	anti-Mullerian hormone
Ctgf	505	716	1.42	NM_022266	connective tissue growth factor
Fgf8	22	40	1.80	NM_133286	fibroblast growth factor 8
Gdf9	626	951	1.52	NM_021672	growth differentiation factor 9
Stc1	644	392	0.61	BF552244	stanniocalcin 1
Development					
Lrrn3	66	107	1.62	NM_030856	leucine rich repeat protein 3, neuronal
Metabolism & Transport					
Arg2	239	396	1.66	NM_019168	arginase 2
Aqp9	185	115	0.62	NM_022960	aquaporin 9
Ldhc	62	99	1.61	NM_017266	lactate dehydrogenase C
Sardh	92	57	0.63	AI103641	sarcosine dehydrogenase
LOC684802 /// LOC685152	76	49	0.65	BI273855	similar to Probable phospholipid-transporting
RGD1565367_predicted	124	66	0.53	C07059	Similar to Solute carrier family 23, member 2
Receptor & Binding Pr					
Il17re	127	83	0.65	BM383766	interleukin 17 receptor E
Trpc3	248	153	0.62	NM_021771	transient receptor potential cation channel,
Transcription/Epigenetic					
Arid1b	136	86	0.63	AI556402	AT rich interactive domain 1B (Swi1 like)
Irx3_predicted	333	213	0.64	AI713965	Iroquois related homeobox 3 (Drosophila)
EST's					
RGD1308734	84	140	1.66	BI285951	similar to RIKEN cDNA 1100001H23
---	103	61	0.59	BM391972	CDNA clone IMAGE:7366335
RGD1310110_predicted	116	176	1.52	AI501165	similar to 3632451O06Rik protein (predicted)
RGD1307390_predicted	80	123	1.54	BI295093	similar to BC282485_1 (predicted)
RGD1309888	164	107	0.65	BG371863	similar to RIKEN cDNA 1500002O20
---	159	101	0.64	BF396928	Transcribed locus
---	122	79	0.65	BE109251	Transcribed locus
---	104	61	0.59	BF283840	Transcribed locus
---	123	201	1.64	BM386842	Transcribed locus, strongly similar to XP_001086149.
---	165	96	0.58	BE117343	---
---	457	257	0.56	AI058976	---

DISCUSSION

Ovaries from four-day old rats were cultured in vitro with and without exogenous GDNF. After culture, morphometric analysis showed that ovaries treated with GDNF displayed higher percentages of developing follicles, indicating that the rate at which arrested primordial follicles left the primordial pool and underwent primordial to primary follicle transition was stimulated by exogenous GDNF. The increased percentage of developing follicles in GDNF treated ovaries was not due to changes in overall follicle pool size, as there was not a significant difference in total follicle numbers per counted section between control and GDNF-treated ovaries. The slight decrease in follicle number observed after treatment could not account for the degree of stimulation in primordial follicle development. This observation is similar to that previously observed with several other stimulatory growth factors (Parrot *et al.* 1999; Kezele *et al.* 2005a; Nilsson *et al.* 2001, 2006, 2007). GDNF treatments stimulated primordial follicle development to the same extent as KL, a known promoter of primordial to primary follicle transition (Parrott, *et al.* 1999; Nilsson, *et al.* 2004). Localization of GDNF in neonatal rat ovaries further supported the evidence from organ culture experiments that GDNF promotes early follicle development. Immunohistochemical localization revealed that GDNF was present in the oocyte cytoplasm of primordial follicles and was in oocytes, cumulus granulosa cells and theca cells of antral (late-stage developing) follicles. This localization in granulosa cells near the oocyte suggests that factors produced and secreted by the oocyte may be differentially regulating GDNF expression in the cumulus granulosa cells of the antral follicle. GDNF expression in the theca cells tended to be limited to the theca externa layers. The presence of GDNF protein in antral follicles supports previous research localizing GDNF message to pre-ovulatory follicles (Golden, *et al.* 1999; Aravindakshan, *et al.* 2006; Linher, *et al.* 2007). Immunohistochemistry localized GFR α 1

