

Hepatocyte Growth Factor-Modulated Rat Leydig Cell Functions

JESSICA DEL BRAVO,* ANGELA CATIZONE,* GIULIA RICCI,† AND MICHELA GALDIERI†

From the *Department of Histology and Medical Embryology, School of Medicine, "Sapienza" University of Rome, Rome, Italy; and †Department of Experimental Medicine, Histology and Embryology Laboratory, School of Medicine, Second University of Naples and National Institute of Biostructures and Biosystems Interuniversity Consortium—Unit of Sections of Naples, Naples, Italy.

ABSTRACT: Hepatocyte growth factor (HGF) regulates many cellular functions acting through c-Met, its specific tyrosine kinase receptor. We previously reported that in prepuberal rats HGF is secreted by the peritubular myoid cells during the entire postnatal testicular development and by the Sertoli cells only at puberty. We have also demonstrated that germ cells at different stages of development express c-Met and that HGF modulates germ cell proliferation and apoptosis. In the present article, we extend our study to the interstitial compartment of the testis and demonstrate that the c-Met protein is present on Leydig cells. The receptor is functionally active as demonstrated by the detected effects of HGF. We report in this article that HGF significantly increases the amount of testosterone secreted by the Leydig cells and decreases the

number of Leydig cells undergoing apoptosis. The antiapoptotic effect of HGF is mediated by caspase-3 activity because the amount of the active fragment of the enzyme is decreased in Leydig cells cultured in the presence of HGF. However, treatment with the growth factor does not modify the expression levels of caspase-3 mRNA. These data indicate that HGF regulates the functional activities of Leydig cells. Interestingly, the steroidogenic activity of the cells is increased by HGF in cultured explants of testicular tissues as well as the antiapoptotic effect of HGF. Therefore, our data indicate that HGF has a crucial role in the regulation of male fertility.

Key words: *c-met*, HGF, testosterone, caspase-3, apoptosis, testis.

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Hepatocyte growth factor (HGF) is a cytokine with proliferative and motogenic capacities that is able to regulate the functional activities of many different cellular types (Zarnegar and Michalopoulos, 1995; Matsumoto and Nakamura, 1996; Lail-Trecker et al, 1998). The multiple actions of HGF are mediated by its receptor, c-Met, a transmembrane glycoprotein with tyrosine kinase activity, encoded by the *MET* proto-oncogene (Weidner et al, 1993; Hartmann et al, 1994). As we previously demonstrated, the HGF receptor is expressed in the postnatal rat testis and is detectable in the interstitial tissue and peritubular myoid cells of the seminiferous tubules (Catizone et al, 1999, 2001). In Sertoli cells, c-Met expression is developmentally regulated, first detectable in cells isolated from puberal animals, and HGF is involved in postnatal testis development and function (Catizone et al, 2005). We also reported that in rats, as in humans (Depuydt et al,

1996; Herness and Naz, 1999), c-Met is localized on testicular and epididymal spermatozoa, and we have shown that HGF positively influences sperm motility (Catizone et al, 2002). More recently we demonstrated that mitotic and meiotic rat germ cells express c-Met and that HGF regulates proliferation and apoptosis of prepuberal rat germ cells (Catizone et al, 2006). HGF is also expressed during embryonic development (Sonnenberg et al, 1993), and we previously demonstrated that in the embryonic mouse testis HGF induces testicular cell proliferation and acts as a morphogenetic factor (Ricci et al, 1999, 2002, 2004).

Leydig cells, the testosterone-producing cells of the mammalian testis, are differentiated cells that rarely proliferate in the adult (De Kretser and Kerr, 1994; Saez, 1994). During postnatal development of the testis, Leydig cells undergo a series of morphologic and functional transformations and act as proliferating precursors in prepuberal rats and as immature and mature adult Leydig cells in puberal rats (Ariyaratne and Mendis-Handagama, 2000; Haider, 2004). In both stages, the cells are able to produce testosterone but synthesize different levels of the hormone (Haider, 2004). Leydig cell proliferation and endocrine functions are regulated by several hormones, including interleukins (Svechnikov et al, 2001; Walch and Morris, 2002), transforming growth factor β (Khan et al, 1992; Dickson et al, 2002), insulin-like factors (Khan et al,

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Correspondence to: Michela Galdieri, Dip. Istologia ed Embriologia Medica, Via A. Scarpa 14, Roma 00161, Italy (e-mail: michela.galdieri@uniroma1.it).

