Characterization of Oocyte and Follicle Development in Growth Differentiation Factor-9-Deficient Mice

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Female mice null for the oocyte-specific gene product, growth differentiation factor-9 (GDF-9), a member of the transforming growth factor-β superfamily, exhibit primary infertility due to failed ovarian follicular development. The purpose of this study is to characterize oocyte and follicular differentiation as a function of animal age using cell culture and fluorescence, confocal, and electron microscopy. Analysis of follicles from GDF-9 homozygous mutant mice indicates that GDF-9-deficient oocytes grow more rapidly than control oocytes and that follicle growth ceases at the type 3b stage. Based on germinal vesicle (GV) chromatin patterns, fully grown oocytes isolated from GDF-9-deficient mice progress to advanced stages of differentiation equivalent to those found in antral follicles of control (heterozygous) mice. In vitro maturation of oocytes from homozygous mutant mice revealed that most oocytes are capable of resuming meiosis, with the ability to achieve meiotic completion reaching the highest levels in 6-week-old mice. Among the characteristic ultrastructural features of oocytes from homozygous mutant mice are perinuclear organelle aggregation, unusual peripheral Golgi complexes, and a failure to form cortical granules. Modified interconnections between granulosa cells and oocytes were also observed by ultrastructural (EM) and fluorescence microscopic analysis of follicles from GDF-9-deficient mice. These modifications included a decrease in the number of actin-based transzonal processes and modifications of microtubule-based projections that over time gave rise to invasion of the perivitelline space with eventual loss of oocyte viability. These cell–cell aberrations suggest a critical role for GDF-9 in the regulation of growth in preantral follicles through a mechanism involving bidirectional somatic cell–germ cell interactions.© 1998 Academic Press

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INTRODUCTION

The coordination of follicular development in the ovary with the growth and differentiation of its gametic cargo involves a bidirectional communication system between the oocyte and companion somatic cells. Transcellular communication within the follicular compartment occurs as a result of direct physical contacts (intercellular junctions) and the local production of soluble factors that are believed to act in an autocrine or paracrine fashion (Gougeon, 1996; Richards, 1994; Buccione et al., 1990). In mammals, where preantral follicle growth occurs by a process that is independent of extraovarian hormonal stimuli, it is likely that the bidirectional communication controls this early phase of folliculogenesis. As folliculogenesis proceeds, follicles become dependent on pituitary-derived gonadotropins at the time when both fluid accumulation in the antrum and granulosa cell proliferation contribute to the volume increase associated with preovulatory follicle growth (Richards, 1994). Oocytes in antral follicles secrete many factors that influence the proliferative and differentiative properties of granulosa cells (Buccione et al., 1990). At these later stages of follicle develop-
ment, oocyte-derived factors appear to modulate production of steroids (Vanderhyden et al., 1993; Vanderhyden and Tonary, 1995), hyaluronic acid (Tirone et al., 1997), plasminogen activator (Canipari et al., 1995), and LH receptors (Eppig et al., 1997). Furthermore, it has long been known that granulosa cells can regulate the state of the meiotic cell cycle by direct transfer of meiosis-arresting signals through heterocellular gap junctions (Tsafriri and Channing, 1975). The relative contribution of soluble factors and junction-mediated modes of communication during preantral follicle development remains unresolved, in large part due to the difficulty in experimentally separating these mechanisms of signaling.

The initial phase of folliculogenesis entails a protracted period of limited granulosa cell proliferation, recruitment of a thecal investment around the follicle, and in rodents the bulk of oocyte growth and differentiation (Pedersen, 1969). Evidence is available to suggest that growth promoting factors are derived from both the oocyte and granulosa cells. Stem cell factor or Kit-ligand (SCF, KL) mRNA expression has been demonstrated in granulosa cells and acts in a paracrine fashion through an interaction with the c-kit receptor expressed at the oocyte surface (Horie et al., 1991). Moreover, KL (Packer et al., 1994), in addition to other yet characterized factors (Ceccone et al., 1996), appears to have a direct effect on in vitro oocyte growth. Likewise, oocyte-derived factors have been directly implicated in the proliferation of granulosa cells during both preantral and antral follicle development (Vanderhyden et al., 1992). More recently, an oocyte-specific factor, growth differentiation factor-9 (GDF-9), has also been identified and is thought to participate in the early stages of follicle development.

GDF-9 is a member of the transforming growth factor-β superfamily that is expressed in growing mouse oocytes but not in oocytes of quiescent primordial follicles (McGrath et al., 1995). We have shown that a targeted deletion of exon 2 of the GDF-9 gene causes infertility in homozygous mutant female mice, whereas homozygous mutant males are fertile (Dong et al., 1996). Adult female GDF-9-deficient mice exhibit a hypogonadal phenotype characterized by primary follicle arrest and oocyte degeneration associated with follicular transformation. This hypogonadal state results in increased secretion of pituitary gonadotropins, but the degree to which this condition contributes to the gonadal phenotype reported previously is unclear.

