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Regulation of Steroidogenesis by p53 in Macaque Granulosa Cells and H295R Human Adrenocortical Cells

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Ovulation and formation of a functional corpus luteum in primates involve cascades of events, including increased progesterone synthesis and changes in granulosa cell proliferation. However, critical gaps remain in our understanding of how an ovulatory gonadotropin surge initiates these processes. To more fully elucidate changes in the cell cycle during luteal formation, the actions of the tumor suppressor p53 were examined. Rhesus macaque granulosa cells were isolated during controlled ovarian stimulation protocols before (nonluteinized) or after (luteinized) an ovulatory gonadotropin stimulus. Phosphorylated p53 protein was detected in the cytoplasm of granulosa cells before and after human chorionic gonadotropin (hCG) treatment, whereas granulosa cells from hormonally controlled rats did not express p53 before or after hCG. Treatment of nonluteinized macaque granulosa cells with hCG and the p53 inhibitor pifithrin-α (PFT) *in vitro* did not alter markers of the cell cycle, including proliferating cell nuclear antigen, p21, and human double minute (HDM)-2 expression compared with hCG alone. Levels of pregnenolone and progesterone increased 2- and 4-fold, respectively, within 6 h of hCG treatment, whereas PFT completely blocked this hCG-induced effect. Estradiol was increased transiently (>10-fold) by hCG plus PFT relative to levels after hCG alone. PFT also inhibited hCG-induced increases in steroidogenic acute regulatory protein and 3β-hydroxysteroid dehydrogenase mRNAs. Similar results were obtained using the human adrenocortical cell line H295R, suggesting that p53 may have a general function in primate steroidogenesis. These data indicate that p53 plays a key role in luteinization of the primate ovarian follicle though the regulation of steroidogenic enzymes leading to progesterone synthesis. (*Endocrinology* 145: 5734–5744, 2004)

FORMATION OF A corpus luteum (luteinization) involves several cascades of events, including increased progesterone synthesis and attenuation of granulosa cell proliferation (1–3). Progesterone synthesized by the luteinizing follicle in response to an ovulatory stimulus increases the expression of proteolytic enzymes and also acts as a potent antiapoptotic factor (1, 4, 5). In rhesus monkeys undergoing controlled ovarian stimulation (COS), the human chorionic gonadotropin (hCG)-induced increase in progesterone synthesis occurs within 30 min of an ovulatory hCG bolus, and increases in the expression of 3β-hydroxysteroid dehydrogenase (3βHSD) and steroidogenic acute regulatory protein (StAR) mRNA have been detected at 12 h post-hCG, the earliest time yet examined *in vivo* (6–9). Data generated over the past several years have implicated StAR in the rapid response to trophic stimulation in almost all steroidogenic cells, and more recently, steroidogenic factor-1 (SF-1), GATA, and CCAAT/enhancer-binding protein β have been shown to regulate StAR as well as 3βHSD and other steroidogenic enzymes as an early step in steroidogenesis (6, 10, 11). Interestingly, the SF-1, GATA, and CCAAT/enhancer-binding protein β genes are not themselves strongly regulated by hCG, but, rather, are activated via protein kinase A-dependent phosphorylation, rendering them able to interact with transcriptional coregulators (12, 13). Recently, liver receptor homolog-1 (LRH-1) has been shown to be structurally and functionally related to SF-1 in the regulation of steroidogenic enzymes and probably plays a more pronounced role in the regulation of steroidogenic enzymes in the human corpus luteum than does SF-1 (14–16) The regulation of StAR in particular is thus known to be a complex orchestration of multiple transcription factors and signal transduction cascades.

In contrast to steroidogenesis, cell cycle control in luteinizing granulosa cells has received relatively little attention, although recent evidence indicates that cell cycle control after hCG administration is more complex than suspected. For example, administration of an ovulatory hCG bolus to normally cycling adult rats or pregnant mare's serum gonadotropin-primed immature rats transiently increases the proportion of granulosa cells in S phase (17, 18). Similarly, nonluteinized macaque granulosa cells treated with hCG *in vitro* to induce luteinization have a transient increase in [^{3}H]thymidine uptake (19), although in both species granulosa cell proliferation is eventually decreased. An ovulatory stimulus given to rhesus monkeys undergoing COS results in changes in the expression of a number of genes associated with cell cycle arrest, notably the sequential induction of the

Abbreviations: COS, Controlled ovarian stimulation; DMSO, dimethylsulfoxide; hCG, human chorionic gonadotropin; HDM-2, human double minute-2; 3βHSD, 3β-hydroxysteroid dehydrogenase; LRH-1, liver receptor homolog-1; NLGC, nonluteinized granulosa cell; PCNA, proliferating cell nuclear antigen; PFT, pifithrin-α; r-h, recombinant human; P450scc, cytochrome 450 side-chain cleavage enzyme; PVA, polyvinyl alcohol; RPL19, ribosomal protein L19; SF-1, steroidogenic factor-1; StAR, steroidogenic acute regulatory protein; TL, Tyrode's lactate.

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cyclin-dependent kinase inhibitors p21 and p27 (20–22). The tumor suppressor p53 is a key transcription factor for many genes, including p21 and p27, and has been localized to human granulosa cells (23). However, because p53 also increases the expression of proapoptotic genes (24), it is hypothesized that p53 blocks cell cycle progression of granulosa cells after hCG treatment while at the same time augmenting progesterone synthesis to blunt apoptosis and block luteal proliferation. The data presented herein do not support a clear role for p53 in the regulation of granulosa cell proliferation, but do indicate that p53 augments progesterone synthesis by macaque granulosa cells.

Materials and Methods

Animals

Adult female rhesus macaques (*Macaca mulatta*) were housed at the California National Primate Research Center as previously described (25). After the onset of menstruation, adult female rhesus monkeys were treated with recombinant human FSH (r-hFSH; Ares-Serono, Randolph, MA; or Organon, West Orange, NJ; 37.5 IU, im, twice daily) for 7 d. Antide (Ares-Serono; 5 mg/kg body weight, sc, single injection daily) was administered daily to prevent endogenous gonadotropin secretion. Follicles were aspirated the morning after the last dose of r-hFSH or 33 h after an ovulatory bolus of r-hCG (1000 IU im) by an ultrasound-guided procedure as previously described (26). Aspirates were maintained at approximately 35 C within a temperature-controlled isolette at all times. Oocytes were removed by transferring the aspirate to a 24-mm diameter, 70-μm pore size filter (Netwell Inserts 3479, Corning, Inc., Acton, MA), and the tube was rinsed with fresh Tyrode's lactate (TL)-HEPES-polyvinyl alcohol (PVA) medium (TL-HEPES/0.1 mg/ml PVA) (27) that was also poured onto the filter. The filter was rinsed again with fresh TL-HEPES-PVA medium until blood cells were removed. Nonluteinized granulosa cells (NLGC) were recovered from the filter rinse by a modification of the method previously described (28). Briefly, the cell suspension was centrifuged for 5 min at 300 $\times$ *g* to pellet the red cells and then increased to 500 $\times$ *g* for an additional 5 min, resulting in a thin layer of granulosa cells over the red cell pellet. The supernatant was removed, and the layer of granulosa cells was transferred to a 40% Percoll gradient in medium 199 (Sigma-Aldrich Corp., St. Louis, MO) and centrifuged for 30 min at 500 $\times$ *g*. The supernatant was removed, and the granulosa cells were recovered from the surface of the Percoll with a Pasteur pipette and washed once with TL-HEPES-PVA. The cell pellet was resuspended in 1 ml TL-HEPES-PVA and counted on a hemocytometer. An additional 14 ml TL-HEPES-PVA supplemented with 5 μg/ml r-hFSH was added to the cell suspension. Cells were placed in a biohazard shipping container and shipped by overnight delivery at ambient temperature from September to June. Upon receipt, cells were recovered by centrifugation, and viability was determined by trypan blue exclusion.

