Paracrine Actions Of Growth Differentiation Factor-9 in the Mammalian Ovary

Julia A. Elvin, Amander T. Clark, Pei Wang, Neil M. Wolfman, and Martin M. Matzuk

Department of Pathology (J.A.E., A.T.C., P.W., M.M.M.)
Department of Molecular and Human Genetics (J.A.E., M.M.M.), and
Department of Cell Biology (M.M.M.)
Baylor College of Medicine
Houston, Texas 77030

Genetics Institute (N.M.W.)
Cambridge, Massachusetts 02140

Although the transforming growth factor-β (TGF-β) superfamily is the largest family of secreted growth factors, surprisingly few downstream target genes in their signaling pathways have been identified. Likewise, the identities of oocyte-derived secreted factors, which regulate important oocyte-somatic cell interactions, remain largely unknown. For example, oocytes are known to secrete paracrine growth factor(s) which are necessary for cumulus expansion, induction of hyaluronic acid synthesis, and suppression of LH receptor (LHR) mRNA synthesis. Our previous studies demonstrated that absence of the TGF-β family member, growth differentiation factor-9 (GDF-9), blocks ovarian folliculogenesis at the primary follicle stage leading to infertility. In the present study, we demonstrate that mouse GDF-9 protein is expressed in all oocytes beginning at the type 3a follicle stage including antral follicles. To explore the biological functions of GDF-9 in the later stages of folliculogenesis and cumulus expansion, we produced mature, glycosylated, recombinant mouse GDF-9 using a Chinese hamster ovary cell expression system. A granulosa cell culture system was established to determine the role of GDF-9 in the regulation of several key ovarian gene products using semiquantitative RT-PCR. We find that recombinant GDF-9 induces hyaluronan synthase 2 (HAS2), cyclooxygenase 2 (COX-2), and steroidogenic acute regulator protein (StAR) mRNA synthesis but suppresses urokinase plasminogen activator (uPA) and LHR mRNA synthesis. Consistent with the induction of StAR mRNA by GDF-9, recombinant GDF-9 increases granulosa cell progesterone synthesis in the absence of FSH. Since induction of HAS2 and suppression of the protease uPA in cumulus cells are key events in the production of the hyaluronic acid-rich extracellular matrix which is produced during cumulus expansion, we determined whether GDF-9 could mimic this process. Using oocytectomized cumulus cell-oocyte complexes, we show that recombinant GDF-9 induces cumulus expansion *in vitro*. These studies demonstrate that GDF-9 can bind to receptors on granulosa cells to regulate the expression of a number of gene products. Thus, in addition to playing a critical function as a growth and differentiation factor during early folliculogenesis, GDF-9 functions as an oocyte-secreted paracrine factor to regulate several key granulosa cell enzymes involved in cumulus expansion and maintenance of an optimal oocyte microenvironment, processes which are essential for normal ovulation, fertilization, and female reproduction. (Molecular Endocrinology 13: 1035–1048, 1999)

0888-8809/99/$3.00/0
Molecular Endocrinology

INTRODUCTION

The oocyte plays an integral role in regulating folliculogenesis within the mammalian ovary. In particular, the oocyte has been shown to act on granulosa cells to regulate follicle formation perinatally, stimulate granulosa cell proliferation, modulate granulosa cell gene expression, and influence steroidogenesis (reviewed in Ref. 1). Granulosa cells in the preovulatory follicle can be separated into two populations with respect to their proximity to the oocyte; cumulus granulosa cells closely surround the oocyte while the mural granulosa cells are located around the periphery of the follicle separated from the oocyte by an antrum. Cumulus cells secrete a hyaluronic acid-rich matrix during cumulus expansion and are extruded with the oocyte during ovulation. This expanded matrix is a critical factor for reproductive integrity since it binds

the oocyte and cumulus cells together, facilitates follicular extrusion and oviductal fimbria capture, and allows sperm penetration and fertilization (2). On the other hand, mural granulosa cells synthesize proteases important for follicle rupture at ovulation, remain within the ovary after the cumulus cell-oocyte complex is released, and eventually undergo terminal differentiation to form the corpus luteum. These positional and functional differences in the granulosa cell populations suggests that gradients of oocyte-secreted factors modulate gene expression and eventual cell differentiation. *In vitro* studies demonstrate that oocyte-secreted growth factors regulate granulosa cell synthesis of hyaluronic acid, urokinase plasminogen activator (uPA) and LH receptor (LHR) as well as steroidogenesis and luteinization (2–7). However, the identities of the oocyte-derived factors that regulate these somatic cell functions remain largely unknown.

The transforming growth factor β (TGF-β) superfamily is comprised of secreted peptide growth factors which are critical for regulating a variety of developmental events, including cell proliferation, differentiation, matrix secretion, and apoptosis during embryogenesis and in the adult (8, 9). These family members are synthesized as prepropeptides which are processed to form mature, disulfide-linked dimers. Several TGF-β family members have been shown to be expressed in the ovary, including Müllerian inhibiting substance, inhibin α, activin βA, activin βB, growth differentiation factor 9 (GDF-9), bone morphogenetic protein (BMP)-6, and BMP-15, and several of these factors have been shown *in vivo* and/or *in vitro* to play important roles in regulating reproductive function (9–15). Within the ovary, GDF-9, BMP-6, and BMP-15 are expressed specifically in the oocyte (12–15). In particular, GDF-9 and BMP-15 mRNA are expressed specifically in the oocyte of the type 3a preantral follicle (small primary follicle with one-layer of granulosa cells), and expression persists in oocytes throughout all stages of folliculogenesis and in cumulus cell-oocyte complexes after ovulation (Refs. 14 and 15 and J. A. Elvin and M. M. Matzuk, unpublished data). We have shown that a knockout of the GDF-9 gene leads to infertility due to a block at the type 3b (primary) follicle stage, absence of thecal layer formation, and defects in oocyte meiotic competence (16–18). However, the potential role of GDF-9 at later stages of folliculogenesis is unknown.

In this report, we demonstrate that GDF-9 protein is synthesized in oocytes at all stages of folliculogenesis beginning at the one-layer (type 3a) follicle stage coinciding with the expression of the GDF-9 mRNA. Using an *in vitro* granulosa cell culture system, we demonstrate that recombinant mouse GDF-9 can mimic several of the previously reported paracrine effects of the oocyte. GDF-9 can induce cumulus expansion in the absence of the oocyte, stimulate hyaluronan synthase 2 (HAS2), cyclooxygenase 2 (COX-2), and steroidogenic acute regulator protein (StAR) mRNA expression, suppress uPA and LHR mRNA expression, and increase progesterone synthesis in the absence of FSH or when supplemented with low levels of FSH. These effects cannot be duplicated by at least two other known oocyte-expressed TGF-β family members (*i.e.* BMP-6 and BMP-15). These studies demonstrate that receptors for GDF-9 are present on granulosa cells and that GDF-9 plays multifunctional roles in oocyte-granulosa cell communication and regulation of follicular differentiation and function.

RESULTS

Immunohistochemical Detection of GDF-9 in Mouse Ovaries

Using a monoclonal antibody to human GDF-9, GDF-9 protein is specifically detected in mouse oocytes (Fig. 1). At low-power magnification of an immunohistochemically-stained ovary (Fig. 1A), GDF-9 immunoreactivity is detected only in oocytes, whereas oocytes in GDF-9-deficient ovaries do not stain (Fig. 1B). Primordial (type 2) oocytes are negative (Fig. 1C) consistent with the absence of GDF-9 mRNA expression (Ref. 14 and J. A. Elvin and M. M. Matzuk, unpublished data). GDF-9 immunoreactivity is first seen at low (and variable) levels in oocytes of type 3a follicles (follicles with $<$20 cuboidal granulosa cells/cross-section arranged in one concentric layer around the oocyte) and is higher in the oocytes of type 3b follicles and beyond (Fig. 1, D and E). Full-grown oocytes of multilayer preantral follicles (Fig. 1, A, C–E) stain more intensely for GDF-9, and GDF-9 immunoreactivity is clearly detected in oocytes of cumulus cell-oocyte complexes of large antral and preovulatory follicles (Fig. 1, A and F). As expected for a secreted peptide, GDF-9 immunoreactivity is excluded from the germinal vesicle (*i.e.* nucleus). Interestingly, an asymmetric staining pattern is frequently observed within the oocytes likely due to the detection of the precursor forms of GDF-9 within the oocyte endoplasmic reticulum and Golgi complex (19). Thus, GDF-9 mRNA and protein are synthesized by oocytes of all growing follicles, suggesting that it could function at all stages of folliculogenesis.