To gain a more complete understanding of the normal process of folliculogenesis, particularly as related to the putative functions of GDF-9, further analysis of oocyte and somatic cell differentiation will be needed. Juvenile mice provide an optimal model system to clarify the role of GDF-9 in follicle and oocyte differentiation because the hypergonadotropic condition is minimized, follicle development is synchronized at this age, and given the progressive degeneration of follicles with increasing age, more follicles are available for study. In this study we define the growth and differentiation characteristics of oocytes contained within follicles from GDF-9-deficient mice. GDF-9-deficient mice afford a unique opportunity to study the role of a specific gamete-derived factor as a component of the bidirectional communication that operates during preantral follicle development. The results of these studies indicate that early events in oogenesis, including oocyte growth, proceed in a fashion independent of multilayer follicle development but that the completion of oogenesis and continuation of folliculogenesis are interrupted possibly due to disruptions in both soluble factor-mediated and cell–cell-mediated communication pathways.

METHODS

Collection and Measurement of Ovarian Follicles

Analysis of follicle growth was obtained by direct measurement of follicles isolated from 3-week-old unprimed gdf9m1+ (heterozygous) and gdf9m1/gdf9m1 (homozygous) mice (Dong et al., 1996). In all experiments, controls are unprimed heterozygous mice unless otherwise noted. All results obtained from heterozygous mice were consistent with previous studies using wild-type control mice. Ovaries were teased using 27-gauge needles in Eagle’s MEM (collection medium) with Hanks’ salts, and Hepes (Life Technologies, Gibco/BRL, Gaithersburg, MD) supplemented with 100 IU ml−1 penicillin, 100 μg ml−1 streptomycin, and 0.3% bovine serum albumin (BSA). The diameters of freshly isolated follicles and their enclosed oocytes were determined using a Zeiss dissecting stereomicroscope coupled to a Hitachi CCD camera and Sony PVM-100 monitor with a final magnification of 300× as measured with a calibrated micrometer (American Optical). The longest measured follicular diameter was recorded and over the follicle size range examined in this study (60–400 μm in diameter), follicles were spherical. The oocyte diameter was determined between paired points at the vitellus. Follicle growth curves were generated from measurement of at least 75 follicles in each group obtained from 4 different animals (8 ovaries per category). Data are plotted as mean oocyte diameters/follicle diameters within 10-μm increments using KaleidaGraph version 3.0.2 for Macintosh.

Collection and Culture of Mouse Oocytes

Oocytes were obtained from 6 heterozygous (gdf9m1+/+) and 10 homozygous (gdf9m1/gdf9m1) 3-week-old (21–23 days) unprimed mice and 8 gdf9m1+/+ and 10 gdf9m1/gdf9m1 6-week-old (42–45 days) unprimed mice (Dong et al., 1996). Oocytes from each group were isolated by manual dissection using a 27-gauge needle in collection medium (see above). Oocytes were fixed immediately or cultured to assess meiotic competence. Oocytes were cultured for 14 h in Eagle’s MEM with Earle’s salts (Life Technologies, Gibco/BRL), supplemented with 2 mM glutamine, 0.23 mM pyruvate, 100 IU ml−1 penicillin, 100 μg ml−1 streptomycin, and 0.3% BSA in a humidified atmosphere of 5% CO2 at 37°C (Schoeder and Eppig, 1984). Oocytes were fixed and extracted for 30 min at 37°C in a microtubule stabilizing buffer (Messinger and Albertini, 1991) containing 2% formaldehyde, 0.1% Triton X-100, 1 μM Taxol, 10 U ml−1 aprotinin, and 50% deuterium oxide (Herman et al., 1983). Oocytes were washed and stored in phosphate-buffered saline (PBS) blocking solution containing 0.2% sodium azide, 2% BSA, 2% powdered milk, 2% normal goat serum, 2% normal horse serum, 0.1 M glycine, and 0.01% Triton X-100 and placed at 4°C until processed.
Processing of Oocytes for Fluorescence Microscopy