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1992; Ge and Hardy, 1997), and ghrelin (Barreiro et al, 2004). It is also known that Leydig cells enter the programmed cell death pathway in particular situations such as in response to ethylene dimethanesulfonate (EDS) (Kerr et al, 1985; Morris et al, 1997; Kim et al, 2000) or glucocorticoids (Gao et al, 2002), and the regulation of apoptosis could play an important role in the maintenance of the proper number of Leydig cells. We recently demonstrated that in the embryonic testes HGF regulates testosterone production in fetal Leydig cells (Ricci et al, 2006). Interestingly, HGF increases the amount of testosterone secreted in the culture medium of *in vitro* cultures of testes isolated from 18.5-day embryos but does not modulate the amount of testosterone secreted by testes isolated from 15.5-day embryos (Ricci et al, 2006).

In this article, we report that c-Met is expressed by rat Leydig cells isolated from puberal rats, c-Met protein is present on the cells, and the receptor is functionally active. We demonstrate that HGF modifies several metabolic activities of these cells, including their steroidogenic activity.

Materials and Methods

Animals

Wistar rats were housed at the University of Rome "La Sapienza." All animal studies were conducted in accordance with the principles of the University of Rome "La Sapienza" Committee for Animal Welfare and the procedures outlined in the NIH Guide for Care and Use of Laboratory Animals. Rats were killed by CO₂ asphyxia before testes removal. Usually four 30- to 32-day-old rats of 100 to 120 g body weight were used for each experiment.

Leydig Cell Isolation and Culture

Leydig cells were isolated as previously reported (Morris et al, 1997) with slight modifications. Briefly, decapsulated testes were incubated with minimum essential medium (MEM; Invitrogen srl, Milan, Italy) containing 0.25 mg/mL collagenase or trypsin (0.18%) at 32°C in a shaking water bath (90 cycles/min) for 15 minutes. After dissociation, the enzyme was diluted with culture medium, and the seminiferous tubules were removed by gravity sedimentation (4 minutes). Tubules were washed again to detach the interstitia, and the two supernatants were collected and centrifuged at 300 × *g* for 10 minutes at room temperature. The pellet was resuspended in MEM containing 0.1% bovine serum albumin (BSA) and 0.01% DNase, and the cell suspension was loaded on a discontinuous Percoll gradient (20%–86% Percoll) and centrifuged at 800 × *g* for 20 minutes at 18°C. After centrifugation, fractions at 1.056 and 1.068 g/mL were collected, washed with buffer, and counted. Isolated Leydig cells were resuspended in MEM culture medium containing 15 mM HEPES, nonessential amino acids, 5 μg/mL gentami-

cin, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were seeded on Falcon culture plates (Becton, Dickinson and Co, Lincoln Park, NJ) at a concentration of 0.5 to 0.7 × 10⁶ cells/mL of medium. Viability of Leydig cells was assessed by the trypan blue dye exclusion method. Briefly, isolated Leydig cells were mixed with an equal volume of 0.4% trypan blue (Flow Laboratories, Irvine, United Kingdom), incubated for 5 minutes at 37°C, and examined under a microscope. After 24 hours of incubation, Leydig cells were almost totally viable. To assess the effects of HGF *in vitro*, cells were cultured for 26 hours at 32°C in a humidified 5% CO₂–95% air atmosphere in the presence of the growth factor (100 U/mL–30 ng/mL) for the last 24 hours of culture.