Production of Recombinant Mouse GDF-9

To study the function of GDF-9 at later stages of folliculogenesis, it was necessary to produce recombinant GDF-9. Chinese hamster ovary cells (CHO) cells were stably transfected with an expression vector containing both a full-length mouse GDF-9 cDNA and a cDNA for PACE, a prepropeptide sequence-cleaving enzyme. Western blot analysis of medium from CHO cells containing the mouse GDF-9 expression vector, analyzed under denaturing conditions, demonstrated two unique bands of approximate molecular masses 21 kDa and 60 kDa (Fig. 2), which were not present in mock-conditioned medium (data not shown). Proteolytic processing of GDF-9 at its tetrabasic R-R-R-R

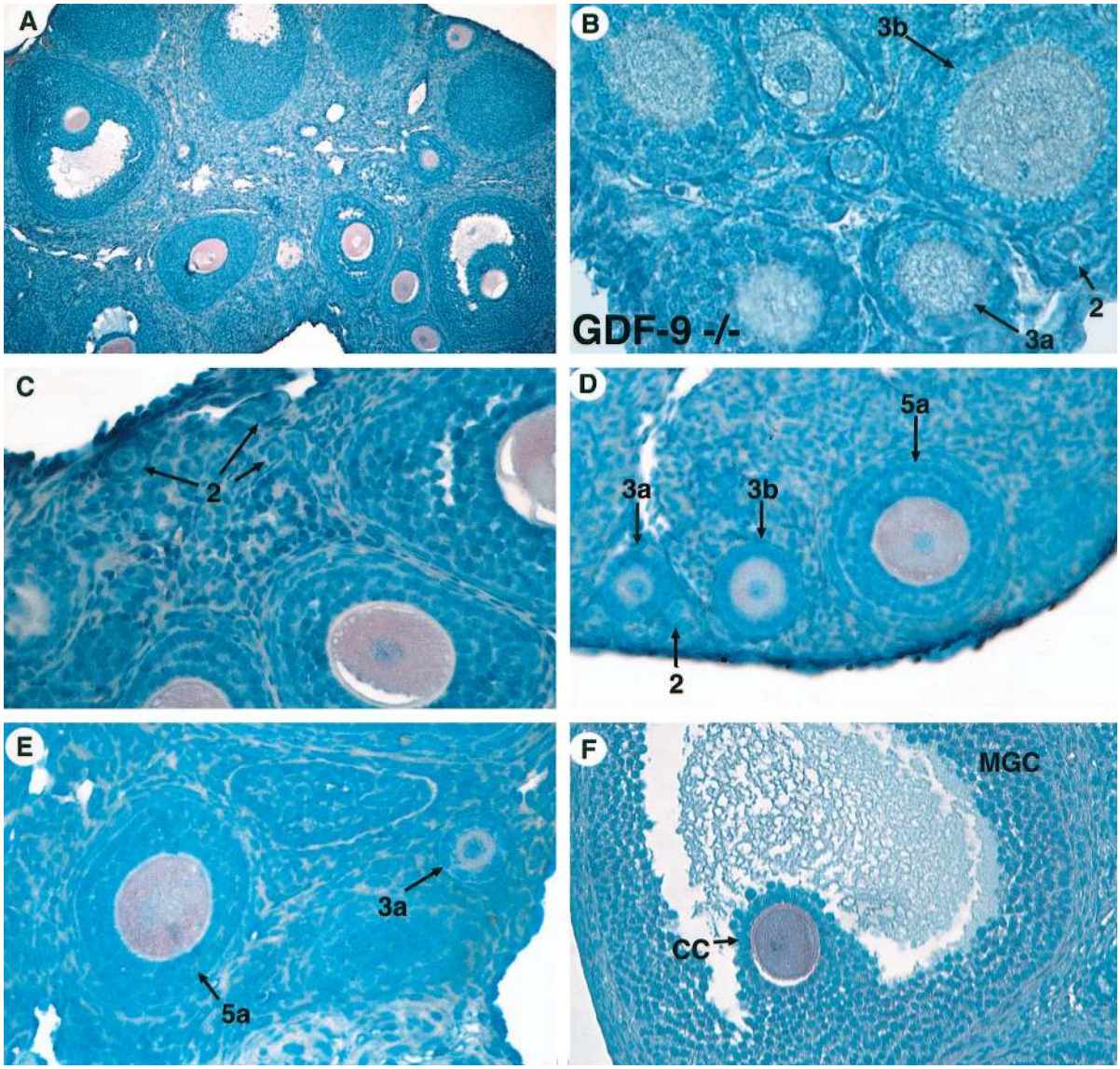

Fig. 1. Immunohistochemical Detection of GDF-9 in Mouse Ovaries

Ovaries from 3- to 4-week-old CD1 female mice were stained with an anti-GDF-9 monoclonal antibody. A, Low-power view of a wild-type ovary stained with the anti-GDF-9 monoclonal antibody shows multiple oocytes within preantral and antral follicles which stain positive (*pink*). Nuclei are *blue* due to hematoxylin counterstaining. B, High-power view of a GDF-9-deficient (GDF-9 −/−) ovary stained with the anti-GDF-9 monoclonal antibody does not show oocyte staining. C–E, High-power views of wild-type ovaries. Oocytes within primordial follicles (type 2) fail to stain positive (C and D). Oocytes from type 3a primary follicles begin to demonstrate weak staining in the cytoplasm (D and E), and higher level staining is detected in the larger preantral follicles such as the three-layer type 5a follicles (D and E). Staining is excluded from the germinal vesicle, which stains *blue*. F, A preovulatory follicle with abundant mural granulosa cells (MGC) and an oocyte surrounded by cumulus granulosa cells (CC) demonstrate persistence of GDF-9 protein synthesis by oocytes of late-stage follicles.

site would be predicted to yield a carboxyl-terminal mature peptide of 135 amino acids with a predicted molecular mass of approximately 15.6 kDa (20). The mature GDF-9 sequence contains a single N-linked glycosylation site (Asn^{325}-Leu^{326}-Ser^{327}). The predominant 21-kDa form would correspond to the cleaved, mature monomeric form of mouse GDF-9 with one N-linked oligosaccharide. This band runs at an identical position as the recombinant human GDF-9 synthesized in CHO cells. The band at 60 kDa would correspond to the glycosylated, unprocessed (prohormone) form (441 amino acids), which is also secreted into the media. To confirm that the increased molecular mass of these two forms is due to N-linked glycosylation, GDF-9-containing medium was treated with N-glycanase to remove the N-linked oligosaccharides. This treatment reduces the size of the 21-kDa band to 16 kDa, the same molecular mass as the GDF-9 mature peptide produced in bacteria, and also reduces the 60-kDa band to 50 kDa. Since the 21-kDa

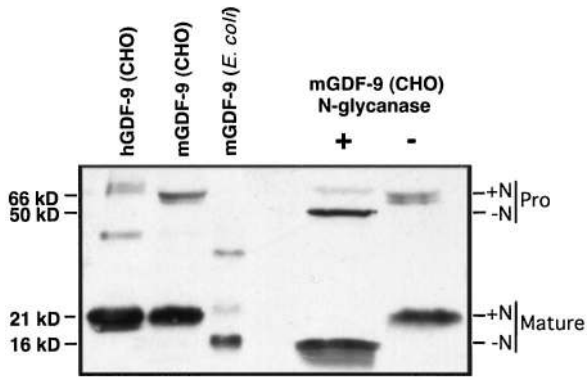

Fig. 2. Western Blot Analysis of GDF-9-Conditioned Media

An antihuman GDF-9 monoclonal antibody was used for detection of GDF-9. The lanes are as follows: lane 1, recombinant human GDF-9 synthesized in CHO cells (~30 ng mature form); lane 2, recombinant mouse GDF-9 synthesized in CHO cells (~20 ng mature form); lane 3, 5 ng mouse GDF-9 mature peptide produced in *E. coli*; lane 4, recombinant mouse GDF-9 media treated with N-glycanase (+) to remove the N-linked oligosaccharides; lane 5, mouse GDF-9 media treated as in lane 4 in the absence of N-glycanase (−). The N-linked glycosylated (+N) or deglycosylated (−N) propeptide (pro) and mature forms of GDF-9 are indicated.

glycosylated GDF-9 form is always the most abundant, this strategy to produce recombinant, glycosylated GDF-9 in mammalian cells in the presence of PACE and under reduced-serum culture conditions appears fairly efficient.

Regulation of HAS2 and uPA mRNA Synthesis by Recombinant GDF-9

Oocytes secrete a growth factor that is known to stimulate hyaluronic acid synthesis, inhibit uPA, and cause cumulus expansion *in vitro* (2). HAS2, which is expressed in mouse cumulus cell-oocyte complexes after the gonadotropin surge and immediately preceding efficient cumulus expansion (21), has been implicated as the major hyaluronic acid synthase involved in cumulus expansion (22). To determine whether GDF-9 is the oocyte-secreted factor responsible for inducing cumulus expansion through increased hyaluronic acid matrix synthesis and decreased hyaluronic acid matrix degradation, we examined the expression levels of HAS2 and uPA in freshly isolated granulosa cells treated with recombinant mouse GDF-9. RT-PCR incorporation of radiolabeled nucleotides into specific products was used to monitor the expression levels of HAS2 and uPA in control and GDF-9-treated granulosa cell cultures. First, a linear range of product amplification for each oligonucleotide pair of the three genes [*i.e.* HAS2, uPA, and hypoxanthine phosphoribosyltransferase (HPRT)] was established. Using radiolabeled [α^{32}P]-dCTP in the PCR reaction, identical samples were amplified for 16–22 cycles and quantitated using photodensitometric analysis of the autoradiographic film (data not shown). Linear increases in amplified product were observed for HPRT over 16–20 cycles, for HAS2 over 16–20 cycles, and for uPA over 18–22 cycles from both control and GDF-9-treated samples. Eighteen to 20 cycles was determined to be optimal and was used to study all three gene products in all further analyses. Similar PCR conditions were performed for the COX-2, LHR, cytochrome P-450 side chain cleavage (P-450 scc), and StAR studies (see below).

Next, we examined the dose-response relationship between recombinant GDF-9 and HAS2 mRNA synthesis. The rate of hyaluronic acid synthesis by cumulus cells or mural granulosa cells exposed to oocyte-conditioned media peaks at 6–12 h in culture (23, 24). Thus, for the dose-response experiment, granulosa cells were cultured for 5 h in the absence or presence of varying concentrations of recombinant GDF-9. Using semiquantitative RT-PCR, we can detect a small increase in HAS2 expression with 10 ng/ml of recombinant GDF-9 (Fig. 3A). Levels of recombinant GDF-9 between 30–300 ng/ml give robust HAS2 induction with a relatively linear dose/response occurring between 30–120 ng/ml. Granulosa cells collected from unprimed immature mouse ovaries also responded similarly to the recombinant GDF-9 (data not shown). In contrast, recombinant mouse BMP-15 (150 ng/ml) or recombinant human BMP-6 (50 ng/ml) was unable to stimulate HAS2 expression or suppress uPA expression when tested in any of the granulosa cell assays (data not shown). These findings demonstrate that these activities are specific to GDF-9 and that other oocyte-secreted TGF-β family members cannot replicate these activities.