To evaluate oocyte differentiation, fluorescence microscopy was used to assay markers of oocyte differentiation, including germinal vesicle chromatin patterns, and cytoplasmic microtubule organization as described by Matson and Albertini (1990). Meiotic competence assays were based on chromosome configuration, spindle organization and location, and the presence or absence of polar bodies. For microtubule localization, oocytes were incubated in a mixture of monoclonal α-tubulin and anti-β-tubulin at a 1:100 final dilution (Sigma Biosciences, St. Louis, MO) for 1 h at 37°C, followed by three washes in PBS-blocking solution. Oocytes were then incubated with a 1:50 dilution of affinity-purified fluoresceinated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 h at 37°C. Primary and secondary antibodies were diluted in blocking solution (see above). To visualize filamentous actin (F-actin), rhodamine-conjugated phalloidin (1 U ml⁻¹, Molecular Probes Inc., Eugene, OR) was included in the secondary antibody reagent. Oocytes were mounted in 50% glycerol/PBS containing sodium azide as an autofading reagent (25 mg ml⁻¹, Bock et al., 1985) and Hoechst 33258 (1 μg ml⁻¹, Polysciences Inc., Warrington, PA) to label chromatin. Labeled oocytes were photographed using a Zeiss IM-35 equipped with fluorescein (Zeiss 487709), Texas Red (Zeiss 487714), and Hoechst (Zeiss 487702) selective filter sets and a 50 W mercury arc lamp using 40× or 63× Neofluor objective lenses. Images were recorded on Kodak Tri-X-pan film using uniform exposure time and processed with Acufine developer (Acufine Inc., Chicago, IL) for 4.5 min at 25°C.

Confocal Microscopy Analysis of Control and GDF-9 Follicles and Oocytes

To analyze the three-dimensional organization of granulosa cell–oocyte interactions, a laser-scanning confocal microscopy was used. Isolated oocytes from 3- and 6-week-old gdf9m1 and gdf9m1 mice and intact follicles from 3-week-old CF-1 and gdf9m1 mice were processed as above for the localization of microtubules and F-actin. Samples were analyzed using the Odyssey XL confocal system (Noran Instruments, Inc., Middleton, WI) mounted on a Nikon Diaphot inverted microscope equipped with a Nikon 40×, 60×, and 100× Fluor objectives. The 488- and 528-nm lines of a krypton-argon laser were used to excite fluorescein and rhodamine markers. Dual-fluor images were collected using Intervision acquisition module software (Noran Instruments, Inc.) using a Silicon Graphics Inc. (Indy 300 workstation (Mountain View, CA). The acquisition module software allows for adjustment of scan mode, sample time, and image-processing parameters. All images were collected using slow scan mode, with a sample time of 400 ns, and real-time image processing with jump averaging (n = 128). A series of Z-axis image stacks was acquired using 1.0-μm steps on intact follicles and 0.5-μm steps for isolated oocytes.

To determine the organization and density of transzonal processes, isolated oocytes were optically sectioned at an optical plane confocal with the germinal vesicle. An image stack consisting of three images was collected at 0.5-μm steps from 3-week-old gdf9m1/+ and gdf9m1/gdf9m1 and 6-week-old gdf9m1/+ and gdf9m1/gdf9m1 mice, six oocytes per experimental group. Image stacks were loaded into 3-D Intervision software and rendered for analysis in the Z axis. Transzonal quantification of reconstructed oocytes was achieved by counting the number of transzonal processes in a 10-μm arc of the zona pellucida. Three separate areas of each oocyte were analyzed. Data are represented as the mean density of transzonal processes per 75 μm³ of zona (based on 10 μm length, 1.5 μm depth, and 5.0 μm zona width).

Electron Microscopy

Electron microscopic analysis was performed on oocytes harvested from 3- and 6-week-old gdf9m1/+ and gdf9m1/gdf9m1 mice. Oocytes were fixed in 2% glutaraldehyde, 3% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4, followed by 1% osmium tetroxide. Following dehydration, samples were embedded in Poly Bed 812 and thin sections were stained with uranyl acetate and lead citrate before viewing. Sections were examined using a Philips CM10 transmission electron microscope (Philips Technologies, Cheshire, CT). Cortical granule (CG) number was calculated as the number observed per 100-μm oocyte surface area and expressed as mean number of CGs ± SEM. The study was conducted on homozygous mutant (n = 23) and heterozygous (n = 11) oocytes.

RESULTS

Characterization of Oocyte and Follicle Growth in Control and GDF-9-Deficient Ovaries

Our previous studies documented that GDF-9-deficient adult mice exhibited an increased oocyte size relative to controls and that follicles exhibited limited overall growth (Dong et al., 1996). In this study, a direct comparison of oocyte growth relative to follicle growth was undertaken using intact dissected follicles in which individual oocyte and follicle dimensions could be accurately monitored. Follicle growth curves were generated from an analysis of follicles from 3-week-old heterozygous (n = 75) and homozygous (n = 75) mice. Data from control mice show that oocyte growth is linear over a follicle size of roughly 60–150 μm, but with further follicle growth, no increase in oocyte size is observed (Fig. 1). In contrast, follicles from GDF-9-deficient mice never exceed a maximum diameter of 130 μm and fail to exhibit an inflection point relative to oocyte size. Unlike the maximum mean diameter of 70 μm recorded in oocytes from control mice, oocytes from homozygous mutant mice reach a maximum diameter of 80 μm. Although follicles were observed having a mean diameter greater than 110 μm, these invariably contained degenerate oocytes. The observation that the maximum diameter achieved by follicles with intact oocytes from GDF-9-deficient mice is 110 μm is further supported by this study, showing that less than 5% of follicles recorded from GDF-9-deficient mice are larger than 110 μm.

