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Ghrelin Inhibits the Proliferative Activity of Immature Leydig Cells *in Vivo* and Regulates Stem Cell Factor Messenger Ribonucleic Acid Expression in Rat Testis

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Ghrelin has emerged as putative regulator of an array of endocrine and nonendocrine functions, including cell proliferation. Recently, we provided evidence for the expression of ghrelin in mature, but not in undifferentiated, Leydig cells of rat and human testis. Yet testicular actions of ghrelin, other than modulation of testosterone secretion, remain unexplored. In the present study we evaluated the effects of ghrelin on proliferation of Leydig cell precursors during puberty and after selective elimination of mature Leydig cells by treatment with ethylene dimethane sulfonate. In these settings, intratesticular injection of ghrelin significantly decreased the proliferative activity of differentiating immature Leydig cells, estimated by 5-bromodeoxyuridine labeling. This response was selective and associated, in ethylene dimethane sulfonate-treated animals, with a decrease in the mRNA levels of stem cell factor (SCF), *i.e.* a key signal in spermatogenesis and a putative regulator of Leydig cell development. Thus, the effects of ghrelin on SCF gene expression were evaluated. In adult rats, ghrelin induced a significant decrease in SCF mRNA levels *in vivo*. Such an inhibitory action was also detected *in vitro* using cultures of staged seminiferous tubules. The inhibitory effect of ghrelin *in vivo* was dependent on proper FSH input, because it was detected in hypophysectomized rats only after FSH replacement. Overall, it is proposed that acquisition of ghrelin expression by Leydig cell precursors during differentiation may operate as a self-regulatory signal for the inhibition of the proliferative activity of this cell type through direct or indirect (*i.e.* SCF-mediated) mechanisms. In addition, we present novel evidence for the ability of ghrelin to modulate the expression of the SCF gene, which may have implications for the mode of action of this molecule in the testis as well as in other physiological systems. (*Endocrinology* 145: 4825–4834, 2004)

GHRELIN IS THE endogenous ligand for the GH secretagogue receptor (GHS-R) (1, 2). This molecule is a 28-amino acid peptide, mainly expressed in the stomach, that acting at central levels has been primarily involved in the control of GH secretion and food intake (1–3). However, emerging evidence indicates that ghrelin carries out additional endocrine and nonendocrine actions, including central regulation of neuroendocrine functions, as well as a wide array of peripheral effects (2–6). The cognate ghrelin receptor, the GHS-R, belongs to the large family of G protein-coupled, seven-transmembrane-spanning receptors (7). Two GHS-R subtypes, generated by alternative splicing of a single gene, have been identified to date: the full-length type 1a receptor and the truncated GHS-R type 1b. GHS-R1a is the functionally active, signal-transducing form of the receptor (7). However, evidence for GHS-R-independent effects of ghrelin as well as of synthetic GHSs has been presented recently (8, 9).

Among other peripheral actions, a role for ghrelin in the autocrine/paracrine control of cell proliferation and cancer has been recently proposed, although conflictive results have been reported (10). Thus, proliferative actions of ghrelin (or its synthetic analogs, the GHSs) have been observed in prostate (PC-3) and thyroid (ARO) cancer cell lines as well as in the cardiomyocyte H9c2 cell line and cultured adrenocortical cells (11–14). However, antiproliferative effects of ghrelin/GHSs have been also reported in several thyroid (NPA and WRO), lung (CALU-1), and breast (MCF7, T47D, and MDA-MB231) cancer cell lines (9, 12, 15). Although the basis for such a discrepancy remains obscure, it is likely that differences in cell types and expression of ghrelin/GHS-Rs may contribute to this phenomenon.

In the testis, two different populations of Leydig cells, *i.e.* fetal and adult types, are identified during development. The adult-type Leydig cells emerge during pubertal maturation through a complex process of proliferation and differentiation of mesenchyme-derived precursors that is under the control of a plethora of endocrine and paracrine signals (16). Conventionally, three stages of postnatal differentiation are detected: undifferentiated Leydig cell precursors, differentiating immature Leydig cells, and fully mature adult-type Leydig cells (17). As immature Leydig cells differentiate into a mature adult-type population, they become mitotically less active, and when a critical mass of mature Leydig cells is

Abbreviations: BrdU, 5-Bromodeoxyuridine; EDS, ethylene dimethane sulfonate; GHS, GH secretagogue; GHS-R, GH secretagogue receptor; hCG, human chorionic gonadotropin; HPX, hypophysectomized; i.t., intratesticular; MIS, Mullerian inhibiting substance; SCF, stem cell factor; SCFm, membrane-bound form of stem cell factor; SCFs, soluble form of stem cell factor.

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achieved, the proliferative activity of the population is almost negligible (16–18). However, administration of the cytotoxic drug ethylene dimethane sulfonate (EDS) induces selective destruction of mature Leydig cells, activating a subsequent wave of proliferation and further differentiation of Leydig cell precursors and the appearance of a new population of Leydig cells within 3 wk (16, 19–21). Because this process is believed to mimic the normal developmental events of Leydig cells during puberty, the EDS-treated rat has been widely used as an experimental model for studies of Leydig cell development (16, 19–22). Indeed, studies using this model have demonstrated a relevant role of stem cell factor (SCF) and Mullerian inhibiting substance (MIS) in the control of Leydig cell proliferation (23, 24).

Our recent evidence suggests a role for ghrelin in the direct control of testicular function. Thus, we have previously reported the presence of ghrelin in the testis, whose expression is restricted to Leydig cells and under the control of pituitary LH (25–27). Similarly, expression of the cognate ghrelin receptor in rat and human testis has been demonstrated (25, 27, 28), and the ability of ghrelin to modulate stimulated testosterone secretion *in vitro* has been reported (25). It is of note that ghrelin expression in Leydig cells appeared critically dependent on their differentiation state, because ghrelin levels were low to undetectable in immature Leydig cells during puberty and after EDS treatment (26). Moreover, ghrelin was not detected in poorly differentiated human Leydig tumor cells, whereas highly differentiated Leydig cell tumors expressed the peptide (27). Considering the proposed role of ghrelin in the local control of cell proliferation (10), we hypothesized that ghrelin may operate as a novel signal in the autoregulation of Leydig cell proliferation. To test this hypothesis, the effects of ghrelin on the proliferative activity of Leydig cell precursors were assessed *in vivo* during pubertal development and after elimination of mature Leydig cells, and the expression levels of several genes involved in Leydig cell proliferation and differentiation were monitored.