A time course analysis (0–24 h) of the HAS2 expression pattern using 100 ng/ml of GDF-9 demonstrated a small induction of HAS2 mRNA at 2 h (data not shown), a peak induction between 3–5 h in culture, and by 9 h in culture, the HAS2 expression level is decreasing (Fig. 3B). Granulosa cells cultured in the absence of GDF-9 for 0–24 h express more than 10-fold lower levels of HAS2 compared with the GDF-9-induced peak level, indicating a very low level of basal activity in these cells. The time course for uPA expression in control granulosa cells cultured in the absence of GDF-9 indicates that uPA levels increase over the first 9 h in culture (Fig. 3B). However, granulosa cells treated with 100 ng/ml of recombinant GDF-9 maintain a detectable but much lower level of uPA expression. At 5 h, the band intensity from the GDF-9-treated sample was approximately 15% of the control, and at 9 h, it was about 9% of the control-treated sample band intensity (normalized to HPRT for each sample).

To confirm the effects of GDF-9 on HAS2 and uPA expression as seen by RT-PCR, we examined the expression of HAS2 in primary granulosa cells by Northern blot analysis in the presence or absence of 50 ng/ml of GDF-9. Total RNA from each sample (at 0 or 5 h incubation in the presence or absence of 50

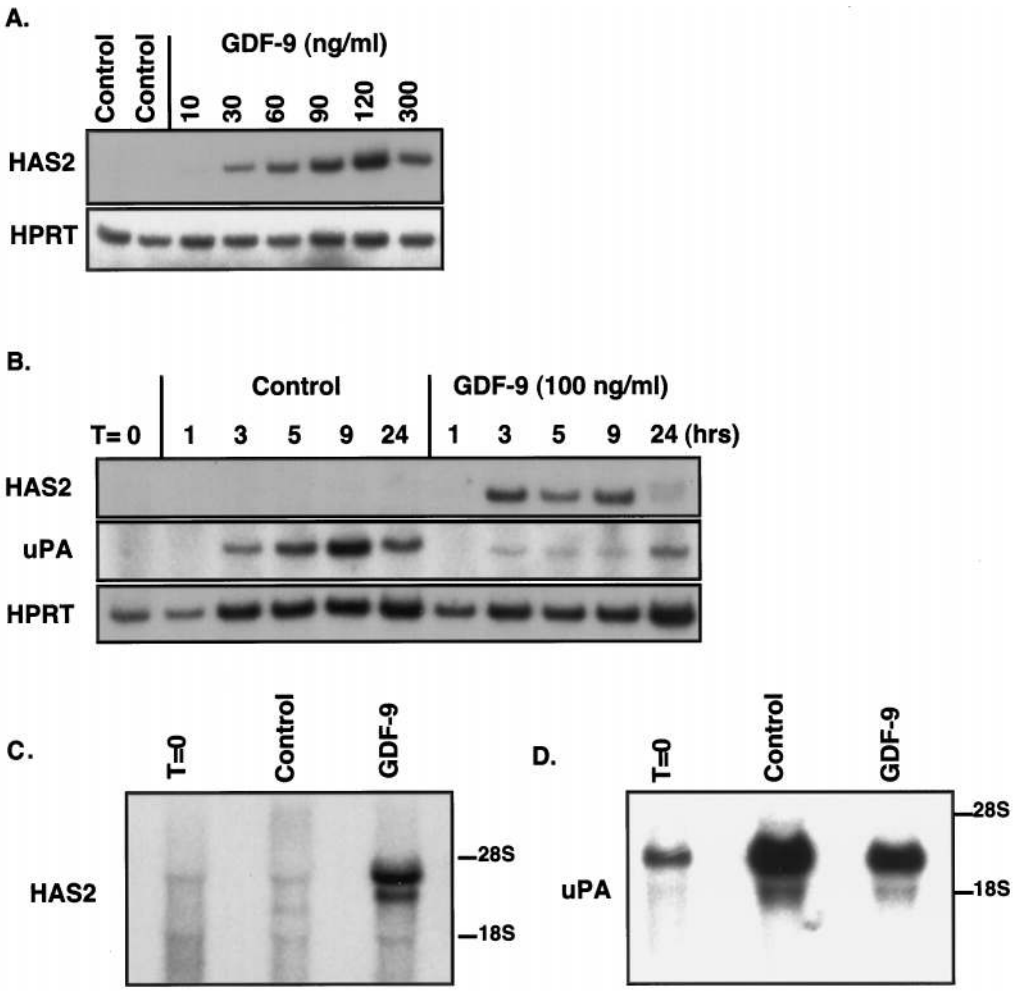

Fig. 3. Differential Regulation of HAS2 and uPA by GDF-9

A, HAS2 dose-response curve. RT-PCR analysis of RNA from granulosa cells cultured for 5 h in the presence or absence (control) of varying concentrations of GDF-9 using primer sets for HAS2 and HPRT. B, HAS2 and uPA time courses. RT-PCR analysis was performed with primer sets for HAS2, uPA, and HPRT using RNA from granulosa cells cultured in the presence or absence of 100 ng/ml of GDF-9 for 0–24 h. C, Northern blot analysis of HAS2. Each lane contains 15 μg total RNA from granulosa cells immediately after isolation (T = 0) or after 5 h of culture in the presence or absence of 50 ng/ml GDF-9. The blot was hybridized first with an HAS2 cDNA probe, and then stripped and rehybridized with a GAPDH cDNA probe. D, Northern blot analysis of uPA. Each lane contains 15 μg total RNA from granulosa cells immediately after isolation (T = 0) or after 5 h culture in the presence or absence of 50 ng/ml GDF-9. The blot was hybridized first with a uPA cDNA probe, and then stripped and rehybridized with a GAPDH cDNA probe.

ng/ml GDF-9) was subjected to Northern blot analysis and hybridized with either an HAS2 or uPA probe and subsequently with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe. The signals were quantitated on a phosphorimager and HAS2 and uPA levels and normalized to GAPDH. HAS2 is barely detectable in mural granulosa cells at 0 h or after 5 h of culture in the control sample (Fig. 3C). However, after 5 h incubation with GDF-9, both the 4.8-kb and 3.2-kb HAS2 mRNA forms (22) are increased 9.7-fold compared with control. In contrast, Northern blot analysis of uPA showed that 50 ng/ml GDF-9 suppresses uPA synthesis to 40% of control cultures (Fig. 3D). Thus, the Northern blot data confirm our RT-PCR analyses.

Recombinant GDF-9 Causes Cumulus Expansion of Oocytectomized Cumulus Cell-Oocyte Complexes

Intact cumulus cell-oocyte complexes were isolated from PMSG-treated immature female mice. Using a transgenic micromanipulation set-up, the oocytes from these complexes were punctured, and the oocyte contents were suctioned. Before culture, these oocytectomized cumulus complexes are spherical objects approximately 100 μm in diameter consisting of several layers of granulosa cells that surround an empty zona pellucida. After 18 h in culture, cumulus cells from 25 of 25 oocytectomized complexes cultured in

control media (*i.e.* deficient in GDF-9 but containing 10% FCS and 5 ng/ml or 150 ng/ml of FSH) adhere to the tissue culture plate and assume a fibroblastic appearance (Fig. 4A). Consistent with previous reports that cumulus cells have low or undetectable levels of LHR mRNA (4), incubation of these oocytectomized complexes (9 of 9) with LH (1 μg/ml) fails to alter their fibroblastic appearance (Fig. 4B). In contrast, 40 of 44 oocytectomized complexes, isolated under identical conditions and cultured in the presence of 100 ng/ml GDF-9, maintain a spherical appearance and expand into a three-dimensional, gelatinous sphere (Fig. 4C). These results are similar to cumulus cell-oocyte complexes with intact oocytes cultured in FSH-containing media (data not shown). These cells have not detached from the plate because of cell death since the majority of cells continue to exclude the vital dye, trypan blue (data not shown). In contrast, incubation of the complexes with recombinant human BMP-6 or BMP-15 did not result in cumulus expansion (data not shown). These observations indicate that GDF-9 specifically stimulates cumulus expansion and is the oocyte-derived factor that normally mediates this process.

Regulation of LHR and COX-2 by GDF-9

Based on the above findings, we determined the effect of recombinant mouse GDF-9 on LHR and COX-2 expression in granulosa cell cultures using semiquantitative RT-PCR (Fig. 5). After collection of the granulosa cells (T=0), a low level of LHR is detected (Fig. 5A). In the absence of GDF-9, the levels of LHR mRNA decrease at 1–3 h but increase from 5 h to 24 h (Fig. 5, A and B). In contrast, incubation of the granulosa cells with GDF-9 (100 ng/ml) suppresses LHR mRNA synthesis at all time points (Fig. 5A). Even in the presence of 10 ng/ml FSH, LHR mRNA is suppressed in the presence of 100 ng/ml GDF-9 (Fig. 5B). In the experiment shown using 10 ng/ml FSH, the control-treated granulosa cells express about 30-fold more LHR than the GDF-9-treated granulosa cells ($P < 0.05$, n = 3). In contrast, COX-2 expression is low in the absence of GDF-9, but demonstrates dramatically elevated levels after incubation for 24 h in the presence of 100 ng/ml GDF-9 (Fig. 5, C and E). While FSH had no significant effect in either the control or GDF-9-treated granulosa cells, GDF-9 caused a more than 50-fold increase in COX-2 expression ($P < 0.05$, n = 6). Analysis of HAS2 expression in these samples (Fig. 5, C and F) is consistent with previous results (Fig. 3).