To further address the growth properties of follicles from GDF-9-deficient mice, direct measurements of granulosa cell number were undertaken to assess the relative contribution of the oocyte or somatic cells to follicle size. Follicles from 3-week-old GDF-9-deficient mice were isolated and randomly grouped according to follicle size as either growing (50–70 μm diameter) or full grown (~100 μm diameter). Using fluorescence microscopy of Hoesch
33258-stained samples, the number of granulosa cells contained within each follicle was determined. Growing follicles had a mean granulosa cell number of 147.4 ± 21.3 (n = 5) from heterozygous mice (+/− ○) and homozygous mice (−/− □) are plotted as a function of follicle size.

FIG. 1. Follicle growth curves were generated from measurements of follicle diameters and oocyte diameters. Data were collected on intact ovarian follicles from control and GDF-deficient mice. The diameters of follicle-enclosed oocytes (n = 75) from heterozygous animals (+/− ○) and homozygous animals (−/− □) are plotted as a function of follicle size.

Characterization of GV Chromatin Patterns and Meiotic Competence Acquisition in Oocytes from Control and GDF-9-Deficient Mice

Previous studies have demonstrated that modifications of germinal vesicle chromatin and microtubule organization occur during oocyte growth and meiotic competence acquisition in the mouse (Mattson and Albertini, 1990; Wickramasinghe et al., 1991). Analysis of oocytes from GDF-9 heterozygous mice by fluorescence microscopy demonstrated germinal vesicle chromatin and microtubule patterns consistent with previous reports (Mattson and Albertini, 1990). Germinal vesicle stage oocytes from GDF-9-deficient mice exhibit typical unrimmed and rimmed chromatin patterns in full-grown oocytes (diameter ≥ 70 μm; Figs. 2A and 2C). Dense interphase microtubule arrays were observed in all oocytes from homozygous mutant mice independent of whether germinal vesicle chromatin patterns were rimmed or unrimmed (Figs. 2B and 2D). Rimmed GV-stage oocytes were occasionally observed that contain variable numbers of somatic cells within the perivitelline space (Figs. 2G and 2H). This unusual relationship was characterized by the close apposition of somatic cells to the oocyte surface where circular microtubule arrays were evident (Fig. 2H). The presence of somatic cells in the perivitelline space was consistently noted in GV-stage oocytes from GDF-9-deficient animals. A substantial fraction of oocytes, isolated from 3-week-old GDF-9-deficient mice, exhibit signs of spontaneous parthenogenetic activation (see below). Activated oocytes typically displayed a single polar body, interphase nuclei with multiple nucleoli, and interphase microtubule arrays (Figs. 2E and 2F). All spontaneously activated oocytes contained somatic cells within the perivitelline space, suggesting that the loss of normal "contacts" between the oocyte and the surrounding granulosa cells was inducing this activation (see below).

FIG. 2. Germinal vesicle chromatin patterns in oocytes from GDF-9-deficient mice. Corresponding micrographs of chromatin (left) and microtubule (right) patterns of freshly isolated oocytes. Oocytes were labeled with Hoechst 33258 for chromatin configurations and antitubulin (right) to evaluate microtubule organization. Oocytes contain either unrimmed (A) or rimmed (C) germinal vesicle-stage oocytes that exhibit an interphase cytoplasmic microtubule array (B, D). Activated oocytes contain multinucleolar chromatin arrays consistent with a two-cell-stage embryo (E) and a dense interphase array of microtubules that also reveal first polar body formation (F). Several granulosa cell nuclei (E) are also observed within the perivitelline space. Somatic cell invasion of the oocyte was also observed in a subset of oocytes from GDF-9-deficient mice (G and H). Arrowheads indicate circular microtubule arrays of somatic cell bodies juxtaposed to the oocyte surface. Scale bar, 20 μm.
As mentioned previously, germinal vesicle chromatin remodeling is a useful marker for oocyte growth and meiotic competence acquisition. To monitor the extent of nuclear differentiation, staging of germinal vesicle chromatin patterns was undertaken on oocytes from heterozygous and homozygous mutant mice at 3 and 6 weeks of age. Oocytes from 3-week-old heterozygous and homozygous mutant mice exhibited approximately a 1:1 ratio of unrimmed (GVu) to rimmed (GVr) germinal vesicles as shown in Table 1. While 99% of oocytes from heterozygous mice are at the GV stage, a reduced percentage of GV-stage oocytes (72%) was observed in GDF-9-deficient mice. The remaining oocytes had either reinitiated meiosis (6%) or had undergone spontaneous parthenogenetic activation in vivo (22%). This finding suggests that oocytes of 3-week-old GDF-9-deficient mice are prone to parthenogenetic activation. In 6-week-old mice, both heterozygous and homozygous mutant mice display a high percentage of rimmed oocytes (GVr). Surprisingly, there were no oocytes in the 6-week-old GDF-9-deficient mice which had undergone parthenogenetic activation (see Discussion). Collectively, these data demonstrate that oocytes from GDF-9-deficient mice undergo changes in chromatin remodeling consistent with normal oocyte differentiation and the acquisition of meiotic competence.