Materials and Methods

Animals and drugs

Wistar male rats bred in the vivarium of University of Cordoba were used. The day the litters were born was considered d 1 of age. The animals were maintained under constant conditions of light (14 h of light, from 0700 h) and temperature (22 C) and were weaned at d 21 of age in groups of five rats per cage with free access to pelleted food and tap water. Experimental procedures were approved by the Cordoba University ethical committee for animal experimentation and were conducted in accordance with the European Union normative for the care and use of experimental animals. Rat ghrelin was obtained from Bachem AG (Bubendorf, Switzerland). Highly purified human chorionic gonadotropin (hCG; Profasi) and recombinant FSH (Gonal-F) were purchased from Serono (Madrid, Spain). 5-Bromodeoxyuridine (BrdU) was obtained from Sigma-Aldrich Corp. (St. Louis, MO), and a monoclonal antibody anti-BrdU was from Dako Diagnostica (Hamburg, Germany). EDS was synthesized in our laboratory and dissolved in dimethylsulfoxide/water (1:3, vol/vol).

Experimental designs

For the analysis of ghrelin effects on Leydig cell proliferation, an *in vivo* procedure of intratesticular injection was used, in line with previous references (24, 29). In detail, intratesticular (i.t.) injections of ghrelin (15 μg in 50 μl/24 h for 2 d) were applied in two models with a high rate of proliferation of Leydig cell precursors: the pubertal and the EDS-treated rat. In our settings, each animal received unilateral i.t. injections of ghrelin (right testis); the contralateral (left) testis was injected with 50 μl vehicle, thus serving as the control. In experiment 1, the effects of ghrelin were monitored in testes from rats at puberty. Considering the previously reported profile of Leydig cell proliferation during puberty (16, 17), and our data on the developmental expression of GHS-R1a in the rat testis (28), ghrelin was i.t. injected unilaterally on d 30 and 31 postpartum, and testis samples (n = 6/group) were obtained at 32 d of age. For BrdU labeling, the animals were ip injected 1 h before sampling with 50 mg/kg BrdU in 0.1 M Tris-HCl-buffered saline, pH 7.6., and testicular samples were processed as described below. In experiment 2, the EDS-treated rat was used. In this setting, selective elimination of mature Leydig cell was achieved by systemic administration of the cytotoxic drug EDS (single dose of 75 mg/kg, ip), as previously described (19, 22, 23). Considering previous references on the waves of proliferation of Leydig cell precursors after EDS administration (18, 23), d 21 after EDS administration was selected for analysis, because at this time point a peak of active proliferation of immature Leydig cells (which are roughly similar, in terms of state of differentiation, to those on d 30–32 postpartum) is detected. Thus, ghrelin was i.t. injected unilaterally to adult rats on d 19 and 20 after EDS, and testis samples (n = 6/group) were obtained on d 21. Procedures for BrdU labeling and sampling of testis tissue for RNA analysis were similar to those described in experiment 1. Additional groups of testis samples (n = 6) were taken in EDS-treated animals (on d 21 after EDS) at 3, 6, 12, and 24 h after unilateral i.t. injection of ghrelin (15 μg in 50 μl) and processed for RNA analysis as described below.

In the next series of experiments, the effects of ghrelin on testicular expression of SCF gene were evaluated. In experiment 3, a single i.t. injection of ghrelin (15 μg in 50 μl) was applied to intact (60 d old) adult rats. Groups of animals (n = 5) were sequentially killed at 3, 6, 12, and 24 h after injection, and testis samples were obtained and processed for RNA analysis as described above. In addition, in experiment 4, a static culture system of seminiferous tubule preparations at different stages of the epithelial cycle was used to monitor the effects of ghrelin on SCF mRNA levels *in vitro*. Microdissection of seminiferous tubule segments of testes from Sprague Dawley rats was carried out as described in detail previously (30). Specific stages of the cycle were pooled in four major groups corresponding to stages II–VI, stages VII–VIII, stages IX–XII, and stages XIII–I. After exhaustive washing, staged seminiferous tubule preparations were challenged for 24 h with 10^{-6} M ghrelin, and samples of tubular tissue were processed for RNA analysis as described below. In our analyses, four independent experiments were performed, with four replicate samples in each. Finally, in experiment 5, modulation of the effects of ghrelin on testicular SCF mRNA levels by pituitary hormonal signals was monitored *in vivo* using the hypophysectomized (HPX) rat as a model. Thus, a single i.t. injection of ghrelin (15 μg in 50 μl) was applied to long-term HPX adult rats, *i.e.* 4 wk after pituitary removal, with or without gonadotropin replacement: hCG (10 IU/rat·24 h; as superagonist of LH) or recombinant FSH (7.5 IU/rat·24 h) for 7 d before sampling. Animals (n = 5) from the different experimental groups were killed at 6 h after ghrelin injection, and testis samples were obtained and processed for RNA analysis as described above.

RNA analysis by semiquantitative RT-PCR

Total RNA was isolated from testicular samples using the single-step, acid guanidinium thiocyanate-phenol-chloroform extraction method (31). Testicular expression of SCF, MIS, and cyclin A_2 and G_1 mRNAs was assessed by RT-PCR, optimized for semiquantitative detection, using the primer pairs and conditions indicated in Table 1. These genes were selected because they have been previously involved in different aspects of Leydig cell proliferation and differentiation (23, 24, 32). As an internal control for RT and reaction efficiency, amplification of a 290-bp fragment of L19 ribosomal protein mRNA was carried out in parallel in each sample, using the primer pair and conditions indicated in Table 1. The primer pairs used for amplification of the different targets (Table 1) were designed to span over intron sequences and, whenever possible, were obtained from previous reports (28, 32, 33).

For amplification of the targets, 2 μg total RNA were used to perform RT-PCR in two consecutive separate steps, as previously described (25, 26, 28). PCRs consisted of a first denaturing cycle at 97 C for 5 min,

Table 1. Primer pairs used for RT-PCR amplification of SCF, MIS, cyclin A_2, cyclin G_1, and RP-L19 transcripts in testis samples

Target	Oligo-primers		Expected size (bp)	PCR cycles
SCF	SCF sense	(5′-GCT TGA CTG ATC TTC TGG ACA AG-3′)	505 (SFCs)	32
	SCF as	(5′-AAC TGC CCT TGT AAG ACT TGG C-3′)	420 (SCFm)	
MIS	MIS sense	(5′-AGT TGC TAG TCC TAC ATC TGG C-3′)	312	28
	MIS as	(5′-AGG CCT GCA GCT GAG CGA TGG T-3′)		
Cyclin A_2	Cyclin A_2 sense	(5′-CCT TGT GGA CTG GCT AGT TGA-3′)	420	26
	Cyclin A_2 as	(5′-AGC GAT GGC AAA TAC TTG AGG T-3′)		
Cyclin G_1	Cyclin G_1 sense	(5′-CCT TCC AAT TTC TGC AGC TC-3′)	280	26
	Cyclin G_1 as	(5′-CTT GGA AAC AAG CTC TTG CC-3′)		
RP-L19	L19 sense	(5′-GAA ATC GCC AAT GCC AAC TC-3′)	290	22
	L19 as	(5′-ACC TTC AGG TAC AGG CTG TG-3′)		