GDF-9 Regulation of Progesterone Synthesis

To study the effects of GDF-9 and FSH on the regulation of progesterone synthesis, we performed dose-response and time course experiments. Semiquantitative RT-PCR was performed to analyze P-450 scc mRNA synthesis and StAR mRNA synthesis (Fig. 6C) and a RIA was used to analyze progesterone secreted into the media. In Fig. 6A, media were collected from granulosa cells cultured in triplicate wells for 4 h containing varying amounts of FSH in the presence or absence of 50 ng/ml GDF-9 and assayed for progesterone by RIA. Under these conditions, granulosa cells cultured for 4 h in the absence or at low levels of FSH produced very little progesterone, whereas granulosa cells cultured with 50 ng/ml GDF-9 produced 5- to 7-fold higher amounts of progesterone (Fig. 6A). In Fig. 6B, granulosa cells were incubated for 24 h in media containing 10% FCS with 0 or 10 ng/ml FSH in the presence or absence of 100 ng/ml GDF-9. After incubation for 24 h, GDF-9 alone can induce significantly higher amounts of progesterone but in the presence of high concentrations of FSH (10 ng/ml), the effect of the GDF-9 is negligible (Fig. 6B). These data suggest that FSH and GDF-9 stimulation of progesterone are not additive.

To determine how FSH and GDF-9 regulate progesterone synthesis in the granulosa cell cultures, the levels of P-450 scc and StAR mRNA were analyzed by semiquantitative RT-PCR after incubation for 24 h in the presence or absence of FSH or the presence or absence of GDF-9 (Fig. 6, C–E). Whereas 10 ng/ml FSH causes a small increase in P-450 scc, the presence of GDF-9 does not have any effect. In contrast, GDF-9 results in 2–5 fold induction of StAR mRNA ($P < 0.05$). These results suggest that FSH and GDF-9 function to regulate progesterone synthesis via different mechanisms.

DISCUSSION

We previously demonstrated that GDF-9 knockout mice have a block in folliculogenesis at the one-layer type 3b follicle stage, demonstrating that GDF-9 protein is essential at this stage (16). Consistent with these studies and the GDF-9 mRNA expression detected by *in situ* hybridization (Ref. 14 and J. A. Elvin and M. M. Matzuk, unpublished data), we show here by immunohistochemical analysis that GDF-9 protein is first detected at low levels within growing oocytes of primary follicles (type 3a follicles), is present at higher levels in full-grown oocytes of type 3b follicles, and is detected in oocytes of every subsequent developmental stage. Critical to the other studies presented in this manuscript, GDF-9 protein is also synthesized by oocytes of large antral and preovulatory follicles in which the oocyte is closely associated with cumulus cells. Although GDF-9 protein is detected in oocytes of type 3a follicles, it only becomes essential for folliculogenesis at the type 3b-type 4 follicle transition. This suggests that the GDF-9 signal transduction cascade is not active before the type 3b follicle stage [*e.g.* GDF-9 receptors or downstream proteins may be absent in granulosa cells at the type 3a stage or GDF-9 prohormone may not be processed correctly to bioactive dimers (see below)].

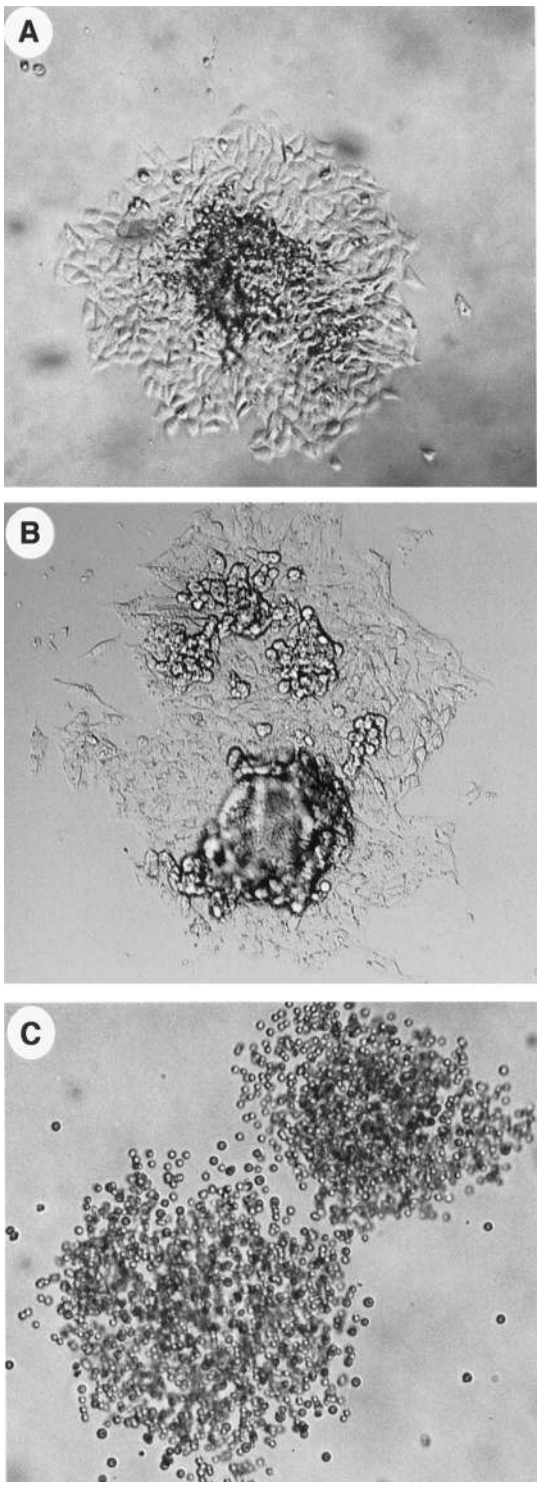

Fig. 4. GDF-9 Induces Cumulus Expansion in the Absence of Oocytes

Morphological comparison of oocytectomized cumulus-oocyte-complexes cultured for 18 h in the absence of GDF-9 or LH (A), the presence of LH (B), or the presence of 100 ng/ml GDF-9 (C). All cultures contained 10% FCS and 100 ng/ml FSH. Cells of the control-treated complexes (A) or the complexes treated with 1 ng/ml LH (B) appear flattened and polygonal and have adhered to the plate forming a monolayer. The oocytectomized complexes incubated with GDF-9 (C) have undergone cumulus expansion: cells appear round and glistening and are suspended in a three-dimensional matrix. The plane of the image is above the level of the culture surface, approximately at the level of the empty zona pellucida.

Previous studies demonstrated an essential paracrine function of the oocyte to stimulate cumulus expansion and to induce hyaluronic acid synthesis (25, 26) and to suppress uPA expression (27) in both cumulus and mural granulosa cells. When cumulus cells are separated from the oocyte, they do not expand and assume an adherent, fibroblastic appearance, producing negligible amounts of hyaluronic acid. When oocytes are added back to the culture, or if the cumulus cells are grown in oocyte-conditioned media (~2 oocytes/μl media), they produce 5- to 10-fold higher levels of hyaluronic acid. Alternatively, mural granulosa cells can be induced to synthesize hyaluronic acid *in vitro* by treatment with oocyte-conditioned media (28). Synthesis of hyaluronic acid in cumulus-oocyte-complexes can be blocked by the addition of actinomycin D, demonstrating a dependence on gene transcription (24). Some of the effects of the oocyte-conditioned media could be mimicked by recombinant TGF-β, but anti-TGF-β antibodies could not block the effect of the oocyte-conditioned media (24, 28). While theca cells synthesize TGF-βs (29), no reports of oocyte-produced TGF-βs exist in the literature. These data suggest that the oocyte-produced growth factor is not one of the TGF-βs but is a related family member that functions in a similar pathway.

In this report, we show that GDF-9 could substitute for oocytes and oocyte-conditioned media in assays analyzing HAS2 induction and uPA suppression typical of processes occurring in preovulatory follicles. Other oocyte-expressed TGF-β family members, BMP-15 and BMP-6, are unable to substitute for GDF-9 in these granulosa cell assays. Mural granulosa cells, isolated from antral follicles treated with recombinant GDF-9, are induced to express HAS2 in a dose-dependent and time-dependent manner. GDF-9 can induce approximately 10-fold higher levels of HAS2 mRNA in mural granulosa cells, which corresponds well to the maximum effect of oocytes on hyaluronic acid synthesis. Additionally, the dose-response curve for GDF-9 is very similar to that of the oocyte-conditioned media. Very low doses (*e.g.* 0.5 oocytes/μl or 10 ng/ml GDF-9) induce very low but detectable increases in hyaluronic acid synthesis or HAS2 expression whereas 1 oocyte/μl or 30–50 ng/ml GDF-9 causes a much more dramatic induction, which plateaus at 2–4 oocytes/μl or 120–300 ng/ml GDF-9 (Ref. 25 and studies presented here). The time course of GDF-9 action also agrees with previous data for the oocyte-produced factor. Whereas HAS2 mRNA is induced by 2 h in culture with GDF-9, oocyte-induced hyaluronic acid becomes detectable at low levels after