to oocyte cytoplasm of primordial and primary follicles, and to oocytes and mural granulosa cells of antral follicles. However, in some antral follicles non-specific oocyte staining was quite prominent, raising the possibility that GFR α 1 levels are low in some oocytes. GFR α 1 was also present in the theca and ovarian surface epithelium. GFR α 1 expression was strongest in the mural granulosa cells (Figure 5O, P) and theca interna layer of cells (Figure 5G, O, P). Further studies are needed to confirm the localization of GFR α 1 to sub-populations of granulosa and theca cells. Western blot analysis was used to verify the specificity of the GFR α 1 antibody (data not shown), which confirms observations from previous studies (Wang, *et al.* 2004; Serra, *et al.* 2005). Localization of GDNF and GFR α 1 in primordial and primary follicles was the same in both cultured neonatal ovaries and adult un-cultured ovaries. This suggests that culture conditions did not affect GDNF and GFR α 1 expression patterns in neonatal ovaries. To confirm the immunohistochemical observations, an RT-PCR procedure localized GDNF mRNA to antral oocytes and granulosa cells, and GFR α 1 mRNA to antral granulosa cells, but not to antral follicle oocytes. Oocytes from antral follicles have some non-specific immunohistochemical staining, so it is possible some antral follicle oocytes have very low levels of GFR α 1 protein. Therefore, the immunohistochemical studies suggest that GFR α 1 is still expressed in antral oocytes, although possibly decreasing in the largest preovulatory follicles. This expression decrease may also explain why GFR α 1 is not detected in antral oocyte samples by RT-PCR. Faint GFR α 1 bands are amplified from antral oocyte samples, although it is not clear whether these bands reflect low oocyte expression or granulosa cell contamination of the samples. Another possibility is that antral oocytes do not themselves produce GFR α 1 protein, but instead GFR α 1 expressed by surrounding granulosa cells is cleaved free of its GPI membrane anchor

and taken up by oocytes, so no oocyte $GFR\alpha 1$ mRNA is detectable. Further studies are needed to confirm the cellular expression pattern for $GFR\alpha 1$.

Combined observations suggest endogenous ovarian GDNF promotes the primordial to primary follicle transition through autocrine cell-cell signaling to $GFR\alpha 1$ receptors on oocytes. In later-stage antral follicles the expression of GDNF changes to include cumulus granulosa cells, while the receptor $GFR\alpha 1$ expression decreases in oocytes. This shift in ligand and receptor expression alters the physiological function of GDNF to a signaling factor with both autocrine and paracrine roles in developing antral follicles (Figure 6D) (Table 1). GDNF may also have an autocrine function for the antral follicle theca cells. Localization of GDNF and its receptor $GFR\alpha 1$ to oocyte cytoplasm presents one of the first examples of autocrine signaling to regulate primordial follicle development (Figure 6C). The mechanism by which GDNF promotes follicle development remains to be fully elucidated.

Interestingly, GDNF expression in the ovary is the opposite of GDNF expression in analogous tissues in the testes. Often paracrine or endocrine signaling ligands have similar localization patterns in the gonads of males and females, such that a particular growth factor is expressed by the germ cells (oocytes and spermatogonia) in both sexes, or in the epithelial cells (Sertoli and granulosa) of both sexes. However, there is a difference between male and female GDNF localization. In males, GDNF is expressed by the Sertoli cells and $GFR\alpha 1$ is in the germ cells. GDNF signaling to spermatogonia stimulates proliferation and self-renewal after division (Naughton, *et al.* 2006; Widenfalk, *et al.* 2000; Wu, *et al.* 2005). A similar function in females is not possible, as postnatal oocytes are arrested in meiosis and not undergoing cell division. This may explain why the site of GDNF production is different in females. Ovarian GDNF and $GFR\alpha 1$ are both present in the germ cell (ie oocyte). It is possible that over the course of

evolution, GDNF's role diverged from spermatogonial self-renewal in the male to autocrine signaling that promotes the primordial to primary follicle transition in the female. Autocrine signaling loops have been shown to promote cell viability in neural, immune, and tumor cells, and to regulate estradiol production by granulosa cells (Meerschaert, *et al.* 1999; Schumacher, *et al.* 2004; Datta, *et al.* 2006; Ireland, *et al.* 2004). Preserving oocyte viability may be an important aspect of autocrine GDNF signaling in oocytes of early stage follicles. Therefore, GDNF is likely essential for germ-cell survival in both the testis and ovary, but the sites of expression are distinct.