Immature female Sprague Dawley rats (26 d old; Harlan, Indianapolis, IN) were primed with pregnant mare's serum gonadotropin (10 IU, sc; Sigma-Aldrich Corp.) for 48 h, followed by hCG (10 IU, sc; Sigma-Aldrich Corp.) to induce periovulatory events. Animals were killed before (0 h) or 1, 2, 4, 6, 8, 10, 12, and 24 h after hCG treatment, and paired whole ovaries were collected, or paired ovaries were used for granulosa cell isolation by follicular puncture as previously described (29).

All animal procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the California National Primate Research Center and Medical College of Georgia animal care and use committees.

Cell culture and treatments

Macaque granulosa cells were plated overnight at 37 C with an initial seeding density of 5 $\times$ 10^5 viable cells/well in 24-well fibronectin-coated plates (Biocoat, Roche, Indianapolis, IN) in 400 μl DMEM/Ham's F-12 medium supplemented with 20 mM HEPES, penicillin/streptomycin (50 U/ml), 2% fetal calf serum, and 25 ng/ml hFSH (Sigma-Aldrich Corp.; F4021). Media were changed the next day to include either 25 ng/ml hFSH (Sigma-Aldrich Corp.) to maintain a nonluteinized phenotype or 20 IU/ml hCG (Sigma-Aldrich Corp.) to induce luteinization. Media were harvested 6 h later and changed every 6 h thereafter to include fresh FSH or hCG for up to 24 h. Treatment groups included FSH or hCG with or without 80 μM of the p53 inhibitor pifithrin-α (PFT; Tocris Biochemicals, Ellisville, MO), where PFT was added only for the initial 6 h after hCG or included only from 6–24 h after hCG treatment. The vehicle for PFT is dimethylsulfoxide (DMSO), and all cultures not receiving PFT were balanced with 0.4% DMSO. To examine early effects on steroidogenic enzyme expression by hCG and p53, macaque NLGC were plated as described above in the presence of FSH with or without PFT or hCG with or without PFT, and total RNA and protein were isolated 6 h later as described below.

Retroviral delivery of E6 oncoprotein, which targets p53 for ubiquitination and degradation (30), was used to corroborate the actions of PFT. The E6 producer line PA317 LXSN 16 E6 (gift from Dr. Andrew Phillips, Medical College of Georgia) was grown in DMEM with 4.5 g/liter glucose and 10% fetal bovine serum. When the E6 producer line reached confluence, media were changed to the granulosa cell media described above for 4 h, after which time media containing shed virus were added directly to the granulosa cell cultures and supplemented with 25 ng/ml FSH. Infection with virus continued for 24 h in the presence of FSH, with fresh media and virus added every 8 h. After 24 h of FSH and E6, media were changed to include hCG and E6 virus for an additional 24 h. Parallel cultures were treated in an identical manner, with the omission of viral particles (n = 2 animals).

H295R human adrenocortical cells (American Type Culture Collection, Manassas, VA) were seeded at 2.5 $\times$ 10^5 cells/well in 24-well plates in DMEM/Ham's F-12 with 15 mM HEPES and 1.2 g/liter sodium bicarbonate, penicillin/streptomycin (50 U/ml), 0.5 mM sodium pyruvate, ITS+1 (Sigma-Aldrich Corp.), and 2.5% NuSerum-I (BD Biosciences, Arlington Heights, IL). Cells were treated with vehicle control with or without 160 μM PFT or 10 μM forskolin (Sigma-Aldrich Corp.) with or without 160 μM PFT and were harvested 5 or 24 h later.

Western blotting and immunocytochemistry

Proteins were isolated by sonication of cells in 20 mM Tris-HCl (pH 7.4), 100 mM KCl, 1 mM EDTA, 10% glycerol, and 1 mM phenylmethylsulfonylfluoride on ice, followed by centrifugation at 14,000 $\times$ *g* for 15 min at 4 C. The protein concentration in the resulting supernatant was determined using the bicinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, IL). Equal amounts of protein (30 μg) were separated by PAGE, transferred to a polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA), blocked with 5% dry nonfat milk, and probed with respective primary and secondary antibodies. Immunoreactive bands were visualized using enhanced chemiluminescence and Western blotting detection reagents (Pierce, Rockford, IL). Primary antibodies raised against p53 (DO-1) and MDM-2 [H-221; cross-reacts with human double minute (HDM)-2] were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-specific antibodies $p53^{Ser15}$, $p53^{Ser20}$, $p53^{Ser46}$, and $p53^{Ser392}$ were purchased from Cell Signaling Technology (Beverly, MA), and proliferating cell nuclear antigen (PCNA) antibody Ab1 was obtained from Labvision Corp. (Fremont, CA).

Immunocytochemistry was performed on cultured cells using anti-p53 (DO1). Briefly, cells were grown on eight-well chamber slides coated with fibronectin (Bio-Coat, Inc., Bedford, MA) using the conditions described above for 24 h, fixed in acetone for 5 min, washed three times in PBS, and blocked for 30 min with normal rabbit serum. Anti-p53 was used at a 1:100 dilution for 1 h in PBS, 0.3% Triton X-100, and 0.2% cargeenan. Slides were washed three times with PBS, followed by goat antimouse Alexa Fluor 594 (Molecular Probes, Eugene, OR) used for 1 h at room temperature in PBS, 0.3% Triton X-100, and 0.2% cargeenan. Slides were washed three times in PBS and coverslipped with Gel/Mount (Biomeda, Foster City, CA).

EMSA

NLGC were briefly sonicated in 20 mM HEPES (pH 7.5), 0.5 M KCl, 2 mM EDTA, and 15% glycerol, followed by centrifugation at 14,000 $\times$ *g* for 15 min at 4 C. Protein concentrations were determined as described above. Protein (20 μg) was preincubated with or without 4 μg anti-p53 (DO1) for 40 min in binding buffer [20 mM HEPES (pH 7.6), 5 mM $MgCl_2$, 50 mM NaOAc, 10% glycerol, 10% DMSO, 1 mM β-mercaptoethanol, 500

μg/ml BSA, and 2 mM spermidine], followed by the addition of ^{32}P-labeled oligonucleotide (100,000 cpm) for 20 min in a final volume of 20 μl. DNA-protein complexes were resolved by electrophoresis through 6% polyacrylamide gels in Tris-glycine buffer. Gels were visualized using the Typhoon 8600 scanner (Molecular Dynamics, Sunnyvale, CA). The oligonucleotides used for the p53 binding site were from the human p21 promoter (5′-CAGGAACATGCCCAACATGTTGAGCT) (31). Raji cells (a p53-positive human Burkitt's lymphoma cell line; American Type Culture Collection) were used as a positive control. Cells were cultured in 24-well plates in RPMI 1640 medium with 2 mM L-glutamine, 1.5 g/liter sodium bicarbonate, 4.5 g/liter glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, and 10% fetal bovine serum supplemented with penicillin/streptomycin (50 U/ml) at an initial seeding density of 2.5×10^5 for 24 h. Lysates were prepared as described above for granulosa cells.

RIA

Estradiol, progesterone, and aldosterone levels were determined with a commercially available RIA kit (Diagnostic Products, Inc., Los Angeles, CA). Pregnenolone concentrations were analyzed by ELISA (ALPCO Diagnostics, Windham, NH).