For each target, the primer pair used for amplification is included. In addition, the expected size of the generated cDNA products and the number of cycles selected for RT-PCR analysis are indicated for each signal.

followed by a variable number of cycles of amplification, defined by denaturation at 96 C for 30 sec, annealing for 30 sec, and extension at 72 C for 1 min. A final extension cycle of 72 C for 15 min was included. Annealing temperature was adjusted for each target: 60 C for SCF, 58 C for MIS and cyclins A_2 and G_1, and 56 C for RP-L19 transcripts. Different numbers of cycles were tested to optimize amplification in the exponential phase of PCR. In detail, analysis of the intensity of PCR signals as a function of the number of amplification cycles revealed a strong linear relationship between cycles 26–38 in the case of SCF (with correlation coefficients $r^2 \geq 0.97$); cycles 22–34 in the case of MIS and cyclins A_2 and G_1 ($r^2 \geq 0.97$); and cycles 19–29 in the case of RP-L19 ($r^2 = 0.992$). On this basis, the numbers of PCR cycles indicated in Table 1 were chosen for additional semiquantitative analysis of specific targets and L19 internal control. PCR-generated DNA fragments were resolved in Tris-borate-buffered 1.5% agarose gels and were visualized by ethidium bromide staining. The specificity of PCR products was confirmed by direct sequencing using a fluorescent dye termination reaction and an automated sequencer (Central Sequencing Service, University of Cordoba). Quantification of the intensity of RT-PCR signals was carried out by densitometry scanning using an image analysis system (1-D Manager, TDI Ltd., Madrid, Spain), and values of the specific targets were normalized to those of internal controls to express arbitrary units of relative expression. In all assays, liquid controls and reactions without RT resulted in negative amplification.

Analysis of proliferative activity by BrdU labeling

BrdU labeling of proliferating testicular cells was conducted as previously described (34). In detail, tissue sampling was carried out 1 h after systemic administration of BrdU, because at this time point labeled cells are at the S or G_2 phase of the cell cycle. Upon decapitation of the animals, testicular samples were dissected free of the surrounding fat and were fixed for 24 h in Bouin-Hollande's fluid (0.2 M picric acid, 0.12 M cooper acetate, and 4% formaldehyde in water). For immunohistochemical detection of BrdU, testicular sections (5 μm thick) were subjected to quenching of endogenous peroxidase (by incubation in 0.2% hydrogen peroxide in methanol for 30 min) and DNA denaturation. Tissue sections were then incubated overnight with a mouse monoclonal antibody against BrdU diluted 1:500 in PBS and processed according to the avidin-biotin-peroxidase complex (ABC, Vector Laboratories, Inc., Burlingame, CA) technique following previously described methods (26, 27, 34). Immunostained sections were counterstained with hematoxylin-eosin or toluidine blue.

Proliferating cells (*i.e.* immunolabeled for BrdU) were counted in three equatorial transverse testicular sections per testis, with five testes per experimental group. The whole section was scored using a ×40 objective. The numbers of proliferating cells were expressed as the number of BrdU labeled cells per square millimeter of tissue section. No significant differences in testicular volume were detected between vehicle- and ghrelin-injected testes (data not shown). Identification of the different cell types of the testicular interstitium was conducted, whenever possible, on the basis of morphological criteria, as described in detail previously (35). In testes from pubertal (32-d-old) animals and at 21 d after EDS treatment, proliferating immature Leydig cells were identified as interstitial cells with distinctive morphological features, including round nuclei and eosinophilic cytoplasm, in line with previous references (16, 17). Interstitial mast cells, previously reported to accumulate in the testis from d 15 after EDS onward (18), were metachromatically stained with toluidine blue for identification, as described previously (35). Other interstitial cell types were globally considered as mesenchymal-type cells. Endothelial cells were not considered for quantitative analysis of BrdU-labeled cells.

Presentation of data and statistics

In BrdU labeling experiments, the number of proliferating cells was expressed as the number of labeled cells per area unit (square millimeters) of testis tissue (n = 5 testes/group). Semiquantitative RT-PCR analyses were carried out in duplicate from at least four independent RNA samples of each experimental group. For generation of RNA samples, individual testis specimens were used. Data from BrdU experiments are presented as individual paired data [for each animal: left (vehicle) *vs.* right (ghrelin-injected) testis values] as well as the mean ± SEM. Results were analyzed for statistically significant differences using paired *t* test (SigmaStat 2.0 software, Jandel Corp., San Rafael, CA). Semiquantitative RNA data are presented as the mean ± SEM. Results were analyzed for statistically significant differences using ANOVA, followed by Student-Newman-Keuls multiple range test (SigmaStat 2.0). $P \leq 0.05$ was considered significant.

Results

Effects of ghrelin on proliferative activity of differentiating Leydig cells

The effects of ghrelin on the proliferative activity of Leydig cell precursors *in vivo* were evaluated in two models in which active Leydig cell division is detected: the pubertal and the EDS-treated rat (16–18, 23). To this end, the BrdU labeling technique was used as described in detail previously (34), and identification of the different cell types of testicular interstitium was conducted following previously accepted morphological criteria (16, 35). In pubertal (32 d old) testis, abundant immature Leydig cells, characterized by their round nuclei and eosinophilic cytoplasm, were found as clusters in the interstitial space (Fig. 1A). At this age, most BrdU-labeled cells corresponded to Leydig cells and mesenchymal-type cells (Fig. 1B). A trend for a decrease in the total number of proliferating interstitial cells, which was within the limits of statistical significance, was detected after two consecutive i.t. injections of ghrelin (15 μg in 50 μl/24