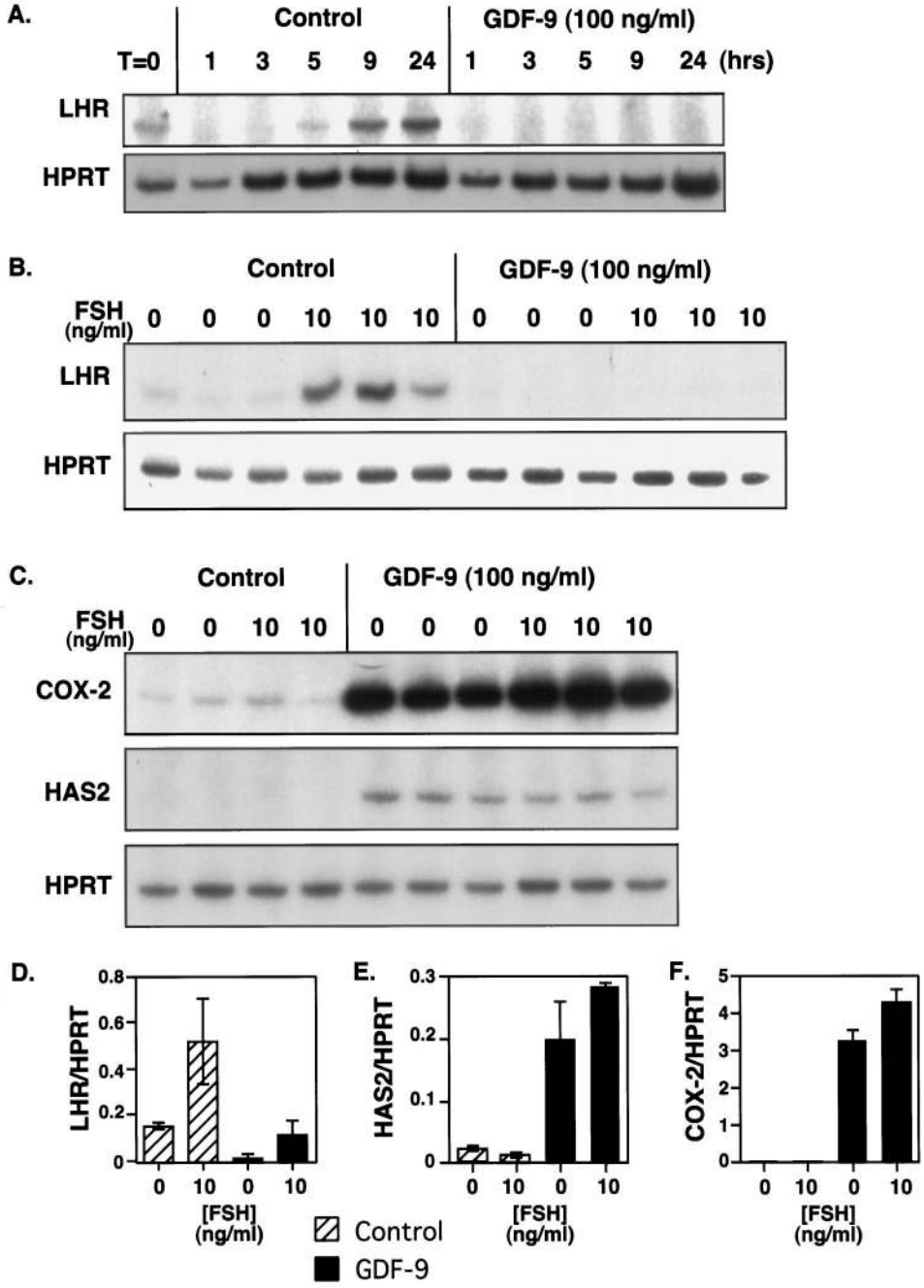

Fig. 5. Regulation of LHR, COX-2, and HAS2 mRNA Synthesis

A, Time course (in hours) of LHR mRNA synthesis in granulosa cells using semiquantitative RT-PCR. Granulosa cells were cultured with 5 ng/ml FSH in the presence or absence (control) of l00 ng/ml recombinant mouse GDF-9. B, Effects of FSH and GDF-9 on LHR mRNA synthesis in granulosa cells after 24 h of culture. Semiquantitative RT-PCR analysis of LHR mRNA in triplicate wells treated ± FSH (10 ng/ml) and ± GDF-9 (100 ng/ml) is shown. C, Effects of FSH and GDF-9 on COX-2 or HAS2 mRNA synthesis in granulosa cells after 24 h of culture. RT-PCR analysis of COX-2 or HAS2 mRNA in duplicate or triplicate wells treated ± FSH and ± GDF-9 is shown. In panels A–C, semiquantitative RT-PCR analysis of HPRT mRNA under identical conditions is used as the internal control. D and E, Quantitation of the LHR, COX-2, and HAS2 mRNA levels from the above experiments normalized to HPRT levels. Each value is the mean ± SEM of duplicate or triplicate wells.

2.5 h (23). GDF-9 induces peak HAS2 mRNA levels between 3–5 h, while the rate of oocyte-induced hyaluronic acid synthesis is maximal between 6–12 h (23). Likewise, oocyte-induced hyaluronic acid synthesis drops after 12 h, and no more hyaluronic acid is made after 18 h (24); GDF-9-induced HAS2 expression is reduced by 24 h and uPA synthesis increases by 24 h. The transient nature of the activation of HAS2

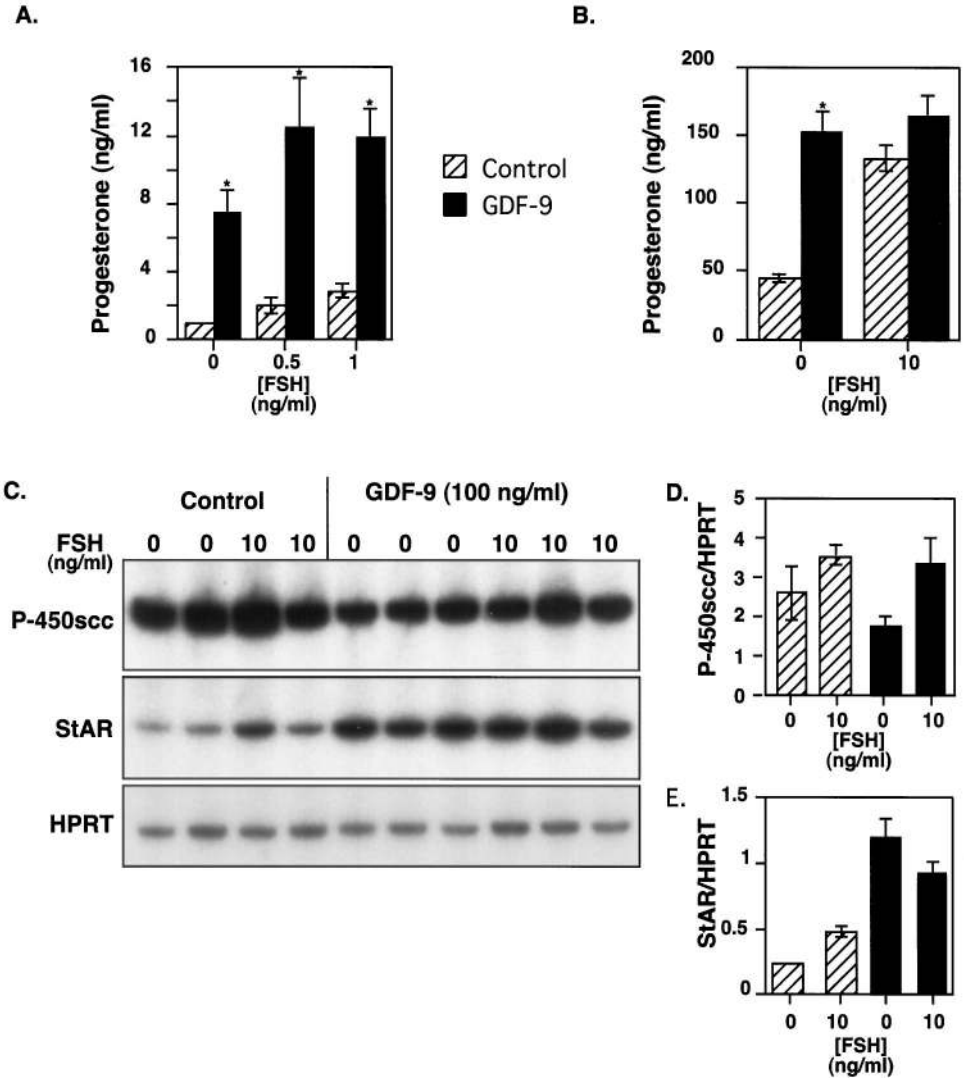

Fig. 6. Progesterone Production and Regulation of Enzymes Involved in Progesterone Synthesis

A, Granulosa cells were cultured for 14 h in serum-free media in the absence or presence of 50 ng/ml GDF-9 in the presence of varying concentrations of 0, 0.5, or 1 ng/ml FSH. Progesterone in the media was assayed by RIA. The error bars represent the SEM. *, $P < 0.05$ for control *vs*. 50 ng/ml GDF-9 at FSH = 0 ng/ml, FSH = 0.5 ng/ml, or FSH = 1 ng/ml. B, Progesterone in the media after 24 h of treatment of cells ± GDF-9 (100 ng/ml) or ± FSH for triplicate samples in 10% of FCS containing media. *, $P < 0.05$ for control *vs.* GDF-9-treated cells cultured without FSH. C, Effects of FSH and GDF-9 on P-450 scc or StAR mRNA synthesis in granulosa cells after 24 h of culture. Semiquantitative RT-PCR analysis of P-450 scc or STAR mRNA in duplicate or triplicate wells treated ± FSH or ± GDF-9. Semiquantitative RT-PCR analysis of HPRT mRNA under identical conditions is used as the internal control. D and E, Quantitation of the P-450 scc and StAR mRNA levels from the above experiment normalized to HPRT levels. Each value is the mean ± SEM of duplicate or triplicate wells.

expression and hyaluronic acid synthesis and the increase in uPA synthesis over this time period may be due to lability of GDF-9 in the media, down-regulation of the GDF-9 receptor, GDF-9-induced differentiation of the granulosa cells, and/or stimulation of a negative-feedback mechanism within the GDF-9 signal transduction cascade. It is interesting to note that the oocyte-secreted factor that regulates several of these processes has been noted to be labile and that continued presence of oocytes in various cocultures with granulosa cells is required for continued activity (4). Lastly, our data suggest that the difference in the *in vivo* phenotype of mural granulosa cells *vs.* expanding cumulus granulosa cells is not intrinsic to the cells themselves but is due to their proximity to the oocyte and the concentration gradient of the oocyte-produced GDF-9.