Evaluation of Isolated Leydig Cell Purity

Purity of the Leydig cell preparation was routinely assessed on the basis of positive staining of cells for the enzyme 3β-hydroxy steroid dehydrogenase (HSD) (Payne et al, 1980). Briefly, an aliquot of Leydig cell fraction was added to a tube containing 0.5 mL of β-NAD (9 mg/mL; Sigma-Aldrich, St Louis, Mo), 0.2 mL of dehydroepiandrosterone (1 mg/mL in methanol), and 0.25 mL of nitroblue tetrazolium (2 mg/mL in phosphate buffer [pH 7.4]; Sigma-Aldrich). The reaction mixture was allowed to stand for 1 hour at 37°C. The percentage of positively (dark blue) stained cells was examined under the microscope. The cell purity was consistently higher than 90%. The purity of our cell populations was also evaluated by immunocytochemistry for cytochrome P450 side-chain cleavage (P450_{scc}). The cells were paraformaldehyde fixed, washed extensively with phosphate-buffered saline (PBS) supplemented with 1% BSA and 0.2% Triton X-100, and incubated for 30 minutes in PBS containing 10% goat serum. Cells were then incubated with a polyclonal antibody against P450_{scc} (AB1294; 1:200 dilution; Chemicon International, Temecula, Calif) for 16 hours at 4°C. At the end of the incubation period, the cells were washed extensively with PBS and incubated for 45 minutes at room temperature with a fluorescein isothiocyanate-conjugated goat anti-rabbit anti-serum (Sigma-Aldrich). Cells were rinsed again with PBS and mounted in buffered glycerol (pH 9). As a negative control, the primary antibody was omitted and substituted with rabbit IgG.

Organ Culture

Fragments of approximately 1 mm³ were isolated from 8 testes of 30- to 32-day-old rats and placed on steel grids previously coated with 2% agar. Grids were then placed in organ culture dishes (Becton, Dickinson and Co) with 0.8 mL of medium necessary to wet the grid. The chemically defined medium is used in Leydig cell isolation and culture section. HGF (150 U/mL; Sigma-Aldrich) was added to the culture medium when indicated. Samples were cultured for 24 hours at 32°C in a humidified atmosphere of 5% CO₂ in air. After culture, the samples were washed twice in PBS, fixed overnight in Bouin fixative, dehydrated, embedded in paraffin, sectioned at a thickness of 7 μm, and used in the *in situ* terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assay. At least three experiments in triplicate were performed, and the number of TUNEL-positive interstitial

cells/500 transverse tubules was evaluated. The morphology of the samples appeared well preserved in all of the experiments.

Immunocytochemistry

Leydig cells prepared as indicated above were fixed in methanol according to the antibody manufacturer's recommendation for 10 minutes at -20°C , treated with 5% BSA (Sigma-Aldrich) for 30 minutes at room temperature to minimize nonspecific binding, and then exposed to a polyclonal antibody against the carboxy terminus of c-Met (sc-162; 1:50 dilution; Santa Cruz Biotechnology, Santa Cruz, Calif) for 16 hours at 4°C . At the end of the incubation period, the cells were washed extensively with PBS and incubated for 45 minutes at room temperature with a fluorescein isothiocyanate-conjugated goat anti-rabbit antiserum (Sigma-Aldrich). The cells were rinsed again with PBS and mounted in buffered glycerol (pH 9). As a negative control, the primary antibody was omitted and substituted with rabbit IgG or 10-fold excess by weight of a blocking peptide (sc-162P; Santa Cruz Biotechnology). Samples were analyzed using a Zeiss Axioplan fluorescence microscope (Oberkochen, Germany).