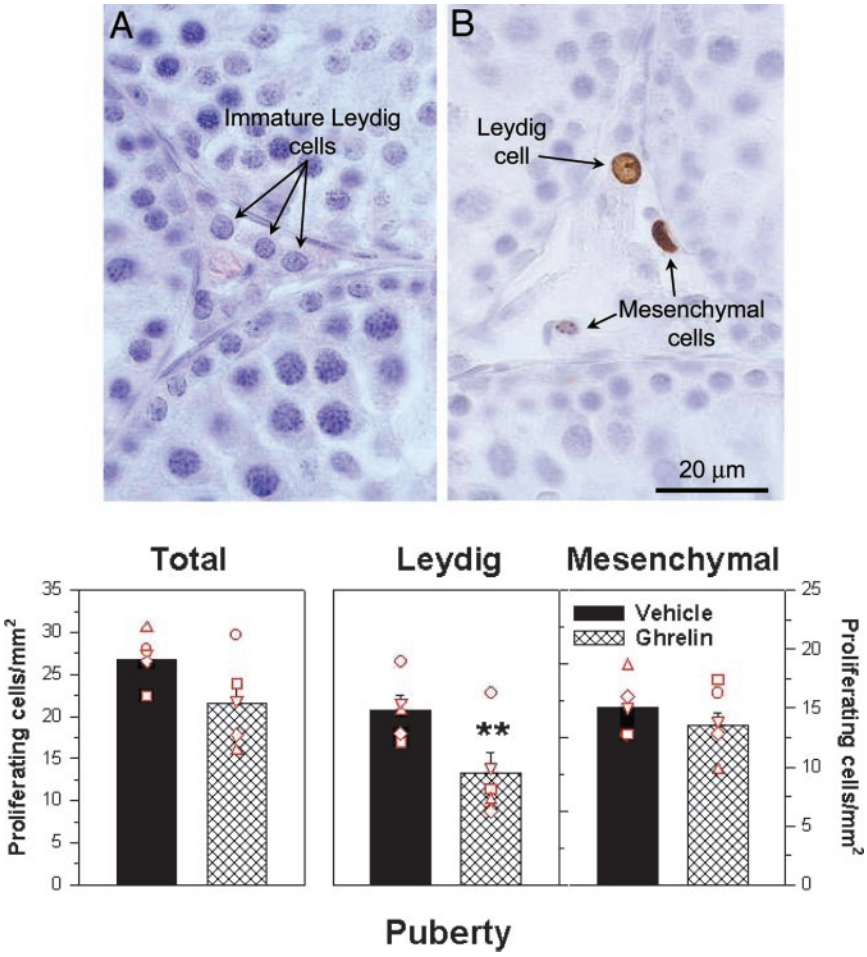

FIG. 1. Effects of ghrelin on the proliferative activity of immature Leydig cells *in vivo* at puberty. In the *upper panels*, representative photomicrographs of BrdU labeling of different cell types of the testicular interstitium in pubertal 32-d-old rat testes are presented. Abundant immature Leydig cells are observed in hematoxylin-eosin-stained sections (A). BrdU labeling is shown in B, where proliferating Leydig and mesenchymal cells can be observed. In the *lower panels*, data on the proliferative activity of different interstitial cell types of the testis in pubertal (32 d old) rats after unilateral i.t. injection of ghrelin are presented. Total as well as specific proliferative rates of immature Leydig cells *vs.* other mesenchymal-type cells of the interstitium are shown. Individual paired data of proliferating cells are presented for each animal [n = 5; left testis injected with vehicle; right testis injected with ghrelin (15 μg in 50 μl/24 h for 2 d)]; paired data from each animal are represented by similar symbols. In addition, data are presented as the mean ± SEM. **, $P < 0.01$ *vs.* corresponding vehicle-injected testes (by paired *t* test).

h). Such an effect was due to a significant decrease ($P \leq 0.01$) in the number of proliferating immature Leydig cells after ghrelin administration. In contrast, no effect of ghrelin on the proliferation rate of mesenchymal-type cells of the interstitium was detected (Fig. 1, *lower panel*).

In the EDS model, BrdU labeling analyses were conducted on d 21 after administration of the cytotoxicant *in vivo*, when a major wave of proliferation of immature Leydig cells is detected (18, 23). Notably, maximum proliferative activity of interstitial testicular cells after EDS treatment has been previously reported at 3 d post-EDS (18). At this point, however, proliferating interstitial cells mostly correspond to fully undifferentiated Leydig cell precursors, which do not express ghrelin (26), and are not similar, in terms of state of differentiation, to prepubertal (32 d old) immature Leydig cells. Thus, this time point (3-d after EDS) was not considered for analysis. At 21 d after EDS treatment, abundant immature Leydig cells, with round nuclei and eosinophilic cytoplasm, were present in the testicular interstitium, where accumulation of mast cells, specifically stained with toluidine blue, was also detected (Fig. 2A). No significant differences in the total number of mast cells were detected between ghrelin-treated and paired vehicle-injected testes (data not shown). Immunohistochemical analyses showed that most of the proliferating BrdU-labeled cells corresponded to immature Leydig cells and mesenchymal-type cells (Fig. 2B). Counterstaining of BrdU-labeled sections with toluidine blue demonstrated that proliferating cells were not mast cells (Fig. 2C), and less than 1% of mast cells were positive for BrdU (Fig. 2C, *inset*). At 21 d after EDS, the total number of proliferating interstitial cells was significantly decreased ($P \leq 0.05$) after two consecutive i.t. injections of ghrelin (15 μg in 50 μl/24 h). As was the case in pubertal animals, this inhibitory effect was selectively observed in immature Leydig cells ($P \leq 0.01$), whereas the number of other proliferating mesenchymal cells was not different between vehicle- and ghrelin-injected testes (Fig. 2, *lower panel*).

Effects of ghrelin on testicular expression of genes involved in Leydig cell development

The above proliferative responses were correlated to changes in the expression levels of several genes recently involved in Leydig cell development. Such analyses were selectively conducted in testes from EDS-treated rats. On the basis of previous references (23, 24, 32), relative mRNA levels of the Sertoli cell products SCF and MIS as well as cyclins A_2 and G_1 were selectively monitored after i.t. injection of ghrelin (15 μg in 50 μl) at 21 d after EDS treatment. In our assays, relative expression levels of MIS mRNA were not altered after i.t. administration of ghrelin at any of the time points (3–24 h) studied. Similarly, cyclin A_2 and G_1 mRNAs levels