The conversion of cholesterol to pregnenolone is the rate-determining step in granulosa cell steroidogenesis. The rate of pregnenolone synthesis depends on the level and activity of the reaction-catalyzing enzyme, P-450 scc, and its access to its substrate cholesterol via stimulation of StAR (30). It is well established that FSH- and LH-induced increases in

intracellular cAMP, leading to subsequent stimulation of P-450 scc and StAR mRNA synthesis and StAR protein phosphorylation, stimulate progesterone synthesis *in vitro* (31–33). In contrast to the effect seen with GDF-9 treatment, activin A decreased basal and FSH stimulated P-450 scc, 3β-hydroxysteroid dehydrogenase, and progesterone synthesis by cultured granulosa cells from the diethylstilbesterol-stimulated rat. Our data confirm that FSH can stimulate P-450 scc mRNA synthesis in mouse granulosa cells but demonstrate that GDF-9 does not significantly affect P-450 scc mRNA synthesis. In contrast, FSH has only a small inductive effect on StAR mRNA, but GDF-9 with or without FSH significantly induces StAR expression. Consequently, both GDF-9 and FSH can independently increase production of progesterone by the granulosa cells and appear to function in the same pathway but via different mechanisms. It would be interesting to determine whether GDF-9 also stimulates phosphorylation of StAR protein through activation of Smads (see below) to increase its activity (33). GDF-9-stimulated local production of progesterone by the cumulus cells may be critical for achieving a perfect microenvironment for the oocyte after ovulation and before fertilization. In support of this, ovulated rat cumulus cell-oocyte complexes secrete measurable levels of both progesterone and prostaglandins (mainly PGE_2) (34), and use of aminoglutethimide, which inhibits conversion of cholesterol to pregnenolone, reduces the number of normal ovine oocytes recovered after *in vitro* follicular maturation.

In situ hybridization analysis of LHR in preovulatory follicles demonstrates that LHR is suppressed in the cumulus cells but not the mural granulosa cells, whereas after LH treatment *in vivo*, COX-2 expression is highest in the cumulus cells (Ref. 18 and J. A. Elvin and M. M. Matzuk, unpublished data). We show here that recombinant GDF-9 also suppresses LHR mRNA but induces COX-2 expression, mimicking the normal expression of these genes in the cumulus cells. In addition, Eppig and colleagues (4, 35) elegantly demonstrated that full-grown oocytes suppress LHR mRNA expression, but that oocytes from preantral follicles, metaphase II-arrested oocytes, or two-cell embryos were not as effective. We have shown in our knockout studies (16, 18) and in the current studies using recombinant GDF-9 that GDF-9 can stimulate changes in cell morphology, gene expression, and steroid production, indicating that granulosa cells, at least from primary follicles and from antral follicles, possess receptors that bind GDF-9 (see Fig. 7 for a summary of our findings). Since GDF-9 dramatically increases the level of COX-2 expression, it is unclear why COX-2 expression is not expressed at earlier stages of folliculogenesis or why earlier stage oocytes (4, 5, 35) are less effective in suppressing LHR. Although GDF-9 protein is detected at the immunohistochemical level at all stages of folliculogenesis, this does not necessarily prove that GDF-9 is active at all of these stages. One possibility is that the GDF-9 precursor is only processed to an active mature dimer at the type 3b stage and at the antral follicle stage. The regulation of the GDF-9-processing enzyme would be one way to regulate the activity of GDF-9 posttranslationally. An alternative explanation is that both GDF-9-regulated and GDF-9-independent transcription factors function together to regulate the synthesis of COX-2. At least one regulator of COX-2 is the transcription factor enhancer-binding protein β (C/EBPβ). C/EBPβ, which is induced between 4 and 7 h after hCG treatment, binds to the COX-2 promoter to down-regulate COX-2 mRNA expression. COX-2 ex-

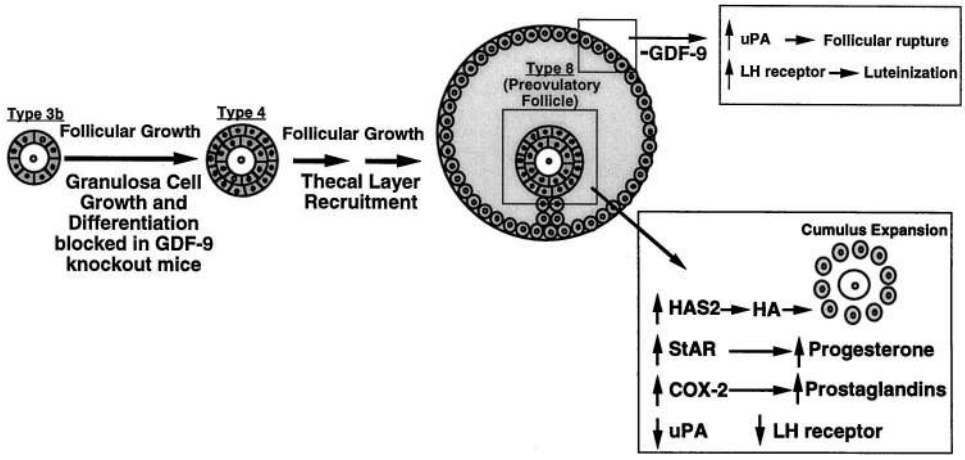

Fig. 7. The Multifunctional Roles of GDF-9 in the Mammalian Ovary (Model)

Based on GDF-9 knockout mouse studies, GDF-9 is required for granulosa cell proliferation and differentiation leading to the formation of the two-layer preantral follicle and subsequent recruitment of the thecal layer. In the preovulatory follicle, GDF-9 induces HAS2 [and subsequently hyaluronic acid (HA)], and suppresses uPA, resulting in cumulus expansion (*bottom box*). GDF-9 may play an important role in cumulus-oocyte-complex survival and fertilization by stimulating cumulus cell progesterone production via induction of StAR and prostaglandin synthesis via induction of COX-2. In the absence of GDF-9 (*top box*), uPA and LHR are induced leading to follicular rupture and luteinization, respectively.

pression in the ovary normally peaks at 4 h after hCG treatment whereas COX-2 protein continues to be present in the cumulus cells after ovulation (36). However, in the C/EBPβ knockout mouse, which are infertile (36a), levels of COX-2 mRNA remain elevated. COX-2 knockout mice are also infertile due to defects in ovulation and impaired oocyte maturation (36). These studies from the C/EBPβ and COX-2 knockout models suggest that appropriate regulation of COX-2 in the cumulus cells is necessary to maintain the optimal microenvironment of PGs around the oocyte. GDF-9 appears to be one of the factors involved in induction of COX-2, and C/EBPβ plays a role in down-regulation of COX-2. Our studies and others (37, 38) also suggest that LH regulates the expression of both COX-2 and C/EBPβ in cumulus cells indirectly because LHRs are not present on these cells. However, it is unclear how this is achieved or how COX-2 is turned on rapidly and C/EBPβ more slowly. Future studies will be necessary to determine the critical interplay between LH and GDF-9 signaling in the preovulatory follicle and their regulation of C/EBPβ and COX-2.

Members of the TGF-β superfamily bind to a distinctive combination of type II and type I serine/threonine kinase receptors and transduce signals through phosphorylation of specific Smad proteins to alter transcription. TGF-βs and activins induce phosphorylation of Smad2 and Smad3, whereas BMPs stimulate phosphorylation of Smad1 and Smad5 (39). Multiple TGF-β superfamily type II receptors (*e.g.* TGF-β type II, activin receptor type II, activin receptor type IIB), type I receptors [*e.g*. Alk2, Alk4 (activin type IB), Alk5 (TGF-β type I)], and Smads (*e.g.* Smad2 and Smad4), and unique receptors (*e.g*. the inhibin receptor) are detected in mouse, rat, and human granulosa cells or granulosa cell tumors (40–42). GDF-9 may be binding to and signaling through already characterized type II and type I receptors and Smad proteins, or through a novel GDF-9-specific signaling pathway. The lack of phenocopy of GDF-9-deficient mice (*i.e.* block at the one-layer primary follicle) and the activin type II receptor-deficient mice (*i.e.* block at the antral follicle stage) (43), indicates that GDF-9 is not signaling exclusively through the activin receptor type II and suggests that GDF-9 and activins signal through distinct receptor and Smad pathways in the same granulosa cells. Further identification and characterization of the GDF-9 receptors, the intracellular Smad signal transduction cascade, and the downstream target genes for GDF-9 in granulosa cells is one continued interest of our laboratory.

MATERIALS AND METHODS

Immunohistochemistry

Ovaries from CD1 (ICR) mice (generated at Baylor College of Medicine from a stock from Charles River Laboratories, Inc., Wilmington, MA) were fixed in 10% neutral buffered formalin, processed, and embedded in paraffin. Ovarian sections (4 μm thick) were dewaxed then rehydrated in a graded series of ethanol solutions. Nonspecific binding was reduced by preincubation for 30 min in 1$\times$ universal blocking buffer (BioGenex Laboratories, Inc., San Ramon, CA) diluted in 0.1 M PBS and 0.1% BSA. The primary antibody, mouse antihuman GDF-9 monoclonal antibody was added to each section and incubated for 2 h at a final concentration of 30–60 ng/μl. Sections were washed twice in 0.1% BSA in PBS for 5 min followed by incubation for 20 min in biotinylated goat antimouse IgG (BioGenex Laboratories, Inc.). After washing as above, the sections were incubated for 20 min in alkaline phosphatase-conjugated streptavidin (BioGenex Laboratories, Inc.), washed twice in 0.1% BSA in PBS, and incubated with New Fuchsin alkaline phosphatase substrate as per manufacturer's instructions (BioGenex Laboratories, Inc.). After detection of a positive reaction, sections were counterstained with hematoxylin and mounted in glycerol.