RNA Isolation and Reverse Transcription Polymerase Chain Reaction Analysis

Total RNA was extracted from cultured cells using a Perfect RNA Eukaryotic Mini kit (Eppendorf, Hamburg, Germany). The purity of isolated mRNA was evaluated spectrophotometrically using the $A_{260/280}$ ratio. To reduce contamination by genomic DNA, total RNA was treated with ribonuclease-free DNase I for 15 minutes as recommended by the manufacturer (Invitrogen srl). Samples of total RNA (200 ng) were reverse transcribed with reverse transcriptase (Invitrogen srl) in the presence of oligo(dT) primers (Invitrogen srl) for 50 minutes at 37°C , and the reaction was terminated by heating for 15 minutes at 70°C . Polymerase chain reaction (PCR) was performed using HotMaster Taq DNA polymerase (Eppendorf) and the following primers: *c-met* sense 5'-AATGTGTCAGGAGGTGTTTGG-3' and antisense 5'-GAATAATCGGGAGGGTAGGAAG-3', *S-16* sense 5'-TCCAAGGGTCCGCTGCAGTC-3' and antisense 5'-CGTTCACCTTGATGAGCCCAT-3'. The amplification program for *c-met* consisted of one denaturing cycle at 94°C for 5 minutes, followed by the following steps: 35 cycles of amplification defined by denaturation at 94°C for 45 seconds, annealing at 57°C for 45 seconds, and extension at 72°C for 1 minute. The final incubation was performed at 72°C for 5 minutes. The amplification program for *S-16* was similar with the exceptions of the number of cycles of amplification (30) and annealing temperature (60°C). Negative controls contained water instead of cDNA. PCR with no reverse transcriptase produced no product, eliminating the possibility of genomic DNA contamination in the RNA preparations. PCR products were separated by 2% agarose gel electrophoresis, visualized by ethidium bromide staining, and quantitated by computer analysis. Reverse transcription PCR (RT-PCR) analysis was conducted with primers for *S-16* to be sure that equal amounts of cDNA were used. A DNA ladder was included in the gels to determine the sizes of the PCR products.

Testosterone Evaluation

Leydig cells were cultured for 24 hours in the presence of different doses of HGF (25–300 U/mL), and the culture medium was tested for testosterone levels by radioimmunoassay. The Access immunoassay system (Beckman Coulter Inc, Fullerton, Calif) was used for the testosterone determination (Wilson and Foster, 1992).

TUNEL Assay

Leydig cells were cultured for 24 hours in the presence of different doses of HGF (25–300 U/mL). Apoptotic cells were detected by the TUNEL method using the ApopTag Peroxidase kit (Q-BIOgene, Irvine, Calif). As positive controls, Leydig cells or testicular sections were treated with DNase I. We omitted the terminal deoxynucleotidyl transferase enzyme in the reaction mixture for negative controls. The samples were counterstained with hemalum and analyzed using a Zeiss Axioscope microscope. Four experiments in duplicate were performed, and the number of TUNEL-positive cells/3000 cells was evaluated.

Protein Extraction and Western Blot Analysis

Freshly isolated Leydig cells were lysed with ice-cold PBS (pH 7.4) containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail (1:100 dilution; Sigma-Aldrich). Lysates were sonicated, and the protein content was determined by a bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, Ill). Equal amounts of protein (40 μg) were resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes. After the membranes were blocked, they were incubated for 16 hours at 4°C with 0.2 mg/mL rabbit polyclonal anti-human caspase-3 antibody that recognizes full-length caspase-3 and proteolytic fragments (Upstate Biotechnology, Lake Placid, NY) and then incubated for 60 minutes at room temperature with a horseradish peroxidase-conjugated secondary antibody (1:7000 dilution; Amersham Biosciences UK Ltd, Buckinghamshire, United Kingdom). Peroxidase activity was visualized with the SuperSignal West Pico Trial kit (Pierce Biotechnology) according to the manufacturer's instructions. The caspase-3 protein content was determined densitometrically. The nitrocellulose membranes were also probed with an anti-tubulin monoclonal antibody (Sigma-Aldrich).

Statistical Analysis

All experimental data were expressed as $\bar{X} \pm \text{SE}$ of at least 3 separate experiments. Statistical analysis was performed by Student's *t*-test. Differences were considered significant at $P < .05$. Analysis of variance followed by Duncan's test for multigroup comparison was also employed to evaluate the significance of differences.

Results

C-Met Expression in Purified Leydig Cells

Leydig cells were isolated from 30- to 32-day-old rats as indicated in the "Materials and Methods" section, and