RT-PCR

Total RNA was extracted using the RNAqueous-Micro kit (Ambion, Inc., Austin, TX) and reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA). For macaque granulosa cells, real-time RT-PCR (Cepheid, Sunnyvale, CA) was performed for StAR, 3βHSD, SF-1, and LRH-1. Primers and 6-carboxy fluorescein-labeled probes were synthesized by Applied Biosystems (Foster City, CA). Primers and carboxy-X-rhodamine (ROX)-labeled probe for the endogenous control ribosomal protein L19 (RPL19) were synthesized by Biosearch Technologies (Novato, CA). For relative quantification of mRNA levels, a standard curve was generated using a pool of macaque granulosa cell or H295R cDNA (Applied Biosystems User Bulletin 2; P/N 4303859). By using probes labeled with different fluorescent dyes (6-carboxy fluorescein or ROX), both the target gene of interest and the endogenous control gene were detected in the same reaction. For all samples, the target gene was normalized to RPL19. Twenty nanograms of cDNA were used in a reaction volume of 25 μl (29). Semiquantitative RT-PCR was performed by coamplification of cytochrome P450 side-chain cleavage enzyme (P450scc), aromatase, or 17βHSD-I, with β_2-microglobulin as an internal standard. Data were analyzed and expressed as the ratio of steroidogenic enzyme to internal standard. All reactions were in the exponential phase of amplification for both primer sets and were linear with respect to increasing amounts of cDNA (32). For H295R cells, all genes (StAR, SF-1, LRH-1, 3βHSD, and 17α-hydroxylase) were analyzed by real-time RT-PCR. Primers and probes are listed in Tables 1 and 2.

TABLE 1. Primer sequences for semiquantitative RT-PCR

Target	Primers (5′–3′)
P450scc	For: GGGTCGCCTATCACCAGTATTACC
	Rev: CCCAGGAGTCTGTAGAGCATGCCACGG
17βHSD-I	For: AGGGCCGCGTGGACGTGCTGGTGTGTAAC
	Rev: CCATCAATCCTCCCACGCTCCCGG
P450 aromatase	For: ATACCAGGTCCTGGCTACTG
	Rev: GGGCCTGACAGAGCTTTCAT
p21	For: ACTGTGATGCGCTAATGGC
	Rev: ATGGTCTTCCTCTGCTGTCC
HDM-2	For: GCTGAAGAGGGCTTTGAT
	Rev: TGGTGTAAAGGATGAGCT
β_2-Microglobulin	For: TTAGCTGTGCTCGGGCTACTCTCTC
	Rev: GTCGGATGGATGAAACCCAGACACA

For, Forward; Rev, reverse.

TABLE 2. Real-time probe and primer sequences

Target	Primers/probe (5′-3′)
SF-1	For: CGCACGGTGCAGAACAAC
	Rev: CGCTGCGTCTTGTCGATCT
	6FAM-CACTACACGTGCACCGAGAGCCAGAG
3βHSD	For: CCAGAACGGCCACGAAGA
	Rev: AGCTTTTTGCTGTACGGGTATG
	6FAM-AGCCTCTGGAAAACACATGGCCCA
StAR	For: CCACCCCTAGCACGTGGAT
	Rev: TCCTGGTCACTGTAGAGAGTCTCTTC
	6FAM-CGGAGCTCTCTACTCGGTTCTC
LRH-1	For: CCCTTTGTAACATCCCCCATTA
	Rev: GGAAAGTGGCCATATGTTTGGTAA
	6FAM-TGCCCCCTCACGGCAGCC
RPL19	For: CCCCAATGAGACCAATGAAATC
	Rev: CAGCCCATCTTTGATGAGCTT
	ROX-ATGCCAACTCCCGTCAGCAGATCC

For, Forward; Rev, reverse.

Apoptosis detection

The relative amount of apoptosis was determined using the DNA intercalating dye YOPRO-1 (Molecular Probes, Eugene, OR) (33). Media were replaced with 5.6 μg/ml YOPRO-1 in 100 μl PBS. The reaction was incubated for 30 min on ice, and the amount of fluorescence was determined on a GENios spectrofluorometric plate reader (Tecan, Durham, NC), with excitation and emission wavelengths set at 485 and 535 nm, respectively.

Statistical analysis

All data were tested for heterogeneity of variance with Bartlett's χ^2 test. Data that were not normally distributed were transformed to (log base 10 + 2) before analysis by ANOVA with one repeated measure, followed by the Newman-Keuls means test. Differences were considered significant at $P < 0.05$, and all values are presented as the mean ± SEM.

Results

Expression of p53 in macaque granulosa cells

Whole cell extracts of granulosa cells isolated from monkeys before hCG (NLGC) or 27 h after *in vivo* hCG administration (luteinized granulosa cells (LGC)] contained high levels of p53, with no evidence for increased or decreased expression following hCG in thee different animals. Conversely, granulosa cells isolated from immature rats primed with pregnant mare's serum gonadotropin, followed by an ovulatory bolus of hCG did not express detectable levels of p53 by Western blot analysis or by immunoprecipitation followed by Western blotting (Fig. 1, A and B). Whole ovary p53 was detectable and was increased transiently by an ovulatory dose of hCG (Fig. 1B).

Because p53 undergoes extensive post-/cotranslational modifications, we examined whether several of these p53 forms were present in macaque granulosa cells isolated after *in vivo* hCG administration. Granulosa cells contained several post-/cotranslational p53 forms, specifically phosphorylation of Ser^{20}, Ser^{46}, and Ser^{392}, but not Ser^{15}. The proportion of phosphorylated to total p53 was not different in LGC *vs.* NLGC. HDM-2 (a p53-regulated gene whose protein product targets p53 for degradation) was not detectable by