Production of Recombinant Mouse GDF-9

A full-length mouse GDF-9 cDNA (44) was subcloned into the expression vector pHTop containing the processing gene PACE (a gift from Dr. Monique Davies, Genetics Institute). The GDF-9 expression vector was lipofectin transfected into CHO cells under standard conditions (Gibco BRL, Grand Island, NY). Expression of mouse GDF-9 in CHO cells was subsequently driven by a tetracycline-regulatable promoter while an SV40 promoter regulated expression of PACE. Stable, positive clones were selected in the presence of 0.02 μM methotrexate in α-modified Eagles' medium (α-MEM) containing 10% heat- inactivated dialyzed FBS, 100 μg/ml G418-sulfate (Gibco BRL; Life Technologies) and the antibiotics gentamicin, penicillin, and streptomycin. After clonal selection and expansion in 0.02 μM methotrexate, the GDF-9-expressing cells were incubated for 24 h in Opti-MEM- reduced serum collection media containing 100 mg/ml heparin (Sigma Chemical Co., St. Louis, MO). The media were harvested, and GDF-9 protein levels were determined by SDS-PAGE with subsequent immunoblotting (see next section). N-linked oligosaccharides were removed by incubation with N-glycanase (Oxford GlycoSciences, Wakefield, MA) overnight at 37 C according to manufacturer's protocol.

Western Blot Analysis

Samples of GDF-9-containing media were electrophoresed on a 5% stacking/15% resolving SDS polyacrylamide gel in a Mini-Subcell apparatus (Bio-Rad Laboratories, Inc., Hercules, CA) as previously described (45) and subsequently transferred to polyvinylidenedifluoride membrane. The membranes were blocked overnight in a 5% nonfat milk in 1$\times$ Tris-buffered-saline with 0.05% Tween 20. Mouse antihuman GDF-9 monoclonal antibody (described above) was used at a 1:1000 dilution in blocking solution, and an antimouse secondary antibody conjugated to horseradish peroxidase (Southern Biotechnology Associates, Birmingham, AL) was used at a 1:2500 dilution in blocking solution. Chemiluminescence using enhanced chemiluminescence Western detection reagents (Pierce Chemical Co., Rockford, IL) and autoradiographic film detected signal. Bands were quantitated using a densitometer (Molecular Dynamics, Inc., Sunnyvale, CA) and Imagequant software, and the concentration of GDF-9 in the conditioned media was determined by comparing the signal intensity of GDF-9 in the conditioned media to known concentrations of GDF-9 standards run concurrently. Several batches of recombinant mouse GDF-9 were produced during the course of these studies, all of which appeared to have similar activities based on Western blot quantitation (*i.e.* immunoreactivity correlated with bioactivity).

Isolation and Culture of Granulosa Cells

Female CD-1 (ICR) mice 21–24 days of age (Baylor College of Medicine) were injected with 7.5 IU Gestyl (Diosynth B.V., Oss, Holland), and ovaries were harvested 44–48 h later, dissected free of fat and surrounding tissue, and placed in minimal essential media with 25 mM HEPES supplemented with 0.3 mg/ml L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin (Gibco BRL), and 0.3% BSA (Sigma Chemical Co.). Mural granulosa cells were released by puncturing large antral follicles. Oocytes and cumulus cell-oocyte complexes (COCs) were carefully removed (see below). Granulosa cells from multiple ovaries were pooled, centrifuged, and resuspended in 2× granulosa cell culture media (GCM): α-MEM (Gibco BRL) with 0.6 mg/ml L-glutamine, 200U/ml penicillin, 0.2 mg/ml streptomycin, and 2× insulin-transferrin-selenite (Gibco BRL) in the presence or absence of 20% FBS (HyClone Laboratories, Inc., Logan, UT), and the presence or absence of ovine FSH (NIDDK-o-FSH-20 kindly provided by Dr. Parlow of the National Hormone and Pituitary Program). GDF-9-containing media or control conditioned media were diluted to 2× the final concentration in α-MEM. Equal volumes of 2× GDF-9-containing media or control media were combined with granulosa cells in 2× culture media and cultured at 37 C in a humidified atmosphere with 5% CO_2. After varying periods of culture, nonadherent cells were pelleted from the media, and the media were stored at −20 C. Granulosa cells were lysed, and total RNA was isolated using RNA Stat-60 (Leedo Medical Laboratories, Houston, TX) following the manufacturer's protocol.

Semiquantitative RT-PCR Analysis

Oligo-dT-primed cDNA from each RNA sample was synthesized using Superscript reverse transcriptase (Gibco BRL) following the manufacturer's protocol. One microliter of each RT reaction (1/20 of total) was used in each 25 μl PCR reaction primed with gene-specific oligonucleotides. Mouse HAS2 mRNA expression was detected using 5′-GCTTGACCCTGCCTCATCTGTGG-3′(sense) and 5′-CTGGTTCAGCCATCTCAGATATT- 3′ (antisense) primers (21), which span a 1.4-kb intron. A PCR product of 403 bp is amplified from RNA, easily distinguished from amplification of contaminating DNA. Mouse uPA mRNA expression was detected using 5′- GTTCAGACTGTGAGATCACTGG-3′ (sense) and 5′-CAGAGAGGACGGTCAGCATGG- 3′ (antisense) primers that span two introns of 1.4 kb total length. A PCR product of 434 bp is amplified from RNA. Mouse HPRT was amplified using 5′-CCTGGTTAAGCAGTACAGCC 3′ (sense) and 5′-TACTAGGCAGATGGCCACAG-3′ (antisense) primers, which span three introns of unknown sizes and give an expected mRNA-derived product size of 309 bp from RNA. Mouse StAR mRNA expression was detected using 5′-TCGCTTGGAGGTGGTGGTAGAC-3′(sense) and 5′-GCAGGTCAATGTGGTGGACAGT-3′ (antisense) primers, which span multiple small introns and give an mRNA-derived 522-bp product. Mouse cholesterol P-450 scc mRNA expression was detected using 5′-GCCAACATTACCGAGATGC-3′(sense) and 5′-CGAACACCCCAGCCAAAGCC-3′ (antisense) primers and give an mRNA-derived 426-bp product. Mouse COX-2 mRNA expression was detected using 5′-CTCCTTTTCAACCAGCAGTTCC-3′(sense) and 5′-TCTGCAGCCATTTCCTTCTCTC-3′ (antisense) primers and give a 377-bp product. Mouse LHR mRNA expression was detected using 5′-CTTATACATAACCACCATACCAG-3′(sense) and 5′-ATCCCAGCCACTGAGTTCATTC-3′ (antisense) primers, which span multiple introns and give a 516-bp product. PCR products amplified from granulosa cell cDNA were initially isolated, subcloned, and sequenced to confirm that they matched published sequences. In later studies, [α^{32}P]-dCTP was added to each PCR reaction, and products were separated by electrophoresis on a 4% polyacrylamide gel. The gels were dried and exposed to autoradiography, and radioactive bands were quantitated on a Molecular Dynamics, Inc. phosphorimager (Storm 860).

Northern Blot Analysis

Total RNA was isolated from granulosa cells and quantitated by fluorometry using Ribogreen RNA quantitation reagents (Molecular Probes, Inc., Eugene, OR) on a VersaFluor fluorometer (Bio-Rad Laboratories Inc.) using a 485–495 nm excitation filter and 515–525 nm emission filter. Fifteen micrograms of total RNA of each sample were electrophoresed on a 1.2% agarose/7.6% formaldehyde gel and transferred to Hybond N nylon membrane (Amersham, Arlington Heights, IL). Probes for HAS2 and uPA were generated from the aforementioned subcloned PCR products by random priming with [α^{32}P] dATP using the Strip-EZ probe synthesis kit (Ambion, Inc., Austin, TX). The membrane was hybridized, washed, and subjected to autoradiography as described (46). The probe was removed from the membrane using the Strip-EZ removal reagents (Ambion, Inc.) following the manufacturer's protocol. The same blots were then reprobed with GAPDH as a loading control. Signals for each probe were quantitated on a Molecular Dynamics, Inc. phosphorimager.

Progesterone RIA

Progesterone in the culture media was measured in duplicate by a specific, solid-phase RIA using a kit from Diagnostic Products (Los Angeles, CA) according to the manufacturers instructions. The sensitivity of this assay is 0.02 ng/ml, and calibration standards between 0.1 and 40 ng/ml were used.

Expansion of Oocytectomized Complexes

Cumulus cell-oocyte complexes were collected as described above. The oocyte was removed from each complex using a microinjection apparatus as previously described (47). Successful oocytectomy was assessed by the removal of the germinal vesicle along with the majority of ooplasm. Oocytectomized complexes were incubated for 18 h in groups in 20 μl droplets of granulosa cell culture media supplemented with 10% FBS and 5 ng/ml or 100 ng/ml of oFSH with or without 1 μg/ml oLH in the presence or absence of 100 ng/ml GDF-9. Photographs were taken on a Nikon (Melville, NY) inverted microscope.

Acknowledgments

We thank Dr. David Albertini and Mary Jo Carabatsos for teaching us the techniques of follicle and granulosa cell culture; Drs. Kathy Tomkinson and Monique Davies for advice on the production of recombinant GDF-9 and use of anti-GDF-9 antibodies; Dr. T. Rajendra Kumar for critical reading of the manuscript; Kim Paes for help with the figures; Shirley Baker for aid in manuscript preparation; Drs. Monty Krieger and Helena Miettinen for interesting discussions; and Dr. Albert Parlow and the National Hormone and Pituitary Program for the gift of the ovine FSH.

Received February 1, 1999. Revision received March 22, 1999. Accepted March 24, 1999.

Address requests for reprints to: Martin M. Matzuk, M.D., Ph.D., Professor, Department of Pathology, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030. E-mail: mmatzuk@bcm.tmc.edu.

