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Follicle-Stimulating Hormone-Responsive Cytoskeletal Genes in Rat Granulosa Cells: Class I β-Tubulin, Tropomyosin-4, and Kinesin Heavy Chain

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FSH regulates gene expression for granulosa cell differentiation and follicular development. Therefore, FSH-responsive genes are crucial, but only a few genes have been identified for the early stage of follicular development. In particular, little is known about cytoskeletal genes, which likely play essential roles in the morphological changes such as the antrum formation, a major landmark. FSH is also known to induce the differentiation of an immature, undifferentiated rat ovary granulosa (ROG) cell line. Our data show that FSH induced massive yet distinct reorganization of microtubules and the actin cytoskeletons as well as morphological changes. To identify those genes responding to FSH during the differentiation, differential display was performed on ROG cells. Of the 80 FSH-responsive genes identified, there were three cytoskeleton-related genes (class I β-tubulin, tropomyosin 4, and kinesin heavy chain), which are crucial for intracellular morphogenesis, transport, and differentiation. Northern blots show that the level of these gene transcripts reached a peak at 6 h after FSH treatment and subsided at 24 h. FSH induced the similar temporal expression not only in granulosa cells isolated from immature rats, but also *in vivo*. For instance, *in situ* hybridization showed that β-tubulin mRNA was transiently expressed in the granulosa cells of large preantral and early antral follicles. Despite the same temporal expression, the regulatory mechanisms of the three genes were strikingly different. As an example, cycloheximide blocked the β-tubulin mRNA expression, whereas it increased tropomyosin-4 (TM4) mRNA. Yet, it did not impact kinesin heavy chain (Khc) mRNA. In conclusion, FSH induces the massive reorganization of the cytoskeletons and morphological changes by the selective regulation of the gene expression, protein synthesis, and rearrangement of the cytoskeletal proteins in the ROG cells and probably, specific follicles and granulosa cells. (*Endocrinology* 144: 29–39, 2003)

THE FOLLICLE OF the mammalian ovary develops in several distinct stages during folliculogenesis. Primordial follicles are recruited from the resting pool to develop toward the preovulatory stage. The critical factors controlling the initial recruitment of primordial follicles are poorly understood (1). Follicular development through the primary, secondary, antral, and eventually preovulatory stages is controlled by both endocrine and paracrine factors (1–5).

Primordial and primary follicles have an oocyte surrounded by a single layer of granulosa cells. Secondary, antral, and preovulatory follicles consist of several layers of granulosa cells and an outer layer of thecal cells. Granulosa cells are the nurse cells to the oocyte and are the only cells in the follicle able to respond to FSH. Functional FSH receptors are found by d 7 in rats, when well-developed preantral follicles can be detected (4). While follicles at this stage can respond to FSH, they are not absolutely dependent on its presence. For example, follicles of hypophysectomized and GnRH agonist-treated juvenile rats can progress to the antral stage though at a slower pace and in fewer numbers (6). Thus, the development of early follicles is probably under the influence of, but not dependent on, FSH. In contrast, FSH, as well as LH, is crucial for proper follicle development past the antral stage. In fact, FSH is the predominant survival factor at this point (7).

The fate of granulosa cells at each stage of follicular development is determined in part by hormone-regulated gene expression (8). In addition, the response of granulosa cells to FSH is developmental stage dependent. For example, at early stages of follicular growth, FSH supports mitotic activity, whereas a different and specific set of genes are induced by FSH during the final stages of preovulatory development (9, 10). A number of genes regulated by FSH during the later stages of follicle development, *i.e.* the preovulatory follicle, have been identified and characterized (8). However, only a few genes have been identified that are regulated by FSH during the early stages of development. These include inhibin α (11, 12), Wilms' tumor gene (13), and cyclin D2 (14). One of the major landmarks for folliculogenesis is formation of the antrum, which likely involves changes in the cytoskeletons. However, little is known about the expression of cytoskeletal genes during the follicular development, except that actin, the primary component of the actin cytoskeleton, is constantly expressed. This is, in part, due to a lack of a suitable experimental system.

The rat ovarian granulosa (ROG) cells used in these experiments is a homogeneous clonal cell line that was established from immature granulosa cells of the rat ovary (15). These cells grow in a serum-free defined medium containing activin A but not FSH. They maintain many characteristics of undifferentiated immature cells, *i.e.* lack of steroidogenesis and ability to respond to LH. Upon exposure to FSH, the cells

Abbreviations: CHX, Cycloheximide; d, deoxy; hCG, human chorionic gonadotropin; Khc, kinesin heavy chain; PBSB, 1% BSA/PBS; PMSG, pregnant mare's serum gonadotropin; ROG, rat ovary granulosa; SDS, sodium dodecyl sulfate; TM4, tropomyosin-4; UTP, uridine triphosphate.

become postmitotic and highly steriodogenic, similar to mature granulosa cells of a dominant follicle. FSH-stimulated ROG cells also become dependent on the continued presence of FSH and will undergo apoptosis upon its removal (15). In addition, ROG cells form a structure resembling a follicle when cultured in the presence of an oocyte/cumulus cell complex (16). We have previously shown that, in response to FSH, ROG cells undergo massive actin cytoskeleton rearrangements within 3 h, leading to changes in cell-cell interactions (17). To identify cytoskeleton and other genes regulated by FSH during immature granulosa cell differentiation, we performed differential display of mRNA on ROG cells. This method has been successfully used to identify genes involved in apoptosis in ovaries after estrogen withdrawal (18), in FSH regulation of pig ovary granulosa cells (19), and in human chorionic gonadotropin (hCG) regulation of 3α-hydroxysteroid dehydrogenase mRNA expression (20).

In this study, differential display of ROG cells identified 80 genes, including 3 cytoskeletal genes, class I β-tubulin, TM4, and Khc. Their FSH-dependent mRNA and protein expression, generally focusing on β-tubulin in ROG cells, primary granulosa cells, and *in vivo*, is described.

Materials and Methods

Materials

FSH and hCG were purchased from the National Hormone and Peptide Program. DMEM, Ham's F12, and antibiotics for tissue culture were from Life Technologies, Inc. (Gaithersburg, MD) and all general reagents were from Sigma (St. Louis, MO). Restriction enzymes, reverse transcriptase, T_3, T7, and SP6 RNA polymerases, and *Taq* DNA polymerase were obtained from New England Biolabs, Inc. (Beverly, MA). [^{35}S]Uridine triphosphate (UTP)-αS and [^{32}P]-deoxy (d)-CTP were from Amersham Pharmacia Biotech (Piscataway, NJ). Oligonucleotides were synthesized by Sigma (Coralville, IA).

Animals, hormone treatment, granulosa cell isolation, and culture

Animals were handled according to the NIH guidelines for care and use of animals and the University of Kentucky Institutional Animal Care and Use Committee Guidelines. Eighteen- to 21-d-old Sprague Dawley female pups with nursing mothers were purchased from Harlan Breeding Co. (Indianapolis, IN) and housed in a photoperiod of 14-h light, 10-h darkness with lights on at 0500 h. For *in situ* hybridization, rats were injected sc with 15 IU pregnant mare's serum gonadotropin (PMSG) (Sigma) in 0.1 ml PBS on 22–23 d of age. Depending on the experiments, PMSG 48 h-primed rats were injected ip with 10 IU hCG. Ovaries were frozen on dry ice immediately after excision and stored at −80 C. To harvest and culture granulosa cells exhibiting a small antral phenotype (21), immature rats were injected sc with 1.5 mg of 17β-estradiol once a day on d 21, 22, and 23. Ovaries were collected in ice-cold serum-free 4F medium consisting of 15 mM HEPES (pH 7.4), 50% DMEM, and 50% Ham's F12 with bovine transferrin (5 μg/ml), human insulin (2 mg/ml), hydrocortisone (40 ng/ml) and antibiotics, and processed as previously described (22). Cells were washed three times, resuspended in 4F, plated on serum-coated (23) six-well plate (1×10^6 cells in 2 ml per well), and incubated at 37 C for 16 h. FSH (30 ng/ml) or forskolin (10 μM) was added to the cell cultures. For the inhibition of protein synthesis or transcription, 10 μg/ml of cycloheximide (CHX), or α-amanitin (30 μg/ml) was, respectively, added 1 h before hormone treatment.

ROG cells were cultured as previously described (15). Briefly, ROG cells were maintained in suspension in a defined serum-free medium consisting of F12-DMEM supplemented with Activin A (25 ng/ml), insulin (10 μg/ml), transferrin (5 μg/ml), α-tocopherol (0.1 μg/ml), progesterone (10 nM), BSA (0.1%), and aprotinin (25 μg/ml) in the absence of antibiotics. Activin A (25 ng/ml) was replenished every 24 h. The cells were provided with fresh media once a week, pooled every 2 wk by centrifugation at 1000 rpm for 5 min and replated at 1:2.

RT-PCR

RT-PCR was performed essentially as previously described (22). Total RNA (1–2 μg) was reverse-transcribed at 37 C in a 20-μl reaction volume using random hexamer (500 ng) and Moloney murine leukemia virus reverse transcriptase (10 U) (New England Biolabs, Inc.). cDNA samples (2 μl) were added to a total 25-μl reaction mix containing the primers (200 ng each), 0.4 mM dNTP mixture, and *Taq* DNA polymerase (2.5 U) in 1× PCR buffer (10 mM Tris, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 0.01% gelatin). All PCR amplifications were carried out for 20, 25 or 30 cycles on a Minicycler (MJ Research, Inc., Watertown, MA). PCR products were separated by 2% agarose gel electrophoresis, stained with SYBR Green I (Molecular Probes, Inc., Eugene, OR), and visualized by phosphoimaging technology (FLA-2000; Fuji Photo Film Co., Ltd., Stamford, CT). The following primers were used in the present studies: TM4 (5′-gag aac tcc tga ctg aac tgg acg-3′ and 5′-cca tat tcc ctg ctg agc gta g-3′, 282 bp), Khc (5′-aac tga atc gcc tcc aag cag-3′ and 5′-cga act ggc gag aac tgg atg-3′, 195 bp), β-tubulin (5′-cct gct cat cag caa gat tcg-3′ and 5′-gtg gtg agc tta agg gta cgg, 210 bp), and inhibin-α subunit (5′-gct ttc cct ctg ttg acc cac-3′ and 5′-aga tgt tga ggg cag ctc gat-3′, 255 bp). L-19 (5′-ctg aag gtc aaa ggg aat gtg-3′ and 5′-gga cag agt ctt gat gat ctc, 194 bp) oligonucleotide primers were used to amplify ribosomal protein L-19 as an internal control (24).

Differential display

ROG cells were incubated in the absence or presence of FSH (30 ng/ml) for 6 h in triplicate before extracting total RNA. Total RNA was used as a template for differential display analyses using the Delta Differential Display Kit (CLONTECH Laboratories, Inc., Palo Alto, CA). Briefly, total RNA was reverse-transcribed to produce first-strand cDNA by incubating 2 μg total RNA with an oligo $(deoxythymidine)_{12–18}$ primer and Moloney murine leukemia virus reverse transcriptase for 1 h at 42 C. Reactions were performed in triplicate and the cDNAs pooled to reduce variability (25). The pooled first-strand cDNA was diluted by transferring 2 μl to a new tube and adding 78 μl of sterile water (tube B), and then 72 μl sterile water to the original tube (tube A). Thus, each sample is run in duplicate but at different dilutions, reducing variability and false positives. PCR amplification of duplicate cDNA samples was performed in a MiniCycler using a P/T primer pair in the presence of [α-^{32}P]dATP and Advantage KlenTaq polymerase (CLONTECH Laboratories, Inc.). The following amplification program was used: 1 cycle of 95 C for 5 min, 40 C for 5 min, 68 C for 5 min; 2 cycles of 94 C for 2 min, 40 C for 5 min, 68 C for 5 min; 25 cycles of 94 C for 1 min, 60 C for 1 min, 68 C for 2 min; and 1 cycle of 68 C for 10 min. Samples were electrophoresed on denaturing 5% polyacrylamide/8 M urea gels. The gels were dried and exposed to x-ray film overnight at −80 C. Lanes containing vehicle and FSH samples were compared and bands appearing to be differentially regulated in both duplicate lanes were excised from the dried gel and eluted in 40 μl sterile water at 100 C for 5 min. The fragments were then reamplified using the same P/T primer combination and the following amplification profile: 1 cycle of 95 C for 5 min; 20 cycles of 94 C for 1 min, 60 C for 1 min, and 68 C for 2 min; and 1 cycle of 68 C for 10 min. Fragments were then subcloned into the pCR2.1 TA cloning vector system (Invitrogen Corp., Carlsbad, CA). Differential regulation of the subclones was verified by cDNA blot and/or Northern blot as described below. Each verified subclone was sequenced on a Beckman CEQ XL2000 capillary sequencer. Clones were identified using the BLAST algorithm at the National Center for Biotechnology Information (26).

Northern blot

For Northern analysis, 10 μg of total RNA per sample was resolved on 1.5% agarose gels containing 2.2 M formaldehyde. RNA was then capillary-blotted to nylon membranes (Nytran super charge, Schleicher & Schuell, Keene, NH). [α-^{32}P]dCTP-labeled cDNA probes were made from each subclone using the *redi*prime II random prime labeling system (Amersham Pharmacia Biotech) and purified with micro bio-spin chromatography columns (Bio-Rad Laboratories, Inc., Hercules, CA). Blots

were hybridized overnight at 42 C in 50% (vol/vol) formamide, 5× SSPE (0.15 M NaCl, 10 mM Na_2PO_4, 1 mM EDTA), 5× Denhardt's reagent, 0.1% (wt/vol) sodium dodecyl sulfate (SDS), and 200 mg/ml denatured, fragmented herring testis DNA. Filters were washed once at low stringency (5× SSPE, 1% SDS, 25 C) and twice at high stringency (0.1× SSPE, 1% SDS, 62 C) for 45 min and exposed to X-AR film (Kodak, Rochester, NY) for 12–72 h at −80 C.

cDNA blots

Differentially regulated cDNA fragments cloned into the pCR2.1 TA vector were amplified using M13 reverse and T7 primers. The samples were extracted once with phenol:chloroform and precipitated by adding 3 M sodium acetate, pH 5.2 (1:0.1), and 100% ethanol (1:2.5) and centrifuging at 14,000 × *g* at 4 C. Pellets were resuspended in 10 mM Tris-Cl, 1 mM EDTA (pH 8). Each reamplified fragment was denatured by adding 0.1 volume 3 M NaOH and incubating for 1 h at 70 C. Samples were cooled to room temperature and 20× SSPE (3.6 M NaCl; 0.2 M $NaPO_4$, pH 7.7; 20 mM EDTA) was added to a final concentration of 6×. Samples were immobilized on nylon membranes (Nytran super charge) using a Minifold II slot-blotter (Schleicher & Schuell). Each sample was divided equally among the appropriate number of filter membranes depending on experiment size. To probe the slot blots, first strand cDNA was synthesized from 5 μg total RNA using SuperScript II (Life Technologies, Inc., Grand Island, NY) and labeled with [α-^{32}P]dCTP using the *redi*prime II random prime labeling system. Probes were purified with micro bio-spin chromatography. The slot-blots were hybridized overnight in the presence of probe at 42 C in 50% (vol/vol) formamide, 5× SSPE, 5× Denhardt's reagent, 0.1% (wt/vol) SDS, and 100 μg/ml denatured, fragmented herring testis DNA. Filters were washed once at low stringency (5× SSPE, 1% SDS, 25 C) and twice at high stringency (0.1× SSPE, 1% SDS, 62 C) for 45 min and exposed onto Kodak X-AR film for 12–72 h at −80 C. Autoradiograms were digitized using an AGFA SNAPSCAN 1212 flatbed scanner and Adobe PhotoShop 5 software. Images were quantitated using Image J software (written by Wayne Rasband at NIH and available by anonymous FTP from zippy.nimh.nih.gov) and percent increase calculated for three independent experiments.

In situ hybridization

Frozen ovaries were cut in 20-μm sections using a MICROM HM 505 E cryostat (Richard-Allan Scientific, Kalamazoo, MI) and mounted onto Superfrost/Plus Microscope slides (Fisher Scientific, Pittsburgh, PA). Sections were fixed, pretreated, and hybridized with antisense and sense RNA probes as previously described (22). Using T7, T_3, or SP6 polymerase, [^{35}S]UTP-labeled RNA probes were synthesized from clone no. 7 for TM4 no. 25 for Khc, no. 46 for β-tubulin, and clone no. 56 for inhibin α, which have been subcloned into pBluescript II vector (Stratagene, La Jolla, CA). RNA probes (1×10^7 cpm/ml in hybridization buffer: 50% formamide, 5× SSPE, 2× Denhardt's reagent, 10% dextran sulfate, 0.1% SDS, and 100 μg/ml yeast tRNA) were applied to sections and the sections were incubated in a humidity chamber at 47 C for 16–18 h. After hybridization, sections were treated with ribonuclease A (20 μg/ml) at 37 C for 30 min, washed in decreasing concentrations of sodium citrate buffer (0.15 M NaCl, 15 mM sodium citrate) down to 0.1× sodium citrate buffer at 58 C, and dehydrated through an ethanol series. Slides were then exposed to Kodak BIOMAX MR film for 2 d and processed for liquid emulsion autoradiography using NTB-2 emulsion (Kodak) for 3–6 wk. Developed sections were stained with Gill's Formulation no. 2 hematoxylin solution (Fisher Scientific). Tissues were examined with a Nikon Microphot-SA microscope (Nikon, Melville, NY) under bright field and dark field optics. For each gene, a sense riboprobe was used as a control for nonspecific binding.

Immunofluorescent staining

ROG cells were cultured on 12-mm no. 1 glass coverslips in 24-well plates and allowed to attach overnight. Before plating, coverslips were coated with 10 μg/ml poly-D-lysine and 5 μg/ml fibronectin to facilitate cell attachment as per the manufacturer's instruction (Sigma). Under these conditions, cell aggregates will attach loosely to the surface (16). After allowing cells to attach overnight, the medium was removed and the cells were incubated in defined media with or without 30 ng/ml FSH before being fixed and stained. Cells stained for f-actin were washed once in PBS, fixed in 4% paraformaldahyde in PBS at room temperature for 10 min, and permeablized in 0.1% Triton X-100 for 5 min. Cells were then washed three times in PBS and incubated with PBSBT (1% BSA and 0.05% Tween 20 in PBS) for 10 min. f-actin was stained by incubating slides with 0.7 μg/ml TRITC-phalloidin (Sigma) for 20 min.

Cells stained for tropomyosin or β-tubulin antibodies were fixed in −20 C 100% methanol at room temperature for 5 min and air dried. Cells were then washed three times in PBS and incubated with PBSB (1% BSA/PBS) for 10 min. Tubulin or tropomyosin was stained by incubating cells with β-tubulin antibody (1:200) or tropomyosin antibody (1:100) (Sigma) diluted in PBSB for 1 h. Coverslips were washed three times with PBSB followed by incubation with an antimouse fluorescein isothiocyanate conjugate (1:100) (Sigma) for 20 min. The fluorescence-labeled samples were viewed using a Leica Corp. (Buffalo, NY) scanning confocal microscope with a krypton-argon laser. Projections were constructed using the NIH image software (written by Wayne Rasband at NIH and available by anonymous FTP from zippy.nimh.nih.gov) and pasted into Photoshop 5.0.

Results

Differential display of FSH-responsive genes

ROG cells are a clonal cell line derived from a P14 immature rat and, thus, undifferentiated. These cells grow in a defined medium containing activin A but free of serum and FSH. They express the FSH receptor and respond to exogenous FSH with a burst of proliferation, becoming both steriodogenic and terminally differentiated by approximately 96 h post stimulation (15). We have previously shown that ROG cells undergo rapid morphological changes, including formation of lamellipodia and filopodia within 3 h of FSH treatment (17). With these considerations in mind, we set out to identify FSH-responsive genes in granulosa cells early in the path to terminal differentiation. ROG cells were cultured in the absence or presence of 30 ng/ml FSH for 6 h, total RNA was extracted, and differential display was performed (Fig. 1A). Differentially expressed bands (Fig. 1B) were excised, PCR amplified, and electrophoresed. Electrophoresis of amplified samples revealed 80 sets of differential bands (an example of which can be seen in Fig. 1C), of which 54 were up-regulated and 26 were down-regulated.

Identification and characteristics of FSH-responsive genes

The cDNAs were extracted from the gels, cloned, and sequenced. Sequence comparison revealed 61 unique cDNAs and 19 duplicates. To verify the time-dependent FSH-responsiveness of the unique cDNAs, they were slot-blotted and probed with [α-^{32}P]dCTP-cDNA probes. To prepare these probes, mRNA extracted from ROG cells treated with FSH for 0, 6, and 24 h were reverse-transcribed with [α-^{32}P]dCTP. Repeated slot blot analyses revealed 14 genes (Fig. 2, Table 1), whose mRNA expression levels changed consistently and significantly over a 24-h period after FSH treatment. Interestingly, their gene expression was diverse. Some were either up-regulated (Fig. 2A) or down-regulated (Fig. 2B), whereas others were initially up-regulated and subsequently, down-regulated (Fig. 2C). Three of these genes are associated with the cytoskeleton and will be further discussed.

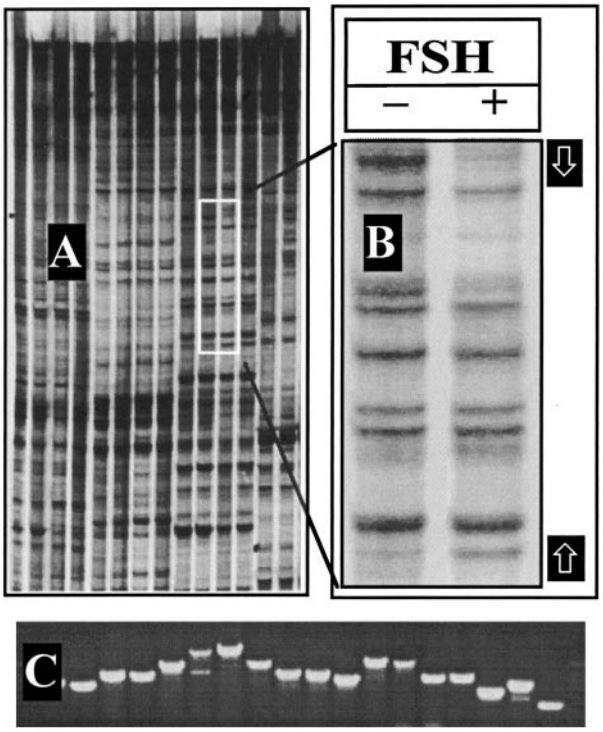

FIG. 1. Differential display autoradiogram. ROG cells were cultured for 6 h in the absence (−) or presence (+) of FSH, and total RNA was extracted and subjected to differential display after RT-PCR (A) on DNA sequencing gels as described in *Materials and Methods*. B, A portion of the gels shows bands that responded to FSH. The *down arrow* indicates the band with a decrease in the intensity upon FSH treatment, and *up arrow* represents the band with increased intensity. C, FSH-responsive bands were excised, PCR amplified, and electrophoresed. The DNAs were isolated and cloned.

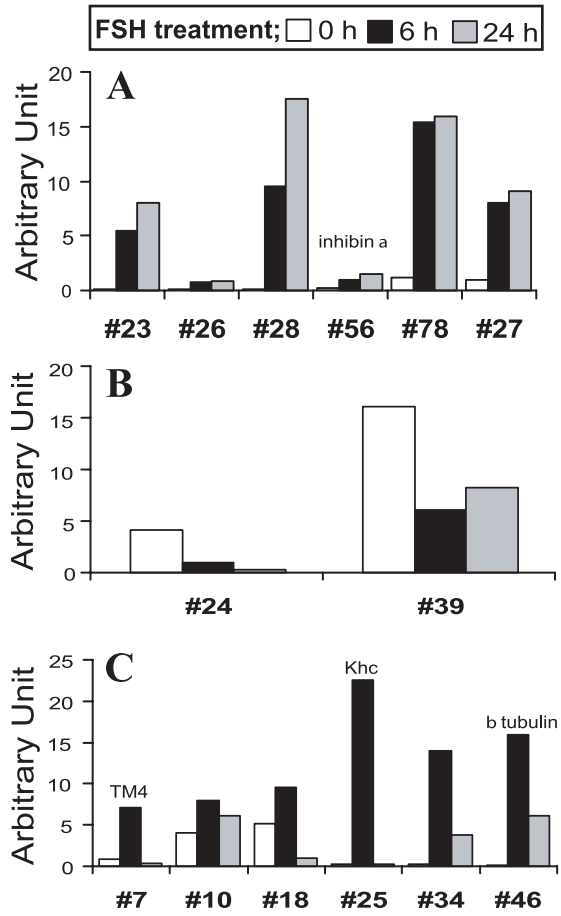

FIG. 2. Temporal expression of mRNAs in ROG cells. The cDNA fragment in each of the clones described in the Fig. 1 legend was amplified and slot-blotted. These blots were probed with [α-^{32}P]dCTP-labeled probes, which were prepared as follows: ROG cells were cultured for 0 h, 6 h, and 24 h in the presence of 30 ng/ml FSH, total RNA was isolated, and reverse transcribed to produce [α-^{32}P]dCTP-labeled cDNA probes, as described in *Materials and Methods*. The experiments were repeated several times, and 14 clones showed consistent patterns of expression. They were grouped according to their temporal expression pattern. A, Six clones were up-regulated by FSH treatment (nos. 23, 26, 28, 56, 78, and 27). B, Two clones showed down-regulation (nos. 24 and 39). C, Six clones were first up-regulated and then, down-regulation (nos. 7, 10, 18, 25, 34, and 46). The identities of the clones are summarized in Table 1.

FSH-responsive cytoskeletal genes in ROG cells

Blast search revealed that clone no. 7 was 100% homologous to rat TM4 (GenBank no. Y00169), no. 25 was 100% homologous to a rat expressed sequence tag clone (GenBank no. AI137325) and 98% homologous to mouse Khc (GenBank no. L27153), and no. 46 was 100% homologous to rat class I β-tubulin (GenBank no. AB011679). It should be noted that all three genes were up-regulated at 6 h post FSH treatment and subsequently down-regulated by 24 h (Fig. 2C). Clone no. 56 was inhibin α, and its expression was induced and sustained by FSH (Fig. 2A). This is consistent with previous reports that the gene is induced and sustained by FSH in immature granulosa cells (27, 28). Therefore, it has been routinely used as a control and representative FSH-regulated gene (29, 30).

Next, we were concerned whether the changes in the three gene transcripts shown in Fig. 2 indeed reflect their mRNA levels or putative experimental variations, in particular PCR dependent amplification. Because the simplest and most direct way to determine mRNA concentrations is a Northern blot, it was chosen. However, it requires substantial amounts of mRNAs. For that purpose, a large quantity of ROG cells were grown and exposed to FSH for 0, 6, or 24 h. This was not trivial because the limited supply of Activin A restricts ROG cell cultures. Total RNA was extracted from these cells and the individual transcripts were directly measured by Northern blot. An equal amount of total RNA was applied to individual sample lanes. The large and small rRNA bands and tRNA band appeared distinctly and their respective band intensities were invariable in the gel lanes as seen in the similar intensity of the 28S rRNA bands in all sample lanes (Fig. 3), suggesting the integrity of the RNA samples. Inhibin α mRNA was also examined as a control. The results confirmed the FSH- and time-dependent expression of the three cytoskeletal genes in ROG cells (Fig. 3). Autoradiograms revealed a 0.9-kb band for TM4, 2.7-kb band for Khc, and 1.6-kb band for the inhibin α-subunit. In contrast to the single band of TM4, Khc, and inhibin α mRNAs, β-tubulin mRNAs showed three isoforms of 2.3-, 3.1-, and 4.4-kb bands. There are striking differences in the mRNA expression of these genes. TM4 and Khc mRNAs were not detectable before FSH treatment, dramatically increased after FSH treatment for 6 h,

TABLE 1. FSH-responsive genes

Clone ID	Gene description	GeneBank accession no.
7	Tropomyosin 4	Y00169
10	Unidentified human gene from brain	BM964053
18	Jbp1: methyltransferase	AF167574
23	Clcn2: chloride channel protein	X64139
24	3α-Hydroxysteroid dehydrogenase	S48540
25	Kif5b: kinesin heavy chain	U86090
26	b4 Intergrin interactor homolog/eIF6	AF047046
27	Unknown	NA
28	Adult male testis cDNA	AK007269
39	Unknown	NA
34	Corel UDP-galactose:*N*-acetylgalactosamine-α-R	NM022950
46	mRNA for class I β-tubulin	AB011679
56	Inhibin α-subunit	NM012590
78	Unknown	NA

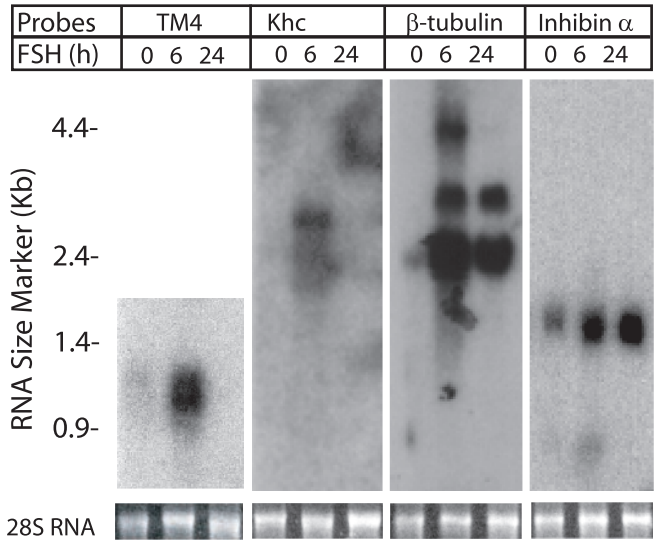

FIG. 3. Northern blot of temporal cytoskeletal gene mRNA in ROG cells. ROG cells were cultured in the presence of 30 ng/ml FSH for 0, 6, and 24 h, and total RNA was isolated. The mRNA expression level was analyzed by Northern blotting. Total RNA (10 μg) was loaded in each lane, electrophoresed, visualized with ethidium bromide, blotted and probed for TM4, Khc, β-tubulin, or inhibin α-subunit. The 28S rRNA bands of all gel lanes that were stained with ethidium bromide are shown in the *lower panel*. The size markers (0.78–4.40 kb RNA ladder, Life Technologies, Inc.) are shown on the *left*. Autoradiograms show one respective band by TM4 (0.9 kb), Khc (2.7 kb), and inhibin α-subunit (1.6 kb) cDNA probes, respectively, and three bands with β-tubulin probe.

and then decreased to background level by 24 h, consistent with results shown in Fig. 2. It is interesting that β-tubulin mRNA shows three isoforms, which diversely responded to FSH. The largest 4.4-kb band of β-tubulin mRNA markedly increased at 6 h of FSH treatment and then, disappeared by 24 h. In contrast, the 2.3-kb and 3.1-kb mRNA bands increased at 6 h and sustained their intensity at 24 h. Therefore, the total intensity of the three β-tubulin mRNA bands markedly increased at 6 h and partially decreased at 24 h, consistent with the observation in Fig. 2. This FSH-dependent differential expression of β-tubulin mRNA is novel and raises an intriguing question on the function and regulation of the three mRNA isoforms and their role in granulosa cell differentiation. Unlike these cytoskeletal genes, inhibin α mRNA gradually increased throughout FSH treatment, which is consistent with previous reports (28).

Expression and localization of β-tubulin and tropomyosin proteins

To determine whether the changes in the mRNA expression are reflected in the protein expression and cell morphology, ROG cells were cultured in the presence or absence of 30 ng/ml FSH and immunostained with anti-β-tubulin antibody or antitropomyosin antibody. The cells were also stained with anti-f-actin antibody as a comparison. Staining of FSH-stimulated ROG cells with antiactin antibody shows the formation of lamellipodia and filopodia as early as 3 h (Fig. 4). By 24 h, the cells around the periphery of the cell clump had spread to form a monolayer, consistent with the previous observation (17). Although β-tubulin staining was light in untreated samples (Fig. 4D), it was intense and distributed throughout the cells by 3 h after FSH treatment (Fig. 4E). The results were the same for 6 h treatment (data not included). These observations suggest that β-tubulin was produced en mass and assembled into an extensive network of microtubules. By 24 h, β-tubulin staining was reduced (Fig. 4F). In contrast to the dramatic increase in the β-tubulin concentration, the f-actin concentration did not change in response to FSH, but there was massive reorganization of the actin cytoskeleton (Fig. 4, A–C). This result is consistent with our observation (17) that the actin mRNA level was constant regardless of FSH treatment (data not shown). Clearly, the organization and regulation of the microtubules differ from those of the actin cytoskeletons including lamellipodia and filopodia in ROG cells.

mRNA expression during follicular development

Because we have dealt with the genes expressed in ROG cells, we were curious whether the three cytoskeletal genes are physiologically relevant to granulosa cell differentiation *in vivo*. To localize the cytoskeletal mRNAs in the ovary and follicle *in vivo*, sections of an adult rat ovary were *in situ* hybridized. The β-tubulin and TM4 mRNAs were detected in a few large preantral and early antral follicles (Fig. 5), suggesting that their expression is dependent on follicular development. On the other hand, Khc mRNA was not detected, probably due to the relatively small size (160 bp) of the Khc riboprobe (data not shown).

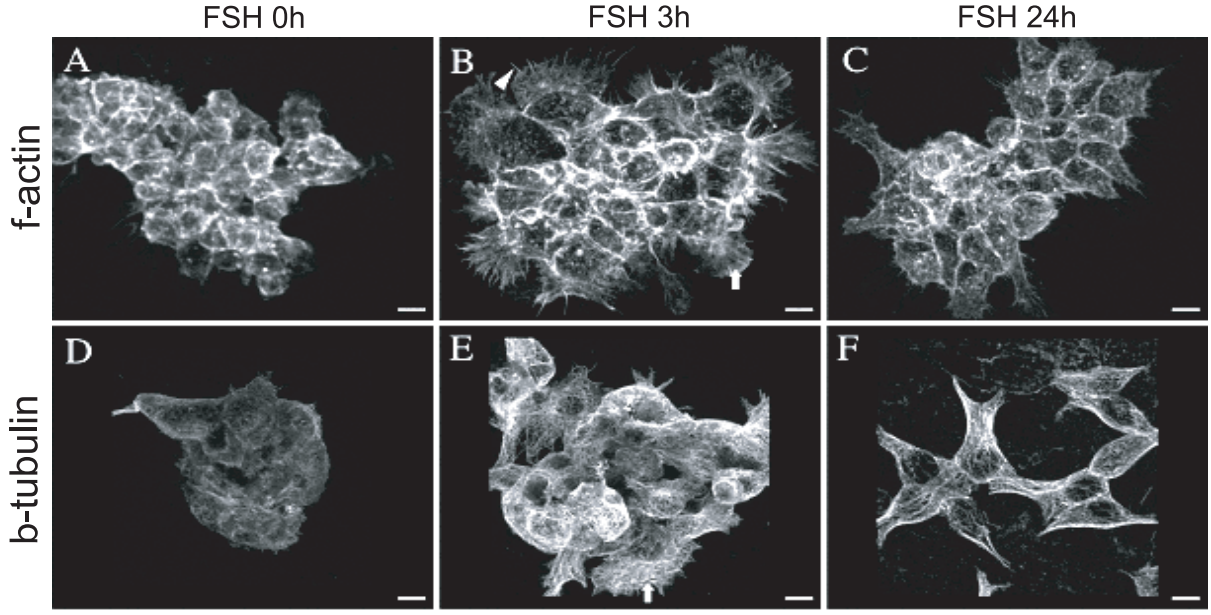

FIG. 4. Fluorescent microscopy of ROG cells stained for β-tubulin and f-actin proteins. ROG cells were grown on poly-D-lysine/fibronectin coated 12 mm no. 1 glass coverslips in defined culture media in the absence of FSH (A and D) or in the presence of 30 ng/ml FSH (B, C, E, and F) for 3 h (B and E) or 24 h (C and F). The cells were stained for f-actin (A–C) or β-tubulin (D–F), and examined under a fluorescence confocal microscope. *Arrows* and *arrowheads* indicate lamellipodia and filopodia, respectively. Note the similar level of fluorescence signal for f-actin (A–C) and the increased signal for β-tubulin protein among the treatment groups (D–F). The *scale bar* represents 15 μm.

We decided to further examine whether β-tubulin expression is dependent on the granulosa cell differentiation and follicular development. Ovaries were excised from immature female rats that were treated with PMSG for 0, 3, 6, 24, or 48 h (Fig. 5). In addition, those primed with PMSG for 48 h were additionally treated with hCG for 3 h, 6 h, or 24 h to promote development of preovulatory follicles (31). β-tubulin mRNA was prominently detected during the early stages of PMSG priming (for example, PMSG priming for 6–24 h), and the labeling intensity was dependent on the follicular development. The large preantral and early antral follicles were the major sites of the β-tubulin mRNA expression. Most of the primary follicles were labeled but the level was moderate. In contrast, the large preovulatory follicles of the ovaries primed with PMSG for 48 h showed either no signal or a markedly lower level of signal. Particularly, it is striking to find that the additional treatment with hCG completely abolished β-tubulin mRNA expression in the preovulatory follicle. The β-tubulin sense probe did not label, showing the specificity of the labeling (data not included). These results clearly show that β-tubulin mRNA expression was dependent on the stage of follicular development.

Because inhibin α has been proven to be regulated by FSH (27, 28) and used as a FSH-regulated marker gene, it was desirable to compare the expression profiles of β-tubulin and inhibin α. For this purpose, adjacent sections of the ovary were hybridized with inhibin α and β-tubulin riboprobes. The expression patterns of the two gene transcripts were similar up to the early antral stage (Fig. 5). However, in preovulatory follicles of the rats primed with PMSG for 48 h, inhibin α mRNA expression was very high, in contrast to suppressed β-tubulin mRNA (Fig. 5, PMSG 48H). A moderate level of β-tubulin mRNA was seen in theca and interstitial cells and the expression persisted in the large antral follicle, even after expression in granulosa cell was diminished (Fig. 6). It should be noted that the basal β-tubulin mRNA level was readily detectable in granulosa cells, but not in ROG cells. The difference may be attributed to the likely exposure of the granulosa cells to FSH before excision from the rat ovaries, whereas ROG cells were not exposed at all.

Expression of the cytoskeletal genes in rat primary granulosa cell cultures

Follicular development requires differentiation of granulosa cells, which is dependent on FSH. Therefore, we set out to investigate whether the cytoskeletal gene expression is associated with FSH-dependent differentiation of granulosa cells. Immature 21-d-old rats were injected with 17β-estradiol for a consecutive 3 d. Granulosa cells were isolated, cultured overnight, and treated with FSH for increasing time periods from 0 h to 48 h. This culture system has successfully been used for the studies on FSH-responsive genes (21, 32, 33). The expression levels of TM4, Khc, and β-tubulin increased to peak at 3 h, subsequently decreased to a trough at 12 h, and increased again to reach another peak at 24 h (Fig. 7). The level of the internal control, L-19, did not change, whereas inhibin α gradually increased to a peak at 24–48 h. The trend of the three cytoskeletal gene transcripts to peak several hours after FSH treatment is consistent both in ROG cells and in primary granulosa cell cultures. In contrast, inhibin α mRNA levels continuously increased in both ROG cells and primary culture. These results underscore the similarity in the gene expression profiles of these two cell types.

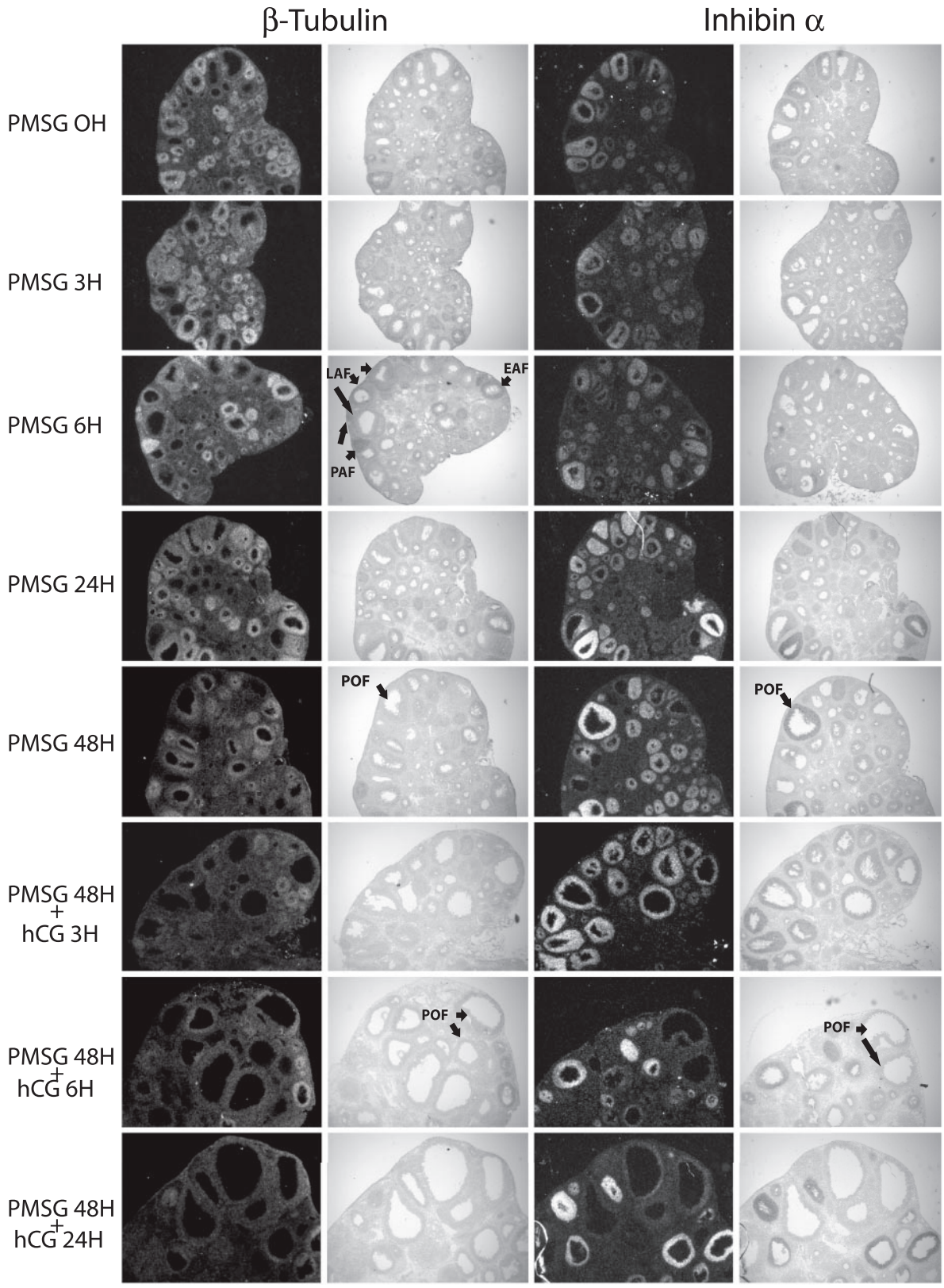

FIG. 5. Temporal expression of β-tubulin mRNA during follicular development. Immature 22- to 23-d-old rats were injected with PMSG (15 IU) and kept for 0, 3, 6, 24, or 48 h. Rats primed with PMSG for 48 h were additionally injected with hCG (10 IU) and kept for 3, 6, or 24 h. Ovaries were excised and sectioned. Adjacent ovarian sections were hybridized with [^{35}S]UTP-labeled β-tubulin (*left panels*) or inhibin α (*right panels*) antisense probe. Bright field images (hematoxylene staining) are aligned in the *right side* of the corresponding dark field images. PAF, Preantral follicle; EAF, early antral follicle; LAF, large antral follicle; POF, preovulatory follicle. Magnification, ×100.

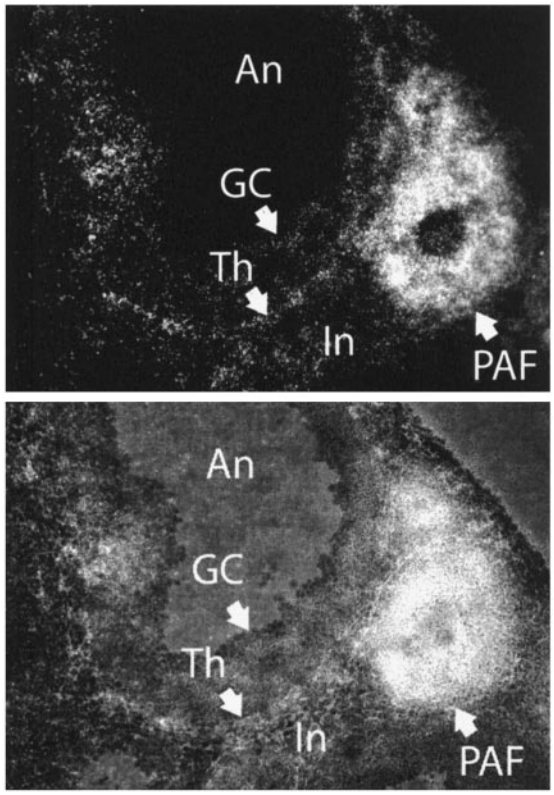

FIG. 6. Cell type-specific expression of β-tubulin mRNA. Ovarian sections of rats primed with PMSG for 6 h were hybridized with the β-tubulin riboprobe. The slide was examined for dark field images (*upper*) and bright field images (*lower*). An, Antrum; PAF, preantral follicle; In, interstitial cells; GC, granulosa cells; Th, theca cells. Photographs are taken at ×100 magnification for A–D and ×400 for E and F.

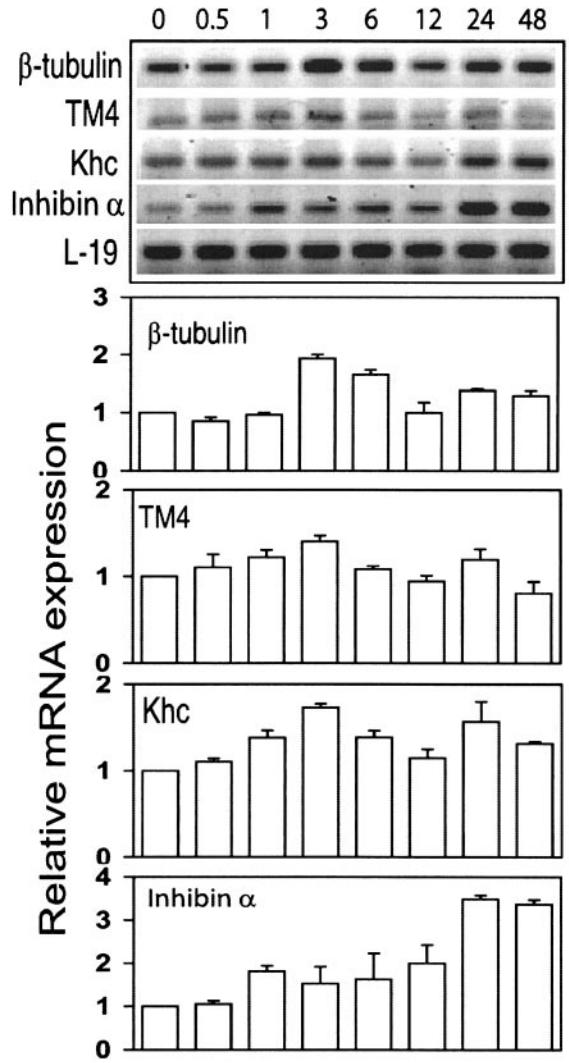

FIG. 7. Temporal expression of cytoskeletal genes in primary granulosa cells. Rat granulosa cells were isolated from the ovaries of 17β-estradiol primed, 24-d-old immature rats described in *Materials and Methods*. They were cultured in the presence of 30 ng FSH/ml for up to 48 h. Total RNA was isolated, amplified for semiquantitative RT-PCR for TM4, Khc, β-tubulin or inhibin α mRNA, resolved on 2% agarose gel, stained with SYBR Green I (Molecular Probes, Inc.), and phosphoimaged as described in *Materials and Methods*. Ribosomal protein L-19 mRNA was used for internal control. Values are the meanfold increase over control (0 h) ± SE. Values were pooled from two separate experiments.

Different mechanisms of cytoskeletal gene expressions in granulosa cells

As a step to determine the mechanisms of the FSH-responsive mRNA expression, the granulosa cells were treated with FSH in the presence or absence of a translation inhibitor, CHX, and a transcription inhibitor, α-amanitin. As shown in Fig. 8, treatment of FSH for 6 h enhanced the expression level of β-tubulin, consistent with the result seen in Fig. 7. It was striking to find that β-tubulin mRNA could not be detected when the cells were exposed to CHX, even in the presence of FSH. In addition, the β-tubulin mRNA level decreased to apparent basal level in response to 24-h exposure to FSH in the presence of CHX (Fig. 8). These results suggest that the *de novo* synthesis of a protein(s) is necessary to sustain the β-tubulin mRNA level in the absence of FSH and increase its level in response to FSH. In addition, the transcription of the β-tubulin gene appears to be involved in the process, as α-amanitin reduced the basal level and blocked FSH-dependent increase of β-tubulin mRNA. Taken together, these results suggest the interesting possibility that the FSH-dependent transcription of β-tubulin mRNA requires *de novo* synthesis of a protein factor(s).

It was surprising to find that the effects of CHX on the expression of the three genes were not the same. CHX lowered the Khc mRNA level, though not completely, in cells treated with FSH for 6 h, suggesting the existence of CHX insensitive mRNA. This differs from the β-tubulin mRNA. In cells treated with FSH for 24 h, the effects of CHX and α amanitin were marginal. In contrast to the CHX-dependent decrease in the levels of the β-tubulin and Khc mRNAs, CHX increased the TM4 and mRNA level of the cells treated with FSH for 6 h (Fig. 8). This suggests that the FSH-dependent increase in TM4 mRNA levels is down-regulated by a protein factor(s). Apparently, in the absence of this protein, the mRNA level went up. Similarly, the inhibin α mRNA level increased in the presence of CHX, suggesting that both types of mRNAs are kept down-regulated by some protein factor(s). However, the similarity ends when the mRNA levels

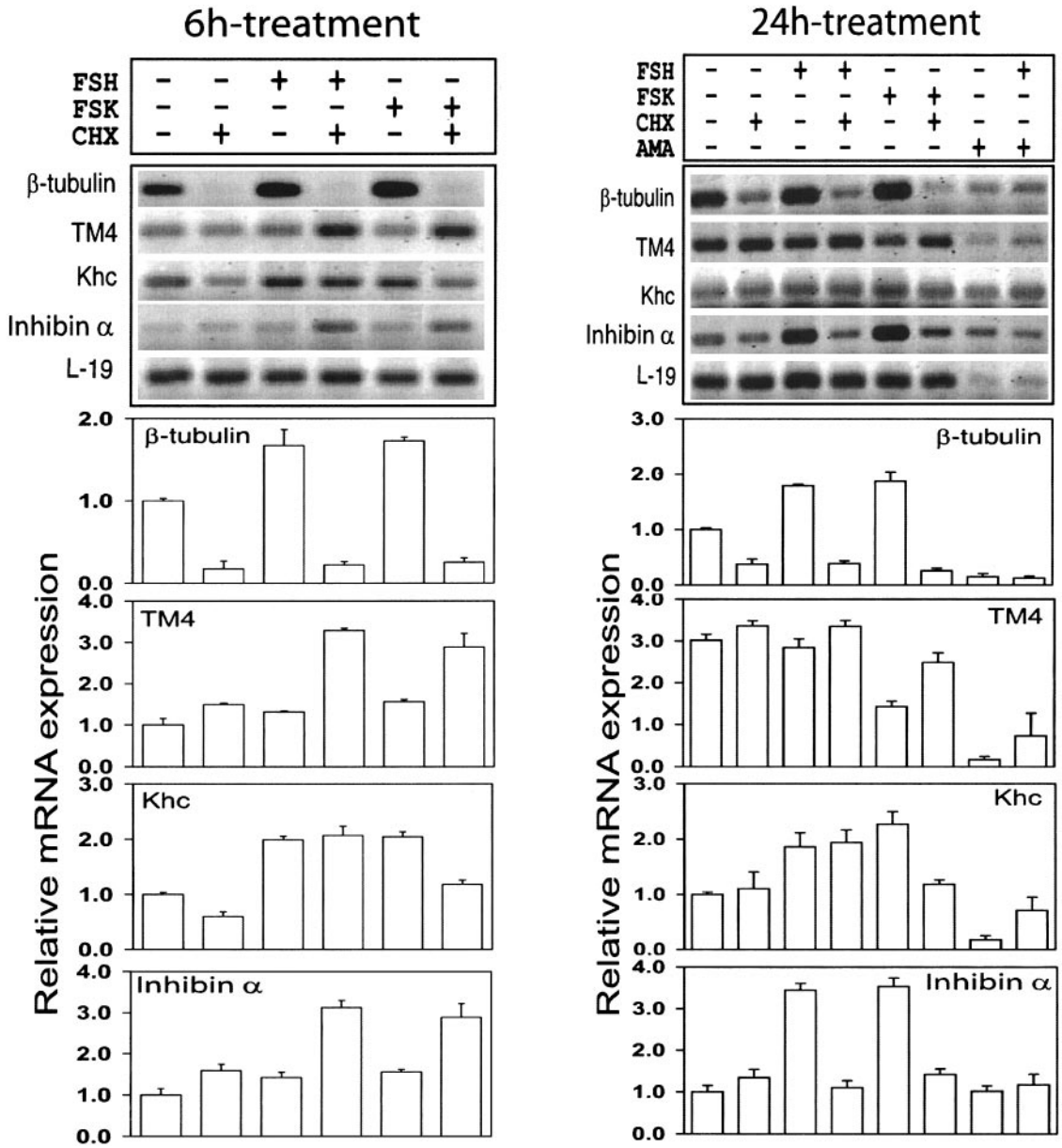

FIG. 8. Effects of CHX and α-amanitin treatment on the FSH/forskolin-induced expression of the cytoskeletal genes. Granulosa cells were isolated from the ovaries of 17β estradiol primed 24-d-old immature rats. They were cultured in the medium containing FSH (30 ng/ml) or forskolin (10 μM, FSK) in the presence or absence of CHX (10 μg/ml) for 6 h (*left*) or 24 h (*right*). In addition, the 24-h cells were also cultured, likewise, in the presence of α-amanitin (30 μg/ml, AMA). Total RNA was isolated and analyzed for mRNAs of TM4, Khc, β-tubulin, and inhibin α as described in the Fig. 7 legend. Values are the meanfold increase over control (no treatment) ± SE. Values were pooled from two separate experiments.

were compared for granulosa cells that were treated with FSH for 24 h. In these cells, the CHX effects were opposite on the mRNA levels of inhibin α and TM4, substantially suppressing the former whereas slightly enhancing the latter (Fig. 8). These opposite effects are of interest because the two gene transcripts showed the second peak at 24 h of exposure to FSH (Fig. 7).

The effects of FSH and forskolin that activates adenylyl cyclase were the same (Fig. 8). These results suggest that FSH induces the expression of the cytoskeletal and inhibin α genes via the adenylyl cyclase. This conclusion is consistent with the previous report that FSH induces the massive reorganization of the actin cytoskeleton through the adenylyl cyclase pathway (17). It should be noted that α-amanitin blocked the FSH-induced mRNA expression of all of the cytoskeletal and inhibin α genes, suggesting the transcriptional regulation.

Discussion

We have demonstrated, using several different methods (differential display, slot blot, and Northern analysis), that three cytoskeletal genes, β-tubulin, TM4, and Khc, were rapidly up- and down-regulated in response to FSH in ROG

cells; not only the mRNA levels but also the β-tubulin and TM4 proteins. Furthermore, these proteins were assembled into lamellipodia and filopodia during the up-regulation. These results underscore their crucial roles in granulosa cell differentiation and follicular development (17). Equally significant is the fact that the change in the cytoskeleton gene expression occurred not only in ROG cells but also *in vivo*. For instance, the similar up- and down-regulation of the three cytoskeletal genes are demonstrated in freshly isolated granulosa cell cultures and developing follicles of the rat ovaries. Therefore, their gene expression is dependent not only on the exposure to FSH but also on the exposure period of the hormone, as well as the stage of follicular development.

The quick increase and peaking in their mRNA levels after FSH treatment are evident by several lines of evidence, the Northern blots and RT-PCR analyses, as well as the *in situ* hybridization studies of both randomly cycling adult rats and immature rats treated for superovulation. This temporal expression of TM4 and β-tubulin mRNAs is confined in the large preantral and early antral stages (Figs. 5 and 6). These results suggest an immediate functional role of the cytoskeletal genes in the FSH-induced granulosa cell differentiation and proliferation.

The cytoskeletons are involved in a variety of cellular functions including intracellular communication, cell polarity, locomotion, establishment and maintenance of morphology, cell-to-cell and cell-to-substratum contacts, and cell division (34). There are three major classes of cytoskeletal structures, actin microfilaments, microtubules, and intermediate filaments. Tropomyosin binds as head to tail aggregates in a groove along the f-actin helix, thus strengthening the filament, and has been intensively studied in muscle cells, where it regulates the binding of myosin heads to f-actin (35). However, in nonmuscle cells, tropomyosin is known to have roles in intracellular granule movement, vesicular transport, mRNA localization, and mitosis (36). TM4 is one of 18 distinct isoforms generated by alternative splicing and alternative promotor usages (37), whose specific function is unknown. β-tubulin is one of the two tubulin subunits, the other being α-tubulin. Polymers of tubulin make up microtubules, which are important in a wide range of cellular processes, including intracellular transport and generation of the mitotic spindle in most of cells (38–40). Kinesin, consisting of two heavy chains and two light chains, binds directly to microtubules and provides anterograde transport of vesicles and organelles (41, 42).

This study is the first to relate these gene transcripts and proteins and their FSH-responsive temporal expression to granulosa differentiation and follicular development. This is consistent with physiological observations. In cycling rats, a few primordial follicles are recruited into the growth cycle by the secondary FSH surge and, under FSH signaling, the granulosa cells of the selected follicles undergo morphological changes, differentiation and proliferation (5). These are accompanied by changes in the intracellular structure, organelle movement, and steroidogenesis. Therefore, these dynamic changes likely require an immediate supply of specific cytoskeletal proteins, and one of the functions of FSH at this early stage of granulosa cell differentiation is to trigger the synthesis of these cytoskeletal proteins by inducing their mRNA expression. It is of interest that the basal mRNA levels of the cytoskeletal genes were notable in primary granulosa cells but not in ROG cells. We wonder whether the granulosa cells were exposed to a low level of endogenous FSH (43, 44) and exogenous 17β-estradiol, resulting in the mRNA expression of the cytoskeletal genes (Figs. 5 and 7). In contrast, ROG cells were derived from a primordial follicle and had never been exposed to FSH (15). The quick surge in the three cytoskeletal genes' transcripts, followed by the secondary mRNA expression peaking at FSH exposure for 24−48 h (Fig. 7), suggests a complex regulation of their gene expression.

Indeed, the FSH-dependent increase in the three genes' mRNAs was regulated by complex and distinct mechanisms, although FSH induced their transcription. For example, CHX blocked the 6-h FSH-induced expression of β-tubulin mRNAs, indicating the requirement of *de novo* protein synthesis. In contrast, CHX enhanced the FSH-induced TM4 mRNA level, suggesting the existence of a negative regulator protein such as RNase. Interestingly, the inhibin α gene responded like wise. On the other hand, the Khc mRNA level was insensitive to the agent. Therefore, although all three genes are up-regulated by FSH, they are all by different mechanisms. It would be interesting to see whether the *de novo* synthesis of the positive and negative regulatory proteins involves their gene transcription. If so, the genes are likely regulated by FSH as well. This diverse transcriptional regulation further diverges at a later stage of differentiation. The effects of CHX were different when the cells were treated with FSH for 24 h (Fig. 8), implying that FSH-dependent gene expression is dependent on the differentiation stage. This is consistent with tissue specific expression of the genes. Because FSH and forskolin, an adenylyl cyclase activator, impacted these genes' expression in the same way, the cytoskeletal genes are regulated by FSH via the adenylyl cyclase/cAMP pathway.

In conclusion, three cytoskeletal genes, β-tubulin, TM4 and Khc, were identified as FSH-responsive genes. They are transiently expressed in granulosa cells at the large preantral and early antral stages of follicular development. Because these stages are around the time of antrum formation and growth, one would raise an intriguing question concerning the relationship of the antrum formation with the dynamic changes in the expression of the three cytoskeletal genes and cytoskeleton structure. Therefore, it is imperative to define the specific function of each of the cytoskeletal genes and the mechanisms that govern the transient expression of the cytoskeletal gene expression.

Acknowledgments

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