Other possible mechanisms for GDNF action in the neonatal rat ovary include interactions with other extracellular signaling factors, thus indirectly promoting follicle development. The known network of extracellular signaling factors involved in primordial to primary follicle transition includes leukemia inhibitory factor (Nilsson, *et al.* 2002), bone morphogenesis protein 4 (Nilsson, *et al.* 2003), platelet-derived growth factor (Nilsson, *et al.* 2006), nerve growth factor (Dissen, *et al.* 2002), and basic fibroblast growth factor (Nilsson, *et al.* 2004), kit ligand (KL) (Nilsson, *et al.* 2004; Parrott, *et al.* 1999) all of which promote development (Skinner 2005). KL may be expressed as either free (KL-1) or membrane bound (KL-2) isoforms. There is evidence that KL-2 is the necessary isoform for oocyte growth and formation of germ cells (Flanagan, *et al.* 1991; Thomas, *et al.* 2007). However, soluble KL-1 can promote primordial follicle transition (Parrott, *et al.* 1999). Anti-Müllerian hormone (AMH) and stromal-derived factor 1 (SDF1) inhibit primordial to primary follicle transition (Nilsson, *et al.* 2006; Ikeda, *et al.* 2002; Holt, *et al.* 2006; Skinner 2005). It was hypothesized that GDNF participated in this network by up-regulating KL or other factors that promote primordial to primary follicle transition. However, data from real-time PCR indicates that exogenous GDNF

does not influence KL expression. This observation indicates that GDNF acts in a signaling pathway that is not dependent on changing KL expression.

The microarray analysis suggests that GDNF activity affects a variety of secreted growth factors, including anti-Mullerian hormone (AMH), connective tissue growth factor (CTGF), fibroblast growth factor 8 (FGF-8), growth differentiation factor 9 (GDF9), and stanniocalcin 1 (Stc1). AMH performs many functions in the ovary, the most relevant being that it inhibits the primordial to primary follicle transition in rat, mouse, and humans (Carlsson, *et al.* 2006; Durlinger, *et al.* 2002; Nilsson, *et al.* 2007). Similarly, GDF9 is also critical for normal follicle development. GDF9 is an oocyte-secreted factor necessary for follicle development from the primary follicle stage through ovulation (Dong, *et al.* 1996; Elvin, *et al.* 1999). The other factors flagged by the microarray analysis have less documented function in early follicle development. FGF8 is expressed in antral follicles in rat and cow. It may also play a role in the development of ovarian and breast tumors (Buratini, *et al.* 2005; Daphna-Iken, *et al.* 1998; Valve, *et al.* 2000; Valve, *et al.* 1997; Zammit, *et al.* 2002). Stanniocalcin 1 was originally shown to have high expression levels in the ovary (Varghese, *et al.* 1998). Further studies described an increase in Stc1 expression during pregnancy and lactation, and a possible function in luteinization inhibition (Ishibashi, *et al.* 2002; Luo, *et al.* 2004). Stc 1 knockout mice develop as phenotypically and reproductively normal (Chang, *et al.* 2005). Connective tissue growth factor (CTGF) is a matrices associated protein with many possible roles in development and differentiation processes (Leask, *et al.* 2003). CTGF is regulated by gonadotropins and is expressed in antral follicles and corpus lutea (Harlow, *et al.* 2007; Landis 2002). Early follicle development is a vital process for female fertility, and GDNF participates in a network of compensatory factors that act as checks and balances. Therefore, GDNF alone only modestly

alters the ovarian transcriptome. Manipulation of the primordial to primary follicle transition for potential therapeutic purposes will likely need to target many factors congruently.

In males, GDNF promotes spermatogonial stem cell self-renewal by signaling through src family kinases (Braydich-Stolle, *et al.* 2007; Oatley, *et al.* 2007). Interestingly, src signaling is necessary in females for antral follicle development (Roby, *et al.* 2005), and in primordial germ cells src kinase activation is vital for migration to the gonad and is a part of the KL signaling cascade (De Miguel, *et al.* 2002; Farini, *et al.* 2007). This leads to the speculation that GDNF may signal through src family kinases to promote primordial to primary follicle transition. Study of GDNF and GFR α 1 knockout animals is difficult as these animals die prior to the onset of primordial to primary follicle transition due to kidney agenesis (Pichel, *et al.* 1996; Sanchez, *et al.* 1996; Cacalano, *et al.* 1998). Further studies are necessary to fully characterize GDNF interactions in other extracellular signaling pathways in primordial follicle development. Since GDNF appears to act in an autocrine manner on the oocyte, other oocyte factors such as bFGF may be involved.

CONCLUSIONS

In summary, experiments using postnatal day-4 cultured rat ovaries were performed to explore how GDNF influences primordial to primary follicle transition. Observations from organ cultures, combined with immunohistochemical and RT-PCR localization, indicate that GDNF does promote the primordial to primary follicle transition, and GDNF and GFR α 1 are present specifically in oocyte cytoplasm of the primordial follicle. Combined data indicates GDNF is a part of the network of extracellular signaling factors that regulates primordial to primary follicle transition. Understanding the regulation of this process may lead to the development of therapies for reproductive ailments such as premature ovarian failure, or to methods to control the transition into menopause. An ability to regulate the primordial follicle pool size will directly influence female reproductive potential and associated endocrine abnormalities.

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