These studies were supported in part by sponsored research grants from Genetics Institute and Metamorphix and

NIH Grant HD-33438 (to M.M.M). Julia A. Elvin is a student in the Medical Scientist Training Program supported in part by NIH Grants GM-07330 and GM-08307 and the Baylor Research Advocates for Student Scientists (BRASS) organization.

REFERENCES

1. Eppig JJ 1994 Oocyte-somatic cell communication in the ovarian follicles of mammals. Semin Dev Biol 5:51–59
2. Salustri A, Camaioni A, D'Alessandris C 1996 Endocrine and paracrine regulation of cumulus expansion. Zygote 4:313–315
3. Vanderhyden BC, Cohen JN, Morley P 1993 Mouse oocytes regulate granulosa cell steroidogenesis. Endocrinology 133:423–426
4. Eppig JJ, Wigglesworth K, Pendola F, Hirao Y 1997 Murine oocytes suppress expression of luteinizing hormone receptor messenger ribonucleic acid by granulosa cells. Biol Reprod 56:976–984
5. Eppig JJ, Chesnel F, Hirao Y, O'Brien MJ, Pendola FL, Watanabe S Wigglesworth K 1997 Oocyte control of granulosa cell development: how and why. Hum Reprod 12:127–132
6. Nekola MV, Nalbandov AV 1971 Morphological changes of rat follicular cells as influenced by oocytes. Biol Reprod 4:154–160
7. El-Fouly MA, Cook B, Nekola M, Nalbandov AV 1970 Role of the ovum in follicular luteinization. Endocrinology 87:288–293
8. Massagué J, Hata A, Liu F 1997 TGF-β signalling through the Smad pathway. Trends Cell Biol 7:187–192
9. Lau AL, Matzuk MM 1999 Transgenic models in the study of reproduction. Gravitational Space Biol Bull, in press
10. Nishimori K, Matzuk MM 1996 Transgenic mice in the analysis of reproductive development and function. Rev Reprod 1:203–212
11. Elvin JA, Matzuk MM 1998 Mouse models of ovarian failure. Rev Reprod 3:183–195
12. Lyons K, Graycar JL, Lee A, Hashmi S, Lindquist PB, Chen EY, Hogan BL, Derynck R 1989 Vgr-1, a mammalian gene related to *Xenopus* Vg-1, is a member of the transforming growth factor beta gene superfamily. Proc Natl Acad Sci USA 86:4554–4558
13. Lyons KM, Pelton RW, Hogan BLM 1989 Patterns of expression of murine Vgr-1 and BMP-2a RNA suggest that transforming growth factor-β-like genes coordinately regulate aspects of embryonic development. Genes Dev 3:1657–1668
14. McGrath SA, Esquela AF, Lee S-J 1995 Oocyte-specific expression of growth/differentiation factor-9. Mol Endocrinol 9:131–136
15. Dube JL, Wang P, Elvin J, Lyons KM, Celeste AJ, Matzuk MM 1998 The bone morphogenetic protein 15 is X-linked and expressed in oocytes. Mol Endocrinol 12:1809–1817
16. Dong J, Albertini DF, Nishimori K, Kumar TR, Lu N, Matzuk MM 1996 Growth differentiation factor-9 is required during early ovarian folliculogenesis. Nature 383: 531–535
17. Carabatsos M, Elvin J, Matzuk M, Albertini D 1998 Characterization of oocyte and follicle development in growth differentiation factor-9-deficient mice. Dev Biol 203: 373–384
18. Elvin JA, Yan C, Wang P, Nishimori K, Matzuk MM 1999 Molecular characterization of the follicle defects in the growth differentiation factor 9-deficient ovary. Mol Endocrinol 13:1018–1034
19. Wasserman PM Albertini DF 1994 The mammalian ovum. In: Knobil E, Neill J (eds) The Physiology of Reproduction. Raven Press, New York, pp 79–122
20. McPherron AC, Lee S-J 1993 GDF-3 and GDF-9: Two new members of the transforming growth factor-b superfamily containing a novel pattern of cysteines. J Biol Chem 268:3444–3449
21. Fülop C, Salustri A, Hascall VC 1997 Coding sequence of a hyaluronan synthase homologue expressed during expansion of the mouse cumulus-oocyte complex. Arch Biochem Biophys 337:261–266
22. Spicer AP, Augustine ML, McDonald JA 1996 Molecular cloning and characterization of a putative mouse hyaluronan synthase. J Biol Chem 271:23400–23406
23. Salustri A, Yanagishita M, Hascall VC 1989 Synthesis and accumulation of hyaluronic acid and proteoglycans in the mouse cumulus cell-oocyte complex during follicle-stimulating hormone-induced mucification. J Biol Chem 264:13840–13847
24. Tirone E, D'Alessandris C, Hascall VC, Siracusa G, Salustri A 1997 Hyaluronan synthesis by mouse cumulus cells is regulated by interactions between follicle-stimulating hormone (or epidermal growth factor) and a soluble oocyte factor (or transforming growth factor beta1). J Biol Chem 272:4787–4794
25. Salustri A, Yanagishita M, Hascall VC 1990 Mouse oocytes regulate hyaluronic acid synthesis and mucification by FSH-stimulated cumulus cells. Dev Biol 138:26–32
26. Eppig JJ, Peters AH, Telfer EE, Wigglesworth K 1993 Production of cumulus expansion enabling factor by mouse oocytes grown *in vitro*: preliminary characterization of the factor. Mol Reprod Dev 34:450–456
27. Canipari R, Epifano O, Siracusa G, Salustri A 1995 Mouse oocytes inhibit plasminogen activator production by ovarian cumulus and granulosa cells. Dev Biol 167: 371–378
28. Salustri A, Ulisse S, Yanagishita M, Hascall VC 1990 Hyaluronic acid synthesis by mural granulosa cells and cumulus cells *in vitro* is selectively stimulated by a factor produced by oocytes and by transforming growth factor-beta. J Biol Chem 265:19517–19523
29. Skinner M, Keski-Oja J, Osteen K, Moses H 1987 Ovarian thecal cells produce transforming growth factor-beta which can regulate granulosa cell growth. Endocrinology 121:786–792
30. Rennert H, Chang YII IJS 1993 Intracellular cholesterol dynamics in steroidogenic cells. In: LC Adashi EY (ed) The Ovary, Raven Press, Ltd., New York, pp 147–164
31. Richards JS, Hedin L 1988 Molecular aspects of hormone action in ovarian follicular development, ovulation, and luteinization. Annu Rev Physiol 50:441–463
32. Clark BJ, Soo SC, Caron KM, Ikeda Y, Parker KL, Stocco DM 1995 Hormonal and developmental regulation of the steroidogenic acute regulatory protein. Mol Endocrinol 9:1346–55
33. Arakane F, King SR, Du Y, Kallen CB, Walsh LP, Watari H, Stocco DM, Strauss JF 1997 Phosphorylation of steroidogenic acute regulatory protein (StAR) modulates its steroidogenic activity. J Biol Chem 272:32656–32662
34. Schuetz AW, Dubin NH 1981 Progesterone and prostaglandin secretion by ovulated rat cumulus cell-oocyte complexes. Endocrinology 108:457–463
35. Eppig JJ, Pendola FL, Wigglesworth K 1998 Mouse oocytes suppress cAMP-induced expression of LH receptor mRNA by granulosa cells *in vitro*. Mol Reprod Dev 49:327–332
36. Lim H, Paria BC, Das SK, Trzaskos JM, Dey SK 1997 Multiple female reproductive failures in cyclooxygenase 2-deficient mice. Cell 91:197–208

36a. Sterneck E, Tessarollo L, Johnson PF 1997 An essential role for C/EBPβ in female reproduction. Genes Dev 11: 2153–2162

37. Lawrence T, Dekel N, Beers W 1980 Binding of human chorionic gonadotropin by rat cumuli oophori and granulosa cells: a comparative study. Endocrinology 106: 1114–1118

38. Meduri G, Vuhai-Luuthi M, Jolivet A, Milgrom E 1992 New functional zonation in the ovary as shown by immunohistochemistry of luteinizing hormone receptor. Endocrinology 131:366–373
39. Heldin C-H, Miyazono K, Dijke Pt 1997 TGF-β signalling from cell membrane to nucleus through SMAD proteins. Nature 390:465–471
40. Sidis Y, Fujiwara T, Leykin L, Isaacson K, Toth T, Schneyer A 1998 Characterization of Inhibin/Activin subunit, activin receptor, and follistatin messenger ribonucleic acid in human and mouse oocytes: evidence for activin's paracrine signaling from granulosa cells to oocytes. Biol Reprod 59:807–812
41. Draper L, Matzuk M, Roberts V, Cox E, Weiss J, Mather J, Woodruff T 1998 Identification of an inhibin receptor in gonadal tumors from inhibin α-subunit knockout mice. J Biol Chem 273:398–403
42. Draper L, Woodruff T, Smad proteins are synthesized in the ovary. Program of the 80th Annual Meeting of The Endocrine Society, New Orleans, LA, 1998 (Abstract P3-300)
43. Matzuk MM, Kumar TR, Bradley A 1995 Different phenotypes for mice deficient in either activins or activin receptor type II. Nature 374:356–360
44. Incerti B, Dong J, Borsani G, Matzuk M 1994 Structure of the mouse growth/differentiation factor 9 gene. Biochim Biophys Acta 1222:125–128
45. Sambrook J, Fritsch E, Maniatis T 1989 In: Ford N, Nolan C (eds) Molecular Cloning: A Laboratory Manual, ed. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
46. Mahmoudi M, Lin VK 1989 Comparison of two different hybridization systems in northern transfer analysis. Biotechniques 7:331–332
47. Buccione R, Vanderhyden BC, Caron PJ, Eppig JJ 1990 FSH-induced expansion of the mouse cumulus oophorus *in vitro* is dependent upon a specific factor(s) secreted by the oocyte. Dev Biol 138:16–25