To determine whether oocytes from GDF-9-deficient mice are able to express meiotic competence, germinal vesicle-containing oocytes were cultured for 14 h under conditions that support meiotic maturation (Schroeder and Eppig, 1984). Following culture, oocytes were fixed and assessed for meiotic progression by analysis of chromosome and spindle organization using fluorescence microscopy. In 3-week-old control mice, the majority of oocytes resume meiosis (82.2%) and are capable of meiotic maturation (45.8%) and arrest at metaphase of meiosis II (Table 2). In homozygous mutant mice, 56% of oocytes resume meiosis; however, few of these (8.8%) reach metaphase of meiosis II. Most oocytes from GDF-9-deficient mice that resume meiosis in culture are unable to complete meiotic maturation, and arrest at germinal vesicle breakdown or meta-

### Table 1

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<th>Animal age and genotype</th>
<th>Percentage of the total number of oocytes examined (number observed)</th>
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<td>3 weeks</td>
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Note. Data are given as the percentage of the total number of oocytes examined. n, total number of oocytes examined; GVu, unrimmed germinal vesicle chromatin patterns; GVr, rimmed germinal vesicle chromatin patterns; GVBD, abnormal germinal vesicle breakdown; M1, arrested at metaphase of meiosis I; MII, arrested at metaphase of meiosis II; Act, spontaneous parthenogenetically activated.

### Table 2

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<th>Animal age and genotype</th>
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Note. Data are given as the percentage of the total number of oocytes examined. n, total number of oocytes examined; GVu, unrimmed germinal vesicle chromatin patterns; GVr, rimmed germinal vesicle chromatin patterns; GVBD, abnormal germinal vesicle breakdown; M1, arrested at metaphase of meiosis I; MII, arrested at metaphase of meiosis II; and Act, spontaneous parthenogenetically activated; MC, percentage resumed meiosis.

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phase of meiosis I, or undergo parthenogenetic activation (Table 2). In all cases, gross defects in spindle and chromosome organization were observed in in vitro matured oocytes from GDF-9-deficient 3-week-old mice. In contrast, oocytes from 6-week-old GDF-9-deficient exhibited quantitative and qualitative improvement in meiotic competence expression. At 6 weeks of age, 29.9% of the oocytes from homozygous mutant mice successfully complete meiosis and arrest at metaphase of meiosis II with normal spindle organization and chromosome alignment (Table 2). Collectively, these results are consistent with the analysis of freshly isolated oocytes (Table 1) in demonstrating that between 3 and 6 weeks of age, there is a progressive increase in the acquisition of meiotic competence and in the ability to maintain competent oocytes in a GV-arrested state in vivo. While there are qualitative differences in the expression of meiotic competence in oocytes from GDF-9-deficient mice (spindle abnormalities, premature arrest), this aspect of oogenesis appears to be relatively normal and suggests that GDF-9 may play a limited role in the regulation of meiotic competence acquisition and expression.

Ultrastructural Characteristics of GDF-9-Deficient Oocytes

Transmission electron microscopy of ovaries from heterozygous mice revealed normal patterns of cell surface organization and organelle density and distribution in full-grown oocytes as documented previously (Wassarman and Albertini, 1994; Zamboni, 1972). In contrast, several novel ultrastructural modifications were observed in oocytes from 3- and 6-week-old GDF-9-deficient mice. As shown in Fig. 3A, clusters of oocyte microvilli are located between large projections from surrounding follicle cells that adhere to nonmicrovillar regions of the oocyte cell surface (Fig 3A, arrowheads). Several other unique organelle alterations are apparent in oocytes from GDF-9-deficient mice. Multiple Golgi complexes are dispersed in the oocyte cortex but these complexes are unusual in lacking stacks of Golgi lamellae (Fig. 3C, arrow). These complexes consist of clusters of single lamellae with associated vesicles (Fig. 3D). Cortical granules are decreased in number (mean, n = 33) in oocytes from homozygous mutant mice (1.78 ± 0.44) compared to oocytes from heterozygous controls (10.2 ± 1.33). Other organelles such as endoplasmic reticulum crystalline lattices, mitochondria, and multivesicular bodies are apparently normal in both number and ultrastructure (Fig. 3C). Gap junctions were observed on the oocyte surface (data not shown). At more advanced stages, follicle cell bodies are commonly found in the perivitelline space. Somatic cell invasion of the perivitelline space is associated with a loss of oocyte viability as indicated by perinuclear organelle aggregation, lipid droplet accumulation, and eventually fragmentation and lysis of the oocyte (Fig. 3B). Thus, most ultrastructural features appear normal in oocytes from GDF-9-deficient mice prior to somatic cell invasion with the exception of the organization of the Golgi and the absence of cortical granules.