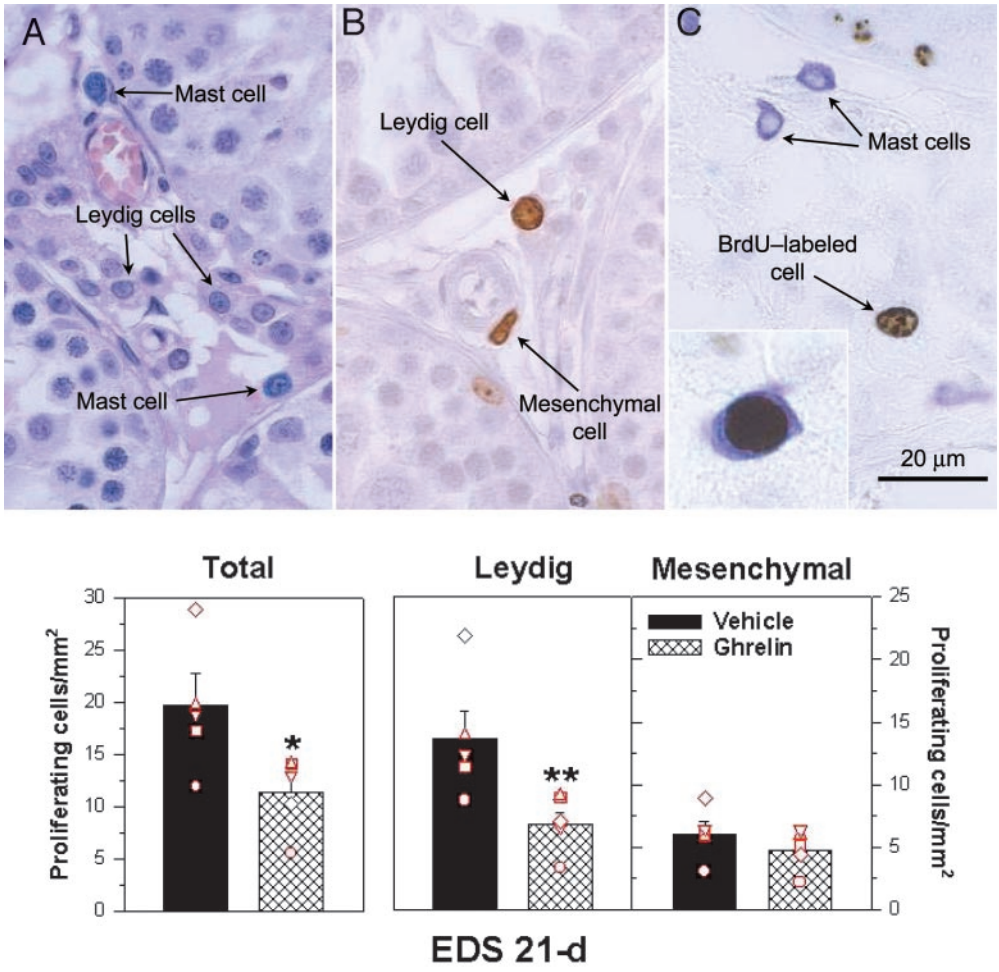

FIG. 2. Effects of ghrelin on the proliferative activity of immature Leydig cells *in vivo* at 21 d after EDS administration. In the *upper panels*, representative photomicrographs of BrdU labeling of different cell types of the testicular interstitium in EDS-treated rats are presented. Testis sections on d 21 after EDS are shown, where abundant immature Leydig cells, as well as mast cells, can be observed in Alcian Blue-hematoxylin- and eosin-stained sections (A). BrdU-immunostained sections, showing proliferating Leydig and mesenchymal cells, are presented in B. C, Counterstaining of BrdU-labeled sections with toluidine blue allows simultaneous identification of proliferating and mast cells; proliferating mast cells (C, *inset*) were scarce. In the *lower panels*, data on the proliferative activities of different interstitial cell types of the testis in EDS-treated rats after unilateral i.t. injection of ghrelin are presented. Differentiating immature Leydig cells were identified on the basis of their morphological features. Total as well as specific proliferative rates of immature Leydig cells *vs.* other mesenchymal-type cells of the interstitium are shown. Individual paired data of proliferating cells is presented for each animal [n = 5; left testis injected with vehicle; right testis injected with ghrelin (15 μg in 50 μl/24 h for 2 d)]; paired data from each animal are represented by similar symbols. In addition, quantitative data are presented as the mean ± SEM. *, $P < 0.05$; **, $P < 0.01$ (*vs.* corresponding vehicle-injected testes, by paired *t* test).

were not affected by ghrelin treatment during the study period. Two SCF transcripts were detected upon RT-PCR: one encoding the soluble form (SCFs) and the other coding for the membrane-bound form (SCFm) of SCF, in keeping with previous studies (33, 36). Contrary to observations for MIS and cyclin transcripts, the mRNA levels of both forms of SCF were significantly decreased ($P \leq 0.01$) at 3, 6, 12, and 24 h after i.t. injection of ghrelin (Fig. 3).

Effects of ghrelin on expression of SCF mRNA in adult testis

The effects of ghrelin on the expression levels of SCF mRNAs were studied in additional experimental models. In keeping with data from EDS-treated animals, in intact adult rats testicular expression of both SCFs and SCFm transcripts was detected, with higher expression of the SCFm messenger, in line with previous reports pointing to a predominant expression of this species in the adult age (36). A single i.t. injection of ghrelin (15 μg in 50 μl) to intact rats decreased the relative levels of SCF mRNAs *in vivo*. This response was statistically significant ($P \leq 0.01$) from 6 h after administration onward, with maximum suppression of SCF mRNA levels at 6 and 12 h after ghrelin injection (Fig. 4). The inhibitory effect of ghrelin on SCF mRNA levels was also detected *in vitro* using cultures of seminiferous tubule fragments isolated at different stages of the seminiferous epithelial cycle. In detail, staged tubular segments were pooled in four major groups corresponding to stages II–VI, stages VII–VIII, stages IX–XII, and stages XIII–I and challenged for 24 h with 10^{-6} M ghrelin. In this setting, significant inhibitory

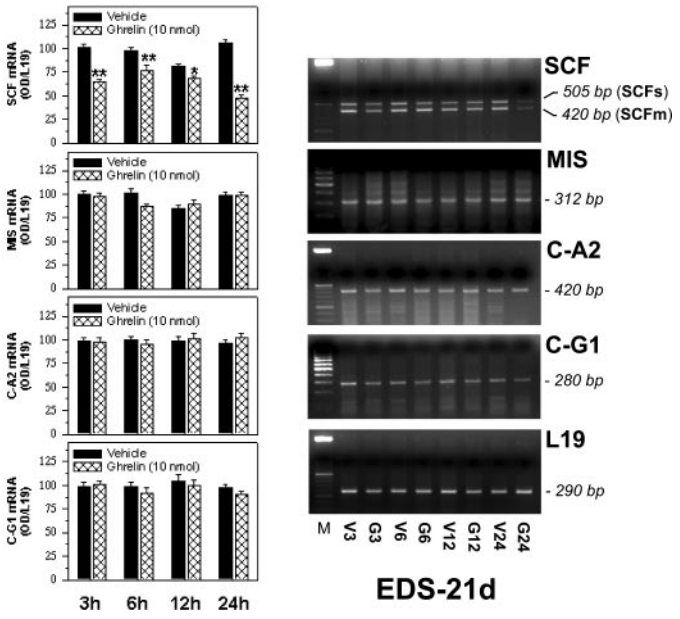

FIG. 3. Expression profiles of SCF, MIS, and cyclin A_2 and G_1 mRNAs in rat testis 21 d after systemic administration of EDS, at different time points (3, 6, 12, and 24 h) after unilateral i.t. injection of ghrelin (G). Contralateral testes injected with vehicle (V) served as controls. Representative RT-PCR assays for each target are presented in the *right panel*. In addition, in the *left panel,* semiquantitative mRNA expression values (mean ± SEM of five samples per group) are shown. Expression levels of SCF are the combined expression levels of both SCFs and SCFm mRNAs. *, $P < 0.05$; **, $P < 0.01$ (*vs.* corresponding vehicle-injected groups, by ANOVA followed by Student-Newman-Keuls multiple range test).