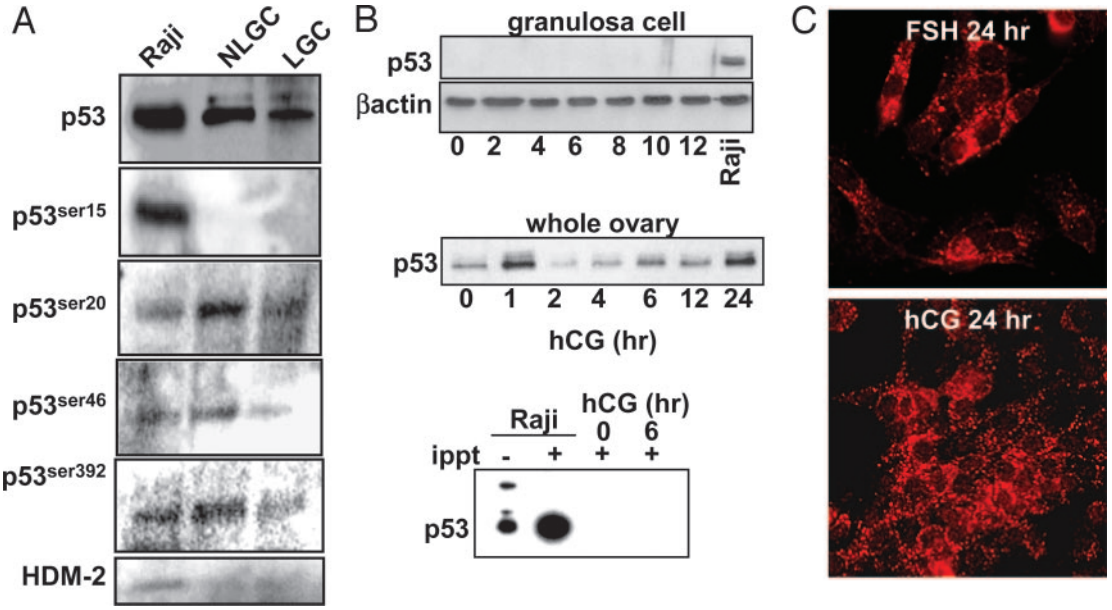

FIG. 1. p53 Protein is expressed in macaque, but not rat, granulosa cells. A, Whole cell lysates were prepared from macaque granulosa cells isolated before (NLGC) or 27 h after (LGC) hCG *in vivo* for use in Western blot analysis with four different phospho-specific p53 antibodies and HDM-2. Raji cells were used as a positive control. Serines 20, 46, and 392 are phosphorylated on p53 before and after hCG, whereas serine 15 is not. Levels of total p53 are not markedly changed after hCG, nor are the relative levels of phospho-specific p53. HDM-2, a p53 target that mediates p53 degradation, is not detectable before or after hCG. B, Immature rats were superovulated, and the resulting ovaries or granulosa cells were isolated before (0 h) or up to 24 h after an ovulatory hCG bolus for Western blot analysis of p53 (*top* and *middle*) or immunoprecipitation of p53 from granulosa cells (*bottom*). Although p53 is present in whole ovary lysates, rat granulosa cells do not express detectable levels of p53 even after immunoprecipitation. C, Immunocytochemistry for total p53 was performed on granulosa cells obtained from rhesus macaques undergoing controlled ovarian stimulation before an ovulatory stimulus. NLGC were treated *in vitro* with FSH (*top*) or hCG (*bottom*) for 24 h to induce luteinization. Nearly all of the p53 was localized to the cytoplasm before and after hCG treatment in a punctate fashion. All data are representative of three animals.

Western blot in macaque granulosa cells before or after hCG (Fig. 1A).

To further characterize the expression of p53 in macaque granulosa cells, we isolated NLGC from rhesus monkeys undergoing COS before an ovulatory stimulus and cultured these cells in the presence of FSH or hCG (19) before immunocytochemistry. Expression of p53 protein was abundant and punctate in the cytoplasm of cells before and after hCG (Fig. 1C). A small fraction of cells (<2%) displayed nuclear p53, although no overt morphologic evidence of apoptosis was seen in this subset of cells (data not presented).

Role of p53 in granulosa cell proliferation

To elucidate the function of p53 in nonapoptotic macaque granulosa cells, nonluteinized granulosa cells were treated with hCG with or without the reversible p53 inhibitor PFT (34). Preliminary dose-response studies in granulosa cells indicated that the 50% effective dose for this compound was approximately 80 μM, and no evidence of apoptosis was observed with any dose of PFT using the intercalating dye YOPRO1 (33) (data not presented). Inhibition of p53 for 24 h did not alter levels of PCNA in the presence of FSH or hCG (Fig. 2A). Treatment of NLGC with hCG for 6 h resulted in a 2-fold induction ($P < 0.05$) of p21 mRNA over corresponding FSH control values (Fig. 2B); addition of PFT to the cultures with hCG did not attenuate the increase in p21 mRNA. To confirm that p53 does not mediate the increase in p21 mRNA after hCG administration, EMSAs were performed using a p53 binding site from the human p21 promoter. Extracts from Raji cells were able to bind the p21 element and were supershifted with the addition of an anti-p53 antibody (Fig. 2C). In contrast, macaque granulosa cells isolated before or after hCG treatment *in vivo* were unable to bind the p53 response element. A number of other p53 response elements from known p53 target genes were tested with similar results [growth arrest and DNA damage-inducible (GADD)45, HDM-2, and p27; data not presented]. Finally, neither FSH nor hCG, with or without PFT, altered levels of the p53 target gene HDM-2 mRNA (Fig. 2D).

Role of p53 in periovulatory steroidogenesis

Treatment of NLGC with hCG for 6 h resulted in a 2-fold increase ($P < 0.05$) in media pregnenolone levels, which returned to pre-hCG levels by 12 h (Fig. 3). Similarly, progesterone concentrations increased 4-fold ($P < 0.05$) by 6 h post-hCG and were maintained thereafter, indicating that the maximal rate of synthesis was reached by 6 h *in vitro*. The addition of PFT for the first 6 h after hCG treatment blocked the induction of both pregnenolone and progesterone, and despite the fact that the media subsequently contained only hCG (without PFT) from 6–24 h, pregnenolone and progesterone levels did not completely return to values with hCG alone. Treatment with PFT starting 6 h after hCG administration resulted in the rapid decline of both pregnenolone and progesterone in the culture media.

Estradiol synthesis by primate granulosa cells was also

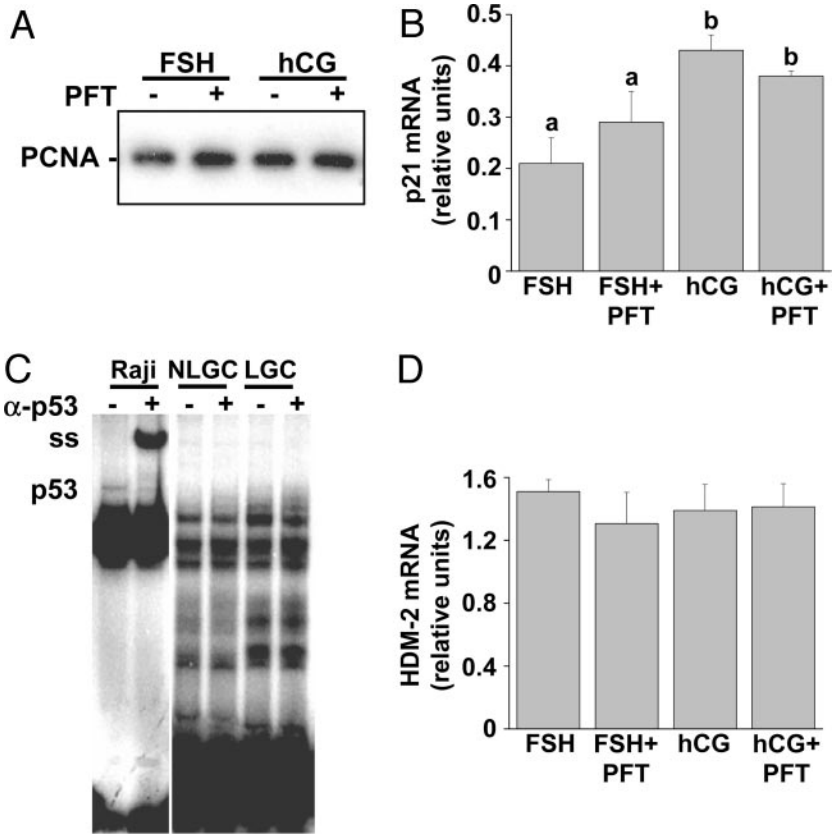

FIG. 2. Inhibition of p53 does not alter markers of cell cycle in macaque granulosa cells. A, NLGC were cultured in the presence of FSH or hCG with or without PFT for 24 h, and expression of the cell cycle antigen PCNA was determined by Western blot analysis. Treatment of cells with PFT did not increase the expression of PCNA, suggesting that granulosa cell proliferation was not altered in response to p53 inhibition. B, NLGC were cultured for 6 h in the presence of FSH with or without PFT or hCG with or without PFT, and levels of p21 mRNA were determined by RT-PCR. Increased p21 was associated with hCG treatment, although PFT did not alter mRNA levels before or after hCG administration. *Different superscripts* denote significant differences (n = 3). C, EMSAs were performed using a p53-responsive element from the human p21 promoter region as the probe. Raji cells served as a positive control protein extract. NLGC were obtained before hCG; LGC were obtained 27 h after hCG *in vivo*. Protein extracts were preincubated with (+) or without (−) anti-p53 (α-p53) antibody to induce a supershift (ss). D, NLGC were cultured for 6 h in the presence of FSH or hCG with or without PFT, and levels of HDM-2 mRNA were determined by RT-PCR (n = 3).

evaluated. Treatment of NLGC with hCG for 6 h *in vitro* resulted in a 3-fold increase ($P < 0.05$) in estradiol, which returned to pre-hCG levels at 12 h (Fig. 3C); this profile was identical to that observed in serum samples after hCG administration (7). Exposure of NLGC to hCG and PFT for 6 h induced a 14-fold increase ($P < 0.05$) in medium estradiol levels, which declined rapidly once PFT was removed from the medium. In contrast to pregnenolone and progesterone, estradiol synthesis was not markedly affected by treatment with PFT starting 6 h after hCG administration.

To verify the role of p53 in steroidogenesis during luteinization, NLGC were infected with an E6-containing virus for 24 h before hCG treatment. The E6 oncoprotein binds p53 and targets it for ubiquitin-mediated degradation (35), making this a good method to compare with PFT treatment. The effects of E6 recapitulated the findings using PFT, albeit with less magnitude, which was almost certainly due to the fact that infection occurs only in cells transiting S phase, which comprise a small fraction of granulosa cells *in vitro*. However, E6 appeared to suppress basal levels of progesterone in NLGC by 2-fold, suggesting some regulation of basal steroidogenesis by p53. Overall, these data indicate that observations stemming from PFT treatment reflect p53 inhibition, rather than nonspecific actions (Fig. 3D).

Regulation of steroidogenic enzyme mRNA by p53

The mRNA expression of genes involved in steroidogenesis was determined 6 h after FSH or hCG treatment with or without PFT in macaque granulosa cells to elucidate the underlying cause of PFT-altered steroid synthesis. Medium levels of progesterone before hCG were not affected by PFT, whereas the hCG-induced increase was significantly attenuated (Fig. 4). The expression of StAR mRNA after 6 h in the presence of FSH and PFT was not different from that with FSH alone. Treatment of NLCG with hCG resulted in a 16-fold increase ($P < 0.05$) in StAR mRNA by 6 h; coadministration of hCG and PFT significantly attenuated (2.5-fold; $P < 0.05$) this induction (Fig. 4). P450scc mRNA was not strongly regulated by PFT in the presence of FSH or by hCG with or without PFT within 6 h. PFT increased 3βHSD mRNA during FSH treatment by 3-fold ($P < 0.05$). The expression of 3βHSD mRNA was increased significantly (500-fold; $P < 0.05$) 6 h after hCG, whereas PFT inhibited (115-fold; $P < 0.05$) this hCG-induced expression. The mRNA expression of 17βHSD type I (17βHSD-I) mRNA was not altered by PFT during FSH treatment. After hCG administration, 17βHSD-I mRNA levels were decreased 2-fold ($P < 0.05$), and PFT completely blocked this down-regulation (17βHSD converts androstenedione to testosterone and estrone to estradiol). P450 aromatase mRNA increased 3-fold ($P < 0.05$) after hCG, and PFT had no effect on its expression either before or after hCG.

Because SF-1/NR5a1 and, more recently, LRH-1/NR5a2 have been implicated in the regulation of multiple steroidogenic enzymes (14, 36, 37), including several of those mentioned above, we sought to determine whether p53 might influence the expression of these critical transcription factors. Granulosa cell LRH-1 mRNA, but not SF-1 expression, increased 2-fold ($P < 0.05$) with hCG treatment (Fig. 4). However, neither LRH-1 nor SF-1 mRNA expression was altered by PFT treatment before or after hCG, and thus, downstream

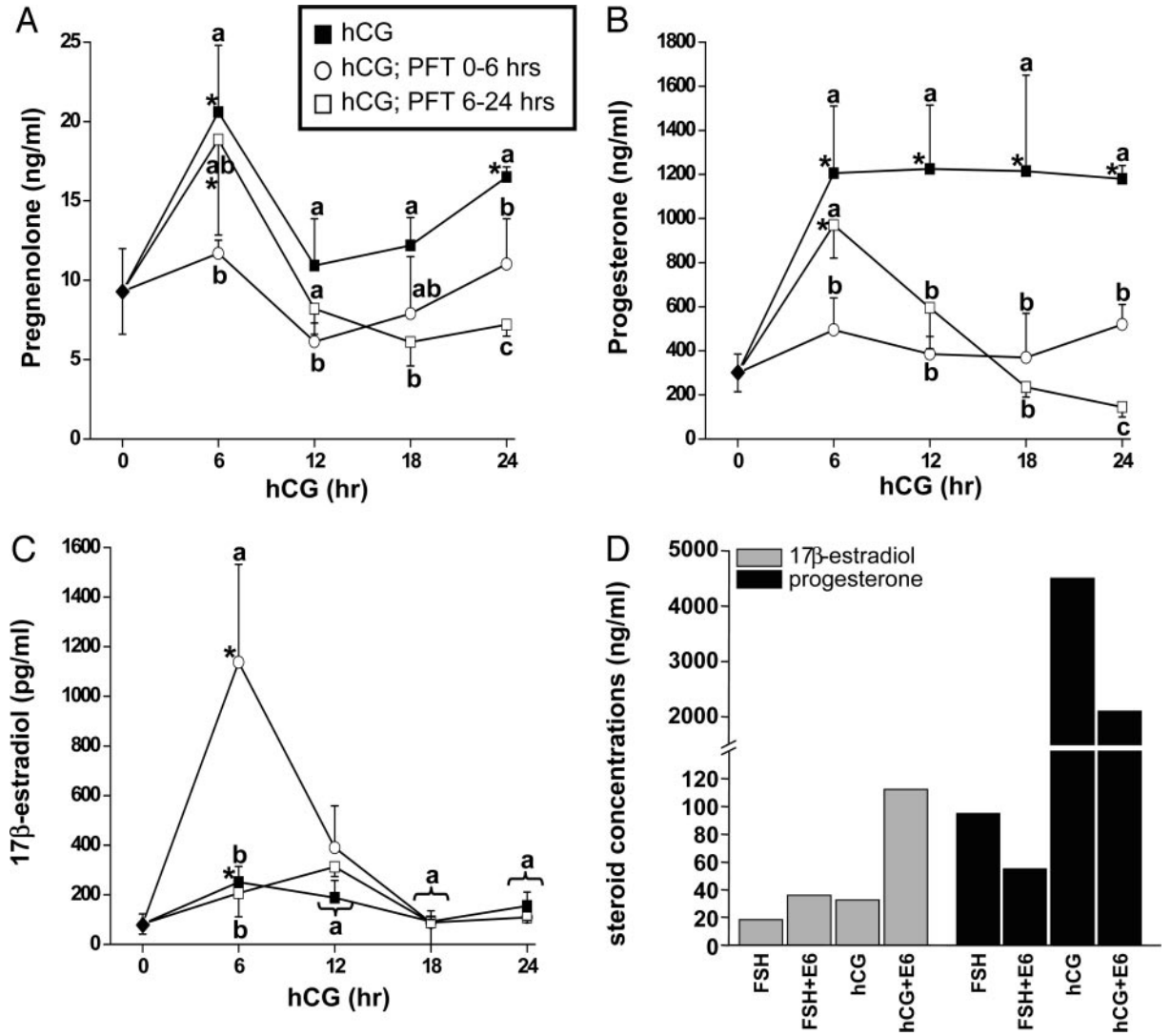

FIG. 3. Inhibition of p53 attenuates hCG-induced steroidogenesis in macaque granulosa cells. NLGC were cultured in the presence of FSH for 6 h (time zero control), followed by hCG for 24 h, with medium changes every 6 h (■). The p53 inhibitor PFT was added for the first 6 h after hCG treatment (○) or from 6–24 h post-hCG (□). Medium levels of pregnenolone (A), progesterone (B), and estradiol (C) were determined by specific RIA (three animals). *, Significant differences *vs.* time zero control (FSH 6 h); lack of a *common letter superscript* indicates significant differences within a time point but between treatments. D, Macaque NLGC were infected with E6-containing virus or sham media; the E6 oncoprotein causes the degradation of p53 and was thus used as an alternate means to inactivate p53. After infection for 24 h, cells were treated with FSH or hCG for an additional 24 h, after which estradiol and progesterone levels were determined by RIA (two animals).

effects of p53 on steroidogenesis cannot be explained by the transcriptional regulation of these factors.

Regulation of H295R adrenocortical steroidogenesis by p53

Because p53 is expressed in most cells, the potential for a generalized function of this protein in steroidogenesis was tested using H295R human adrenocortical cells. The expression of p53 in H295R cells was limited to the cytoplasm before and after treatment with forskolin for 24 h (Fig. 5A). To test the hypothesis that p53 is a key mediator of steroidogenic factor gene expression, H295R cells were treated with or without forskolin and PFT for 5 or 24 h. Basal levels of aldosterone after 5 h were suppressed 2-fold in the presence of PFT alone ($P < 0.05$) (Fig. 5B). Treatment of H295R cells with forskolin for 5 h increased aldosterone levels by 3-fold relative to those in control cultures ($P < 0.05$), and PFT blocked the induction of steroidogenesis. Similarly, PFT reduced medium levels of aldosterone after 24 h in control cultures (2-fold; $P < 0.05$), whereas forskolin increased levels of aldosterone (2-fold; $P < 0.05$). PFT blocked aldosterone synthesis in the presence of forskolin, reducing levels to 24 h control values. Similar to macaque granulosa cells, adrenal SF-1 mRNA was not regulated by PFT before or after forskolin treatment. SF-1 mRNA increased 24 h after forskolin treatment relative to time-matched control values (3-fold; $P < 0.05$). Consistent with published reports, LRH-1 mRNA was expressed at low levels in adrenal cells (data not presented) (15, 38). The expression of StAR mRNA was not affected by PFT in control cultures at 5 or 24 h. Addition of forskolin increased mRNA at 5 and 24 h (2- and 3-fold,

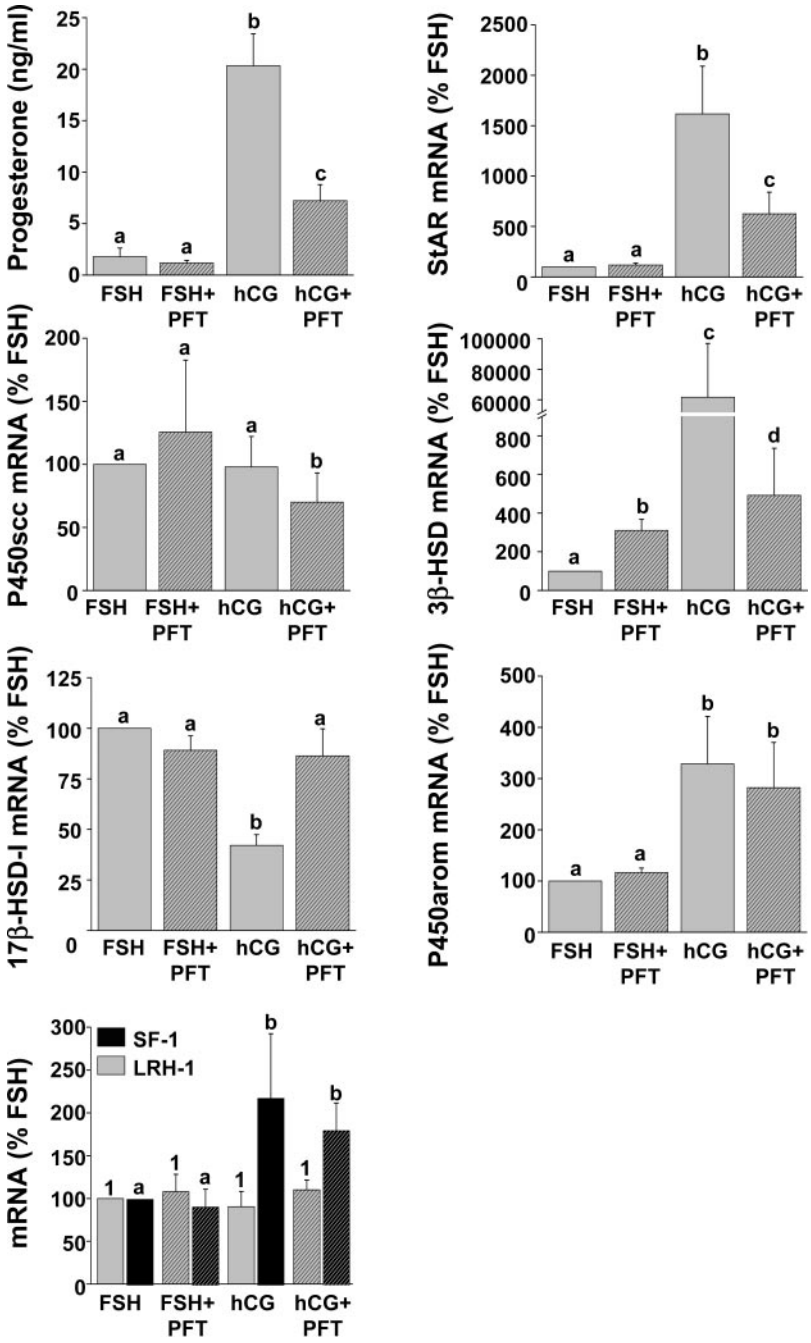

FIG. 4. Inhibition of p53 results in abnormal expression of mRNAs for StAR and steroidogenic enzymes. NLGC were treated with FSH with or without PFT or hCG with or without PFT for 6 h (three animals). Medium levels of progesterone were determined by RIA; levels of StAR, 3βHSD, SF-1, and LRH-1 mRNA were determined by real-time RT-PCR and normalized to RPL19; and levels of P450scc, 17βHSD-I, and P450 aromatase were determined by semiquantitative RT-PCR and normalized to β_2-microglobulin. All data are expressed as a percentage of the FSH control value. *Different superscript letters* indicate significant differences.

respectively; $P < 0.05$), whereas PFT suppressed forskolin-induced StAR expression to control levels. Levels of 3βHSD mRNA were reduced by PFT in control cultures at 5 and 24 h (0.7- and 0.3-fold; $P < 0.05$). Forskolin treatment resulted in a modest, but significant, decline in 3βHSD mRNA at 5 h (0.5-fold; $P < 0.05$) and a slight increase in 3βHSD mRNA at 24 h (1.3-fold; $P < 0.05$). Unlike granulosa cells, H295R cells expressed substantial amounts of 17α-hydroxylase mRNA, although mRNA levels did not change as a result of forskolin or PFT at 5 h. Levels of 17α-hydroxylase mRNA were elevated 2.7-fold ($P < 0.05$) 24 h after forskolin treatment in a p53-dependent manner.

Discussion

The tumor suppressor p53 represents a novel factor in the process of primate, but not rat, luteinization and possibly in primate steroidogenesis in general. In contrast, a clear role for p53 in regulating granulosa cell proliferation during luteinization was not detected. Inhibition of p53 after hCG administration perturbed the expression of several steroidogenic enzymes, including StAR, 3βHSD, and 17βHSD-I, resulting in a block to hCG-induced progesterone synthesis. These observations were extended to human adrenocortical cells (H295R), where StAR and 3βHSD mRNAs were similarly suppressed after trophic stimulation. Immunocyto-

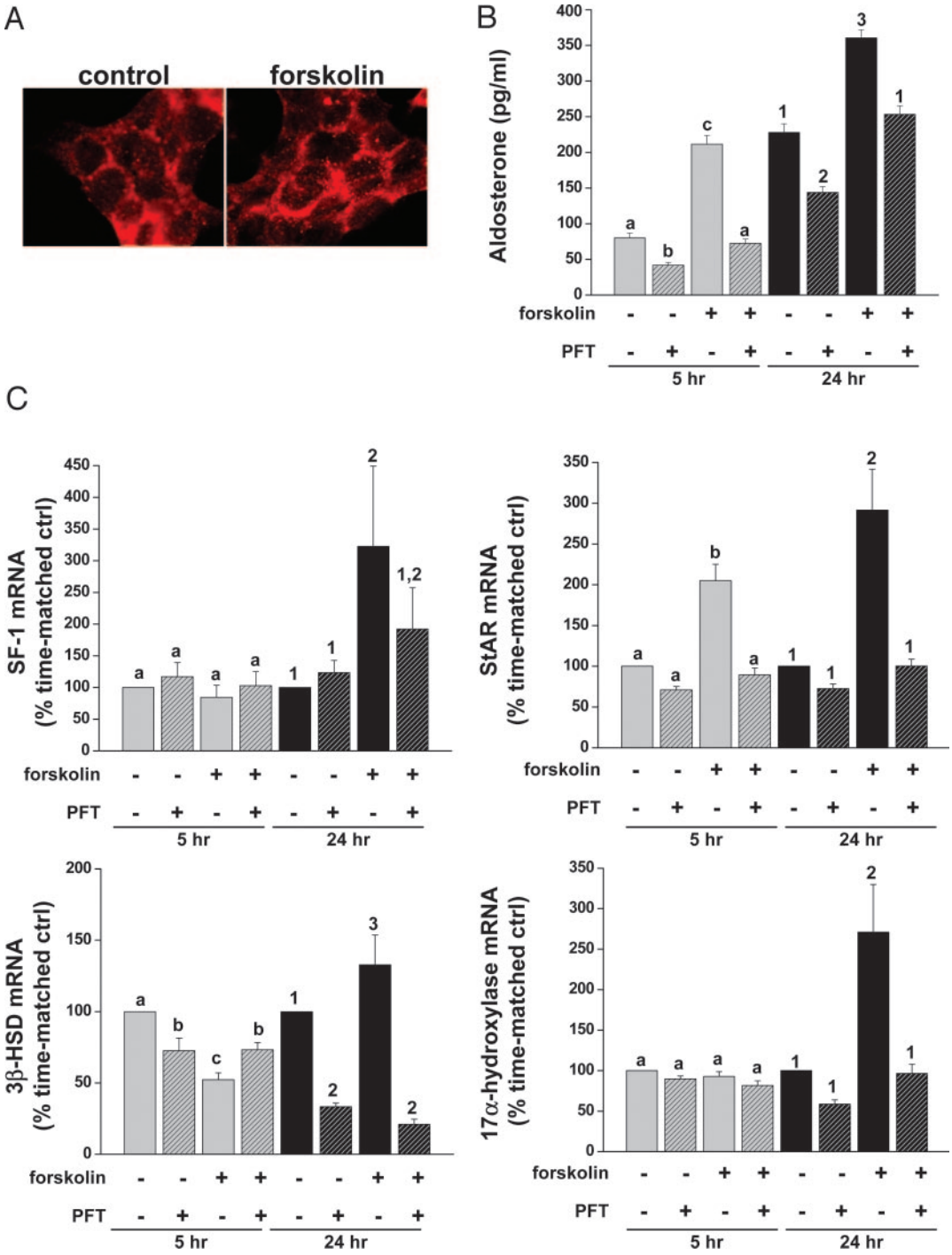

FIG. 5. Regulation of H295R adrenocortical cell steroidogenesis by p53. A, H295R cells were cultured in the absence (control) or presence of 10 μM forskolin for 24 h, and immunocytochemistry was performed using an antibody directed against total p53 (DO-1). A, The expression of p53 was ubiquitous, was localized to the cytoplasm in all cells, and did not change after forskolin treatment (repeated in three animals). To evaluate the effects of p53 on steroidogenesis, H295R cells were cultured for 5 or 24 h in the presence or absence of 10 μM forskolin with or without 180 μM PFT (n = 3). Medium levels of aldosterone were increased by forskolin at both time points, whereas PFT reduced aldosterone with or without forskolin (B). C, The expression of mRNA for enzymes and steroidogenic factors was determined by real-time RT-PCR using RPL19 as an internal control (n = 3). Data were analyzed by ANOVA as a percentage of the time-matched control value. *Superscript letters* refer to significant differences within 5-h treatments, whereas numbers indicate significance during the 24-h treatment interval.

chemical localization of p53 in both cell types showed an exclusive cytoplasmic expression in nearly every cell. These data are among the first to indicate that p53 can assume a function outside of cell cycle and apoptosis.

The current study indicates that p53 protein is expressed by macaque granulosa cells, and based on immunocytochemical evidence, p53 is localized to the cytoplasm. Although other studies have reported p53 in human granulosa

cells, these data have been in relation to apoptosis and follicular atresia, where p53 is nuclear. Makrigiannakis *et al.* (23), for example, showed nuclear p53 in granulosa-lutein cells isolated from *in vitro* fertilization patients after controlled ovarian stimulation plus hCG and cultured for 24 h in serum-free medium to induce apoptosis. In contrast, the culture conditions used in the current study were designed to maximize cell survival, *i.e.* 2% serum and gonadotropin, and may account for the cytoplasmic rather than nuclear localization of p53. The cytoplasmic p53 expressed by macaque granulosa cells in the current study is phosphorylated on at least three serine residues, but markedly not on Ser^{15}, which is associated with apoptosis (39). Although the functions of these posttranslational modifications in granulosa cells are not known, it suggests that p53 may be stabilized/activated before and after an ovulatory stimulus. In contrast, rat granulosa cells do not express detectable levels of p53 protein, although expression is abundant in whole ovary lysates. Also, injection of up to 8.8 mg/kg PFT 2 h before hCG, at the time of hCG, or 2 h post-hCG does not alter serum concentrations of steroids during the periovulatory interval (our unpublished observations). As in primates, it is clear that p53 can be induced during atresia of rat follicles and is associated with granulosa cell apoptosis (40, 41). However, the role of p53 in mouse fertility remains ambiguous, with the initial characterization of $p53^{-/-}$ mice showing normal female fertility (42), whereas subsequent studies of $p53^{-/-}$ mice suggest impaired female reproductive function (43). The regulated expression of p53 in whole ovary from rat observed in the current study supports the hypothesis that p53 may play a role in ovarian function of nonprimate species through nongranulosa cell/nonsteroidogenic actions, although this is speculative.

Data from the current study suggest that p53 does not play an overt role in the control of granulosa cell proliferation. After an ovulatory stimulus to macaque granulosa cells, levels of p21 mRNA are increased (current study and Ref. 20) in a p53-independent manner. Several other p53 targets are increased after hCG in a p53-independent manner, including GADD45 and p27 (our unpublished observations). In addition, whole cell protein extracts from macaque granulosa cells are unable to bind either a consensus p53 binding site or the p53 site found in the human p21 promoter (44). HDM-2 protein is not detectable (nor do levels of HDM-2 mRNA change) in macaque granulosa cells, indicating that the typical feedback loop controlling p53 action is not present in these cells. It is therefore apparent that p53 from macaque granulosa cells is incapable of binding DNA, suggesting that a specific suite of posttranslational modifications occurs that does not facilitate direct transcriptional regulation by p53.

The actions of p53 during luteinization of macaque granulosa cells focus on the conversion of the periovulatory follicle from estrogen to progesterone synthesis and indicate that a broader definition of p53 may be warranted. Consistent with *in vivo* steroidogenesis (1), hCG *in vitro* results in a rapid increase in pregnenolone and progesterone synthesis. It is noteworthy that the maximal rate of synthesis is reached within 6 h of hCG administration, although it is not clear whether this is limited by substrate or enzyme activity. Inhibition of p53 completely attenuates the hCG-induced rise in both steroids, suggesting that p53 regulates early steps in the steroidogenic pathway, probably those involving cholesterol utilization. Consistent with this possibility is the fact p53 mediates hCG-induced StAR expression as well as 3βHSD, thus facilitating metabolism of cholesterol to pregnenolone to progesterone. Both StAR and 3βHSD rely on a number of proximal transcription factors to increase expression in response to trophic stimulation, including LRH-1 and SF-1 (45, 46). Recent evidence in the rat suggests that LRH-1 predominates in granulosa cells, whereas SF-1 is the principal factor in thecal cells, and LRH-1 may be the principal factor in the regulation of 3βHSD expression in human corpus luteum (14, 47). LRH-1 mRNA is increased after hCG in macaque granulosa cells, whereas SF-1 levels do not change, suggesting that LRH-1 is essential for the early increases in StAR and 3βHSD mRNA after hCG treatment in primate granulosa cells. Interestingly, p53 inhibition does not alter LRH-1 mRNA levels, thus ruling out the possibility that changes in LRH-1 expression lead to the observed changes in steroidogenesis after p53 inhibition. However, it remains possible that p53 regulates steroidogenesis though posttranslational activation of LRH-1 (or SF-1) (12).

Synthesis of estrogen increases transiently after an ovulatory stimulus in primates (7). Treatment of macaque granulosa cells with hCG *in vitro* for 6 h increases aromatase mRNA and estrogen 6 h after hCG administration before levels decline. The later decline in estrogen is due partially to substrate limitation (7), but may also result from hCG-suppressed 17βHSD-I mRNA, which converts androstenedione to testosterone and/or estrone precursors to estradiol. Thus, changes in granulosa cell estrogen synthesis may be associated with decreased 17βHSD-I mRNA, possibly as a means to limit estradiol in the face of an early, generalized increase in steroid synthesis. Inhibition of p53 during the first 6 h after hCG treatment caused a marked increase in estrogen, whereas treatment with the p53 inhibitor starting 6 h after hCG had a minimal effect. It is possible that this spike in estrogen is due to the conversion of existing androgens, and as this pool of precursor is depleted (and limited amounts replaced due to suppressed StAR), levels of estrogen decline. Also, aromatase mRNA is not suppressed by p53 inhibition, whereas 17βHSD-I mRNA remains elevated, potentially allowing preexisting androgenic substrate to be converted to estradiol.

Although these are the first data to suggest a direct role for p53 in human granulosa cell steroidogenesis, activation of a temperature-sensitive mutant of p53 in transformed human granulosa cells leads to apoptosis and increased progesterone release (48). The relationship between granulosa cell apoptosis and steroidogenesis has also been shown during other methods of apoptosis induction; however, an association between apoptosis and p53 has been made in human, bovine, and rat granulosa cells (23, 49–51), making it possible that cell stress-induced activation of p53 leads to apoptosis in addition to enhanced progesterone synthesis. If this hypothesis is correct, then granulosa cells possess mechanisms that can dictate whether p53 is prosteroidogenic only or apoptotic and steroidogenic. This is supported by the fact that p53 in nonapoptotic granulosa cells is phosphorylated on multiple residues, but specifically not on serine 15.

It is significant that p53 also regulates steroidogenesis in a nonreproductive steroidogenic cell type, namely H295R adrenal cells, suggesting that the steroidogenic actions of p53 could be widespread and represent a generalized feature of human and primate steroidogenesis. It is clear from mRNA measurements that p53 regulates both StAR and 3βHSD. Other downstream genes leading to aldosterone synthesis may also be p53 targets, notably 21-hydroxylase (our unpublished observation), although a more complete characterization of p53 regulation of these genes is needed. Therefore, the StAR and 3βHSD genes represent a likely mechanism though which p53 acts to regulate steroid synthesis, although a direct action of p53 on steroidogenic enzyme promoters appears unlikely for several reasons. First, the initial 150 bp of the human StAR promoter is cAMP inducible, but does not contain even imperfect p53 sites (52). Second, p53 localizes to the cytoplasm in macaque granulosa cells and human H295R cells, raising the possibility that the steroidogenic actions of p53 could be mediated in the cytoplasm. A nonnuclear role for p53 has been suggested (53, 54), especially with regard to apoptosis, and thus additional studies are needed to fully elucidate the actions of p53 on the regulation of steroidogenesis.

A central question pertaining to the role of p53 as a steroidogenic factor is the action of the protein before trophic stimulation. Both NLGC and H295R cells express substantial levels of p53, raising the possibility that the actions of p53 may not be limited to the luteinizing follicle or during aldosterone synthesis. NLGC are relatively nonresponsive to p53 inhibition, the lone exception being 3βHSD mRNA, which is slightly increased by PFT. Thus, it appears that the actions of p53 on primate granulosa cells are more pronounced after an ovulatory stimulus, although not exclusively limited to luteinizing granulosa cells. This is in contrast to H295R cells, where p53 inhibition in control cells markedly reduces basal aldosterone concentrations and 3βHSD mRNA expression. Whether the differences between NLGC and H295R cells are related to the use of a primary cell *vs.* a tumor cell line or granulosa *vs.* adrenal cell is not known. However, it is clear that some effects of p53 inhibition are specific to stimulated cells, for example, StAR mRNA, whereas other genes are constitutively affected by p53, as in the case of 3βHSD mRNA. Thus it is possible, and perhaps likely, that p53 exerts a constitutive action on genes associated with basal steroidogenesis (*e.g.* 3βHSD) while acting to promote the acute expression of StAR in response to a trophic stimulus. Future studies will address the mechanisms of p53 action in both macaque granulosa cells and H295R adrenocortical cells.

In summary, p53 is expressed by macaque, but not rat, granulosa cells before and after an ovulatory stimulus, although distribution is limited to the cytoplasm. Inhibition of p53 does not alter markers of granulosa cell proliferation, nor does it result in reduced levels of p21 mRNA. However, p53 does play a significant role in hCG-mediated synthesis of progesterone. Also, p53 regulates mRNA for several steroidogenic enzymes, including StAR, 3βHSD, and 17βHSD-I. These findings were extended to the human adrenocortical cell line H295R, where forskolin-mediated aldosterone was suppressed by p53 inhibition, as was StAR and 3βHSD expression. Overall, these data are first to demonstrate a role for p53 in the regulation of primate steroidogenesis.

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