Confocal Microscopy Analysis of Follicular Organization and Transzonal Projections in Control and GDF-9-Deficient Mice

To evaluate the organization and structural integrity of cellular interconnections within follicles from control and GDF-9-deficient mice, laser scanning confocal microscopy was employed. Filamentous actin (F-actin, phalloidin) and microtubule (anti-tubulin) labeling of intact follicles permits a direct assessment of cell boundary and cytoskeletal patterns that are useful in defining contact relationships between cells and their substrates. Control preantral follicles consist of loosely organized granulosa cells with cortical microtubules and occasional focal adhesions between somatic cells (Fig. 4A). In contrast, granulosa cell shape, arrangement, and cytoskeletal organization are markedly altered in follicles from GDF-9-deficient mice (Fig. 4B). In the follicles from GDF-9-deficient mice, granulosa cells are polygonal and densely packed with extensive areas of adhesive contact apparent throughout the follicle. Moreover, colocalization of F-actin and tubulin indicates that an extensive cortical cytoskeleton is present in the granulosa cells of arrested follicles from GDF-9-deficient mice. Analysis of optical sections through intact control follicles reveals two prominent actin-rich zones at the interface between granulosa cells and the oocyte (Fig. 4C, arrowheads). Somatic cell attachments to the outer surface of the zona pellucida form an irregular contour that is clearly separable from the prominent deposition of actin in the oocyte cortex. In contrast, this interface in follicles from GDF-9-deficient mice is conspicuously absent in F-actin at both the outer zona and oocyte cortex (Fig. 4D) which is more evident when isolated oocytes are examined by laser scanning confocal microscopy (see below).

Oocytes from both groups exhibit a dense interphase array of cytoplasmic microtubules that subtends a thick layer of cortical F-actin (Figs. 4E and 4F, arrowhead). Heterozygous mice display an organized array of actin-based transzonal projections that originate from an outer layer of granulosa cell actin associated with the external surface of the zona (Fig. 4E, arrowhead) from which occasional microtubule processes with small multiple projections are apparent (Fig 4E). In oocytes obtained from GDF-9-deficient mice, the organization of the transzonal projections is altered. Transzonal projections appear to be collapsed toward the oocyte, are decreased in number, and are disorganized (Fig 4F, arrowhead).

Reconstructions of six oocytes in control and GDF-9-deficient samples (see Materials and Methods), were used to quantify the density of actin-rich transzonal processes within the zona pellucida. The mean number of actin-rich transzonal processes (TZPs) in oocytes isolated from 3-week old mice is 13.1 ± 0.8 TZPs/75 μm³ zona in heterozygous mice but is decreased to 7.4 ± 1.0/75 μm³ in
Electron microscopy of ovaries from GDF-9-deficient mice. Ultrastructural analysis of oocytes reveals several novel features. (A) Somatic cell invasion of the perivitelline space is characterized by large dense processes that coarse through the zona and contact the oocyte surface at sites that lack microvilli (arrowheads). (B) Oocyte degeneration is characterized by the accumulation of lipid droplets and aggregation of organelles to the membrane of the degenerating germinal vesicle. (C) Although mitochondria and ER (circular membrane structures) appear normal, changes in Golgi organization are observed (arrow). (D) High-power view of the Golgi structures consisting of vesicular aggregates and lacking normal lamellar stacks.
GDF-9-deficient mice. By 6 weeks of age, oocytes from homozygous mutant mice continue to exhibit a reduced density of TZPs (8 ± 0.6/75 μm³) relative to controls. These studies clearly indicate that arrested primary follicles from GDF-9-deficient mice exhibit alterations in adhesive interactions between somatic cells and the oocyte.

**DISCUSSION**

Development of the mammalian ovarian follicle is traditionally viewed as a biphasic process (Hirschfield, 1991). In rodents, primary to secondary follicle growth is protracted (~10 days) and involves an increase in oocyte volume proportional to the increase in follicular volume, with limited granulosa cell proliferation (Pedersen, 1969). This phase of growth occurs in the absence of gonadotropins and is regulated by either direct local interactions between the oocyte and somatic cells or the local production of growth factors by the oocyte and/or the granulosa cells (Halpin et al., 1996). Follicle development from the early antral (tertiary) to Graafian stages is likely influenced by the local production of growth factors as well as gonadotropins. The increase in follicle volume is attributable to rapid granulosa cell proliferation (~2 days) and the accumulation of follicular fluid (Pedersen, 1969). The objective of the present study was to understand the regulation of preantral follicle growth in mice with respect to the putative role of growth differentiation factor-9 in normal and GDF-9-deficient mice.