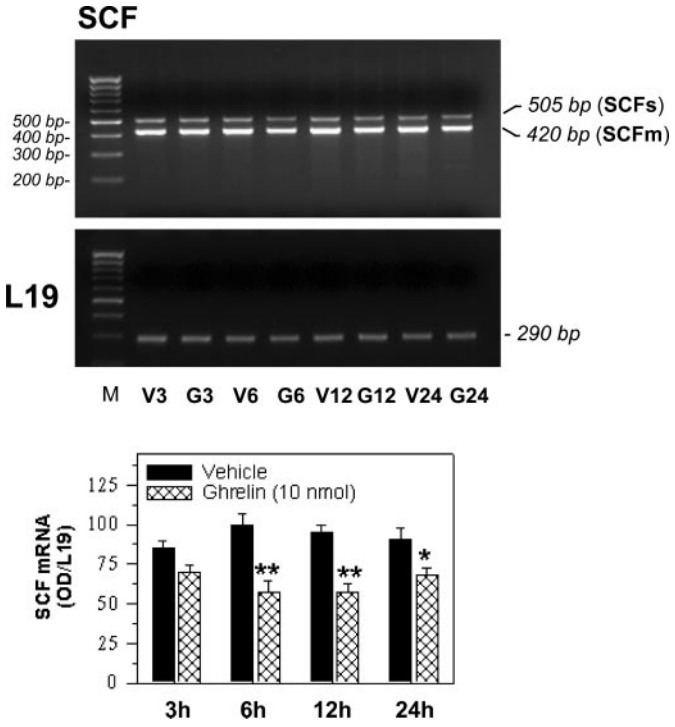

FIG. 4. Expression of SCF mRNAs in adult rat testis at different time points (3, 6, 12, and 24 h) after unilateral i.t. injection of ghrelin (G). Contralateral testes injected with vehicle (V) served as controls. A representative RT-PCR assay is presented in the *upper panel*. In addition, in the *lower panel,* semiquantitative data (mean ± SEM of five samples per group) of the combined expression levels of both SCFs and SCFm mRNAs are shown. *, $P < 0.05$; **, $P < 0.01$ (*vs.* corresponding vehicle-injected groups, by ANOVA followed by Student-Newman-Keuls multiple range test).

responses ($P \leq 0.05$) in terms of SCF mRNA expression levels were detected for both transcripts (SCFs and SCFm) after exposure to ghrelin at all stages of the cycle tested (Fig. 5).

In addition, the ability of pituitary hormones to modulate the inhibitory action of ghrelin on testicular SCF gene expression was evaluated using the HPX rat, with or without gonadotropin replacement, as an experimental model. Because long-term HPX induced dramatic changes in testis weight (HPX, 324.0 ± 19.0 mg/testis; HPX plus hCG, 565.0 ± 35.0 mg/testis; HPX plus FSH, 455.0 ± 12.0 mg/testis; controls, 1025.0 ± 40.0 mg/testis) and considering that the SCF gene is selectively expressed in Sertoli cells (whose net number is not altered by HPX), relative levels of SCF mRNAs were converted to total expression levels per testis after correction of relative values by testis weight (OD per milligram of total weight). In terms of absolute expression per testis, 4-wk HPX induced a significant decrease in testicular SCF levels that was prevented by FSH replacement (7.5 IU/rat·24 h for 7 d), but not by hCG (10 IU/rat·24 h for 7 d; hCG as superagonist of LH) treatment. In this model, i.t. injection of ghrelin (15 μg in 50 μl) induced a significant decrease ($P \leq 0.05$) in SCF mRNA levels in testes from HPX animals supplemented with FSH, in line with results obtained in intact animals. Conversely, however, i.t. injection of ghrelin significantly elevated SCF mRNA levels in HPX rats and in HPX animals supplemented with hCG 6 h after administration (Fig. 6).

Discussion

Compelling evidence demonstrates that Leydig cell development is under the control of a large number of locally produced factors, and changes in the intratesticular environment have been reported to alter (increase or decrease) the proliferation of this cell population (16, 17, 23, 24, 37–39). However, the mechanisms responsible for the loss of proliferative activity in terminally differentiated Leydig cells remain largely unknown. In the present study we tested whether ghrelin, acting in an autocrine or paracrine manner, might contribute to the mitotic arrest of mature Leydig cells. To this end, the effects of ghrelin on cell proliferation were evaluated, by means of BrdU labeling, in two models in which active Leydig cell division is detected: the pubertal and the EDS-treated rat. The effects of ghrelin on Leydig cell proliferation were not monitored in adult testis, given that at this age the mitotic rate of mature Leydig cells is almost null (16, 17). In our experiments, unilateral i.t. injection of ghrelin was used. This setting allows analysis of the roles of locally acting factors (such as ghrelin) *vs.* systemic signals in the control of specific testicular functions (*e.g.* cell proliferation), because both testes share a similar endocrine background, but are exposed to different local inputs (*i.e.* unilateral i.t. injection of ghrelin). Although direct assessment of the physiological testicular levels of ghrelin, which would derive from the systemic, gut-derived hormone plus locally pro-

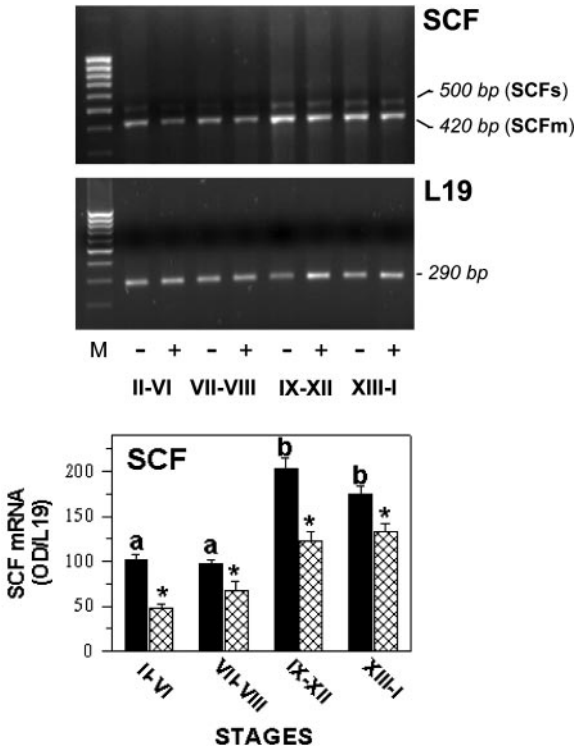

FIG. 5. Expression profile of SCF mRNAs in staged seminiferous tubule preparations after challenge with ghrelin for 24 h. In the *upper panel*, a representative RT-PCR assay is presented of expression levels of SCF mRNAs in tubule preparations isolated at stages II–VI, VII–VIII, IX–XII, and XIII–I of the cycle, after culture in the presence of medium alone (−) or medium containing 10^{-6} M ghrelin (+). In the *lower panel*, semiquantitative data on the combined expression levels of both SCFs and SCFm mRNAs in the different stages are shown. For groups incubated with medium alone, values with different superscript letters are statistically different. *, $P < 0.05$ *vs.* corresponding control preparations cultured in the presence of medium alone (by ANOVA followed by Student-Newman-Keuls multiple range test).