To date, two locally produced and cell-specific growth factors have been implicated in the regulation of preantral follicle growth: Kit ligand (also known as stem cell factor, SCF; steel factor, Sl; mast cell growth factor, MGF; Manova et al., 1993; Matsui et al., 1990) and GDF-9 (Dong et al., 1996 and the present study). Kit ligand is produced by granulosa cells and receptor c-kit, a receptor tyrosine kinase, is expressed at the oocyte surface (Horie et al., 1991). Using blocking antibodies to KL, Packer et al., (1994) have shown that KL signaling is necessary for oocyte growth. Furthermore, Yoshida et al., (1997) observed a c-kit requirement in all stages of follicle development, including follicle recruitment, and all later stages of development (preantral, antral, preovulatory). These studies suggest that one aspect of oocyte growth, as well as the expression of oocyte factors that participate in follicle development and differentiation, is dependent on the activation of an oocyte-induced tyrosine kinase pathway. In these studies, KL appears to be a positive regulator of both oocyte and follicle growth that may need to be counterbalanced by a negative regulator to ensure that folliculogenesis and oogenesis occur in a synchronized fashion. Recently, it has been shown that KL may stimulate theca cell growth and steroid production in the absence of gonadotropins (Parrott and Skinner 1997); however, this effect is more likely to play a role during the later stages of follicle development after c-kit expression is detected in the thecal layer.

Our study implicates GDF-9 as a candidate regulator of follicle growth that may constitute one element of a negative-feedback pathway. This hypothesis is supported by our analysis of oocyte growth and differentiation characteristics in GDF-9-deficient mice. Analysis of the relationship between oocyte size and follicle size in isolated follicles revealed that the rate of oocyte growth relative to follicle size is accelerated in GDF-9-deficient mice when compared to heterozygous controls. Furthermore, oocytes achieve a 70% increase in volume (over controls) in the absence of GDF-9. The relative contribution of oocyte volume to total follicular volume (19%, controls vs 63%, GDF-9-deficient in small (70 μm) follicles and 19% vs 29% in 110 μm follicles) further illustrates the limited growth potential of follicles within this model system. While the transformation from squamous to cuboidal shape occurs normally in granulosa cells of follicles from GDF-9-deficient mice (Dong et al., 1996), our calculations of total cell number indicate that at most four to five doublings occur, indicating limited proliferative potential. This suggests that initially granulosa cell proliferation does not require GDF-9, but that GDF-9 may be required for proliferation and differentiation at subsequent stages of follicle development. Moreover, enhanced oocyte growth may be due to the loss of negative signals either as a direct autocrine influence (i.e., on the oocyte) or as a secondary influence mediated by the granulosa cells. Clearly, the complexity of interactions remains to be elucidated especially with respect to the role of secreted factors and direct somatic cell–oocyte contacts (i.e., gap junctions, adherens junctions). In this regard, it is noteworthy that KL mRNA levels are upregulated in granulosa cells of follicles from GDF-9-deficient mice (Elvin and Matzuk, unpublished data), and as described above, physical interactions between granulosa cells and oocytes are strikingly modified in follicles from GDF-9-deficient mice.

The deficiencies in follicle growth and the increased rate and extent of oocyte growth in the absence of GDF-9 expression also raise questions about the status of oocyte differentiation. To address these issues, we assessed morphological markers of oocyte differentiation and assayed the ability of these oocytes to acquire, modulate, and express meiotic competence. These processes are normally regulated late in the growth phase of oogenesis and are necessary to ensure meiotic progression (Wickramasinghe et al., 1991). At the ultrastructural level, oocytes from GDF-9-deficient mice appear to secrete and assemble the zona pellucida (Fig 3). However, few if any cortical granules are evident in oocytes from GDF-9-deficient mice, and aggregates of vesicular membranes are present rather than the multiple organized Golgi lamellae found in full-grown oocytes in most mammals (Wassarman and Albertini, 1990). The lack of cortical granules and well-developed Golgi complexes suggests that late secretory events in oogenesis are compromised in the absence of GDF-9. Whether this defect is a consequence of the arrested state of
the follicle or an autocrine effect of GDF-9 on the terminal phase of oogenesis remains to be elucidated.

Evaluation of nuclear chromatin configurations demonstrated that a high proportion of oocytes in GDF-9-deficient mice undergo nuclear rimming, an event that normally occurs during antrum formation and precedes the acquisition of meiotic competence (Mattson and Albertini, 1990; Wickramasinghe et al., 1991). These oocytes acquired meiotic competence as evidenced by their ability to resume meiosis in vitro comparable to oocytes obtained from either 3- or 6-week-old control mice. Although many oocytes from GDF-9-deficient mice failed to complete maturation in vitro, instead arresting at metaphase of meiosis I, it is interesting to note that oocytes obtained from 3-week-old deficient mice exhibited a tendency to spontaneously activate either in vivo (22%) or in vitro (16%) (Tables 1 and 2). This finding suggests that after oocytes acquire meiotic competence, follicles in younger GDF-9-deficient mice are unable to maintain meiotic arrest. Studies using the LT/Sv strain of mice have established a precedent for spontaneous activation of oocytes encosed within ovarian follicles (Ep- pig, 1978). Whether an impairment in meiotic arrest in GDF-9-deficient animals is due to decreased granulosa cell number, due to a decreased transfer of a meiotic inhibitor to the oocyte, or due to a change in oocyte-somatic cell contact below a certain threshold is unclear (see below).

It is well established that transzonal projections from granulosa cells to the oocyte form gap junctions through which it is presumed that putative low-molecular-weight inhibitors are delivered (Anderson and Albertini, 1976). For this reason, we examined the organization of TZPs in follicles and oocytes from control and GDF-9-deficient mice by confocal fluorescence microscopy. There are two aspects of TZP organization that are strikingly altered in the ovaries of GDF-9-deficient mice relative to controls. First, fewer TZPs are evident per unit volume of zona pellucida in oocytes from GDF-9-deficient mice than from controls (see Results). This decrease in the Tzp number may be due to the reduced number of granulosa cells present in these developmentally arrested follicles. Second, the structural integrity and fate of the TZPs are highly unusual; these structures are not tightly anchored to the external surface of the zona pellucida and ultimately give rise to fewer large projections that invade the perivitelline space and encase the oocyte by a process that bears similarity to phagocytosis. The appearance of granulosa cell processes encasing the oocyte coincides with the onset of degenerative changes in the oocyte (lipid accumulation, organelle aggregation, loss of microvilli, chromatin compaction), implicating this invasive somatic cell behavior in the overt germ cell loss observed in GDF-9-deficient mice.

The alterations in TZPs in follicles from GDF-9-deficient mice raise the prospect that defects in cell–cell and cell–matrix interactions may contribute to the ovarian phenotype of GDF-9-deficient mice. The present studies document abnormalities in the process of oogenesis in arrested follicles in GDF-9-deficient mice. Previous studies demonstrated that follicles from GDF-9-deficient mice become a steroidogenic corpora lutea-like structures upon oocyte degeneration (Dong et al., 1996). Together these data suggest that once changes in the adhesive interaction between the somatic cells and the oocyte begin, the follicles pursue a novel differentiation pathway that was negatively regulated by the presence of a viable oocyte. In addition to the defect in Tzp organization noted above, other adhesive interactions are apparently modified in GDF-9-deficient mice. Polymerized actin can be used to monitor the adhesive behavior of neighboring cells; the decreased filamentous actin associated with the external surface of the zona suggests that somatic cells are less firmly attached to the zona pellucida in GDF-9-deficient mice (Fig. 4). However, granulosa cell–granulosa cell interactions are more prominent in GDF-9-deficient mice than those observed in control follicles at a comparable stage of development. Finally, adhesion to the basement membrane is strikingly reduced. The increase in somatic cell adhesion relative to the diminished interactions with the basement membrane and germ cell may demonstrate a novel mechanism by which oogenesis proceeds independent of folliculogenesis in this animal model. Thus, the extent to which GDF-9 integrates the processes of oogenesis and folliculogenesis in this early pivotal stage of follicle development will require further examination of direct physical linkages between both the somatic cell and germ cell, as well as soluble factors and their receptors such as KL and c-kit. These studies emphasize the importance of GDF-9 in engaging the somatic cell compartment to temper the rate, extent, and quality of gametogenesis.

**FIG. 4.** Laser scanning confocal microscopy of isolated follicles and oocytes from control and GDF-9-deficient mice. Dual-labeled images of intact follicles and oocytes stained for filamentous actin shown in red and microtubules in green. Three-dimensional (A and B) and two-dimensional (C and D) reconstructions of follicles from control (A and C) and GDF-9-deficient mice (B and D). Control follicles (A and C) consist of loosely organized granulosa cells with cortical microtubule networks. Two prominent actin-rich zones are evident at the oocyte surface and outer zona surface (C, arrowhead). Follicles from GDF-9-deficient animals exhibit a tightly packed epithelium with an extensive overlapping actin and microtubule network within the granulosa cells (B). The two zones of filamentous actin are less pronounced in follicles from GDF-9-deficient animals (D). Three-dimensional reconstructions of isolated oocytes from control (E) and GDF-9-deficient (F) mice illustrate the alterations in transzonal projections. Control oocytes (E) have an organized array of actin TZPs that further illustrates the two zones of actin polymerization (arrowheads). Oocytes from GDF-9-deficient mice have fewer projections that are collapsed toward the oocytes surface (F, arrowheads). Optical sections through the germinal vesicle are shown. Scale bar, 20 μm.
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