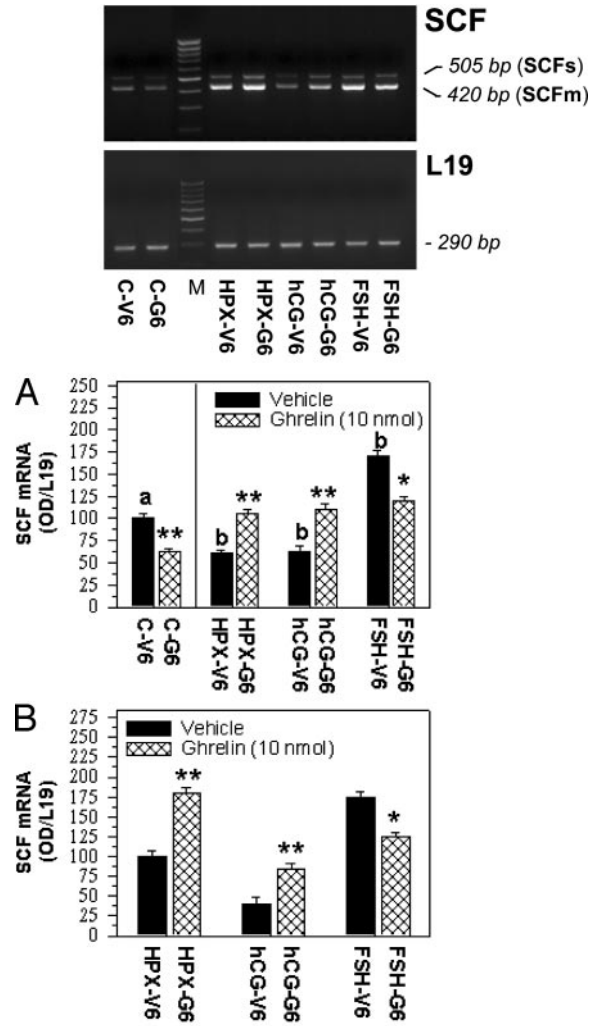

FIG. 6. Analysis of SCF mRNA expression in rat testis from long-term (4 wk) HPX rats, with or without gonadotropin replacement. A representative RT-PCR assay is shown of SCF mRNA levels in testes from control (C) and HPX rats with or without replacement with hCG (10 IU/rat·24 h for 7 d) or recombinant FSH (7.5 IU/rat·24 h for 7 d) at 6 h after unilateral i.t. injection of ghrelin (G). Contralateral testes injected with vehicle (V) served as controls. Semiquantitative data of the combined expression levels of SCF mRNAs in the different experimental groups are shown in the *lower panels*. A, Total levels of expression of SCF gene per testis are presented. These were obtained after correction of relative values for testicular weight (OD per milligrams of total weight). B, Relative levels of expression of SCF mRNA in the different HPX groups are shown. Values are the mean ± SEM of five independent determinations. For vehicle-injected testes (V), values with *different superscript letters* are statistically different. *, $P < 0.05$; **, $P < 0.01$ (*vs.* corresponding vehicle-injected groups, by ANOVA followed by Student-Newman-Keuls multiple range test).

duced ghrelin (25–27), has not been conducted to date, the doses of ghrelin used in the present study are equivalent to those previously reported to inhibit testicular testosterone secretion *in vitro* (25).

Quantitative analysis of BrdU-labeled cells in ghrelin-treated testes showed a significantly lesser number of proliferating immature Leydig cells than in paired vehicle-injected testes both at puberty and on d 21 after EDS. This phenomenon was selectively targeted to Leydig cells, because the proliferative rate of other mesenchymal-type cells in the testicular interstitium remained unaffected after ghrelin administration. This may explain the subtle differences detected in the impact of ghrelin on total proliferative activity of interstitial cells between pubertal and EDS-treated testes. Thus, although only 45% of proliferating interstitial cells in the pubertal (32-d-old) testes corresponded to immature Leydig cells, approximately 70% of BrdU-positive cells in the testicular interstitium at 21 d after EDS were immature Leydig cells. Accordingly, although a clear-cut significant decrease ($P \leq 0.05$) in the total number of proliferating interstitial cells was detected after i.t. injection of ghrelin in EDS-treated testes, only a trend for a decrease (within the limit of statistical significance) in the total number of BrdU-labeled interstitial cells was observed in pubertal testes. Nevertheless, the above results show that ghrelin, in two closely related, but distinct, experimental settings, is able to inhibit the proliferative rate of immature Leydig cells *in vivo*, thus making it tempting to speculate that locally produced ghrelin might contribute to the (self-)regulation of Leydig cell development.

To further characterize the molecular basis for the inhibition of immature Leydig cell proliferation by ghrelin, gene

expression analyses were selectively conducted in testes from EDS-treated rats. Notably, due to the synchronized activation of proliferation and differentiation of this cell population, the EDS model has been extensively used for the analysis of molecular aspects of Leydig cell development (22, 23). In detail, a panel of factors, including SCF, MIS, and cyclins A_2 and G_1 (23, 24, 32), were selected for analysis. SCF and MIS have been previously identified, using the EDS model, as potential modulators of Leydig cell development, with stimulatory and inhibitory effects, respectively (23, 24). In addition, decreased levels of cyclin A_2 and increased levels of cyclin G_1 coincide with the loss of proliferative capacity of immature Leydig cells (32). In our experiments, ghrelin evoked a significant decrease in the relative mRNA levels of SCF, but not MIS or cyclins A_2 and G_1, in rat testis at 21 d after EDS. This suggests a direct effect of ghrelin on Sertoli cells, where the functional ghrelin receptor GHS-R1a has been detected (27, 28), to regulate SCF expression. As SCF has been pointed out as an important growth factor for Leydig cell precursors (23), a ghrelin-induced decrease in SCF mRNA levels might contribute to its inhibitory effects on immature Leydig cell proliferation; yet this association may not be causative. Nevertheless, in addition to indirect actions (*e.g.* SCF mediated), direct effects of ghrelin on immature Leydig cells, which probably express the GHS-R1a (27, 28), cannot be excluded on the basis of our current data.

In contrast, although significant changes in the expression levels of cyclins A_2 and G_1 have been reported in Leydig cells during differentiation (32), and MIS has been recently identified as a putative regulator of Leydig cell number in rodents (40–42), in our experimental setting no effect of ghrelin was demonstrated on their mRNA levels. This may be due to the fact that our pharmacological approach of i.t. injection only allowed us to conduct short-term experiments. In addition, the ubiquitous expression of cyclins in different cell types of the testis may have masked a potential selective effect of ghrelin on Leydig cell expression of cyclin A_2 and G_1 mRNAs, a possibility that needs additional analysis using isolated Leydig cells. Similarly, the effects of ghrelin on the testicular expression of other putative regulators of Leydig cell development, such as IGF-I and TGF-α (16, 43), have not been explored to date and are presently under investigation in our laboratory. Finally, the possibility exists that ghrelin may regulate not only cell proliferation, but also apoptosis of Leydig cells, because SCF has been reported to modulate apoptosis in this cell population (23, 24).

On the basis of our initial data on the ability of ghrelin to modulate SCF gene expression in testes from EDS-treated rats, this phenomenon was evaluated in additional experimental settings. As was the case on d 21 after EDS, ghrelin was able to inhibit SCF mRNA levels in testes from intact rats *in vivo* and evoked a significant decrease in SCF mRNA expression in staged seminiferous tubules preparations *in vitro*. Notably, in these settings, no effects of ghrelin were detected on the expression levels of other Sertoli cell products, such as inhibin-α and inhibin-βB mRNAs (our unpublished observation), suggesting specificity for its action on SCF mRNA levels. Yet the signaling systems for the inhibitory effect of ghrelin on testicular SCF gene expression remain unexplored. Interestingly, however, such an effect *in vivo* appeared to be dependent on the proper input of pituitary FSH. To note, FSH receptors are solely expressed in Sertoli cells, and this gonadotropin is the major regulator of testicular SCF expression (44). Thus, it is apparent that a proper cross-talk between FSH and ghrelin intracellular mediators in Sertoli cells is needed for expression of the inhibitory effects of ghrelin on SCF gene expression. To our knowledge, this is the first report to show a modulatory role of ghrelin on the expression of SCF, which is essential for hemopoiesis, melanogenesis, and spermatogenesis (45). It is of note that in addition to Leydig cell development and survival (23), SCF has been pointed out as the major paracrine stimulator of germ cell development, acting as a survival factor for spermatogonia, spermatocytes, and spermatids in the adult rat seminiferous epithelium (46, 47). Thus, the action of ghrelin on tubular SCF mRNA expression may have implications not only for Leydig cell proliferation, but also in the control of spermatogenesis, a possibility that is presently under investigation in our laboratory. Moreover, besides implications for testicular function, our current data raise the possibility that ghrelin may participate in additional biological systems through modulation of SCF gene expression.

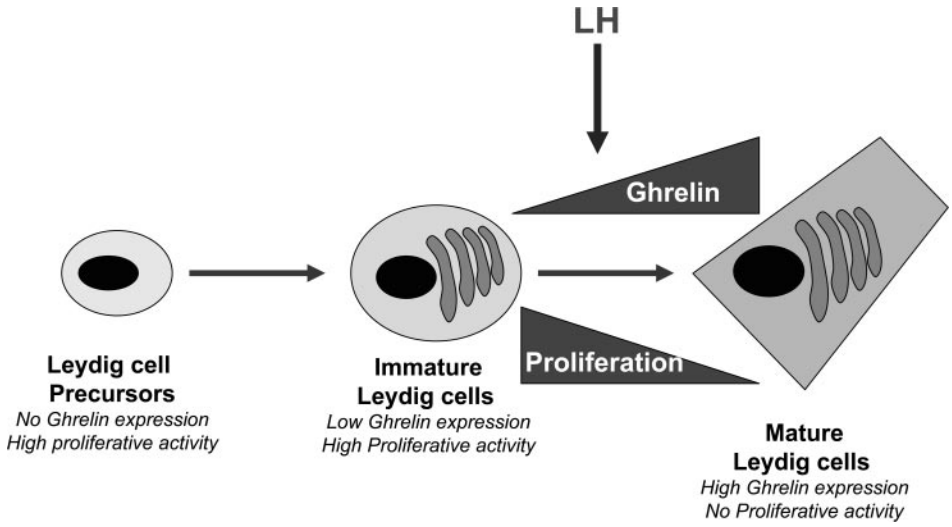

FIG. 7. Proposed model for the role of ghrelin in the local control of Leydig cell proliferation. Ghrelin expression in rat Leydig cells is dependent on their degree of differentiation, with increasing expression along differentiation and maximum levels in fully mature Leydig cells (26). The proliferative rate of immature Leydig cells (both at puberty and on d 21 after EDS) is sensitive to the inhibitory effects of exogenous ghrelin, a phenomenon that may involve direct as well as indirect (*e.g.* SCF mediated) actions. Acquisition of ghrelin expression by Leydig cell precursors during differentiation might thus operate as a self-regulatory signal for inhibition of the proliferative activity of this cell type.

Interestingly, a very recent study reported on the ability of ghrelin to inhibit the development of mouse preimplantation embryos (48), and it is noticeable that SCF had been previously found to be involved in mouse blastocyst implantation (49). Whether these two phenomena are functionally related merits additional investigation. In addition, coexpression of ghrelin and SCF has been demonstrated in other systems, such as placenta (50, 51). The possibility that ghrelin might regulate SCF gene expression in those systems remains to be elucidated.

In conclusion, we have provided evidence for an inhibitory role of ghrelin in the control of immature Leydig cell proliferation both during puberty and after EDS administration. This effect was associated with significant changes in the mRNA levels of SCF, a putative regulator of Leydig cell development. Considering that ghrelin expression in Leydig cells appeared critically dependent on their differentiation state, with low to undetectable levels in immature Leydig cell precursors and increased expression in fully differentiated adult-type Leydig cells (26), it is proposed that acquisition of ghrelin expression by Leydig cell precursors during differentiation may operate as a self-regulatory signal for the inhibition of the proliferative activity of this cell type through direct or indirect (SCF mediated) mechanisms (see Fig. 7). Moreover, considering that pituitary LH is the major elicitor of Leydig cell-specific expression of ghrelin (26), it is suggested that locally produced ghrelin might contribute not only to the mitotic arrest of fully mature, adult-type Leydig cells, but also to the autolimitation of the proliferative actions of LH on this cell population. In addition, we present novel evidence for the ability of ghrelin to modulate the expression of the SCF gene, which may have implications for the mode of action of this molecule in the testis as well as in other physiological systems.

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M.L.B. and F.G. contributed equally to this work and should be considered as joint first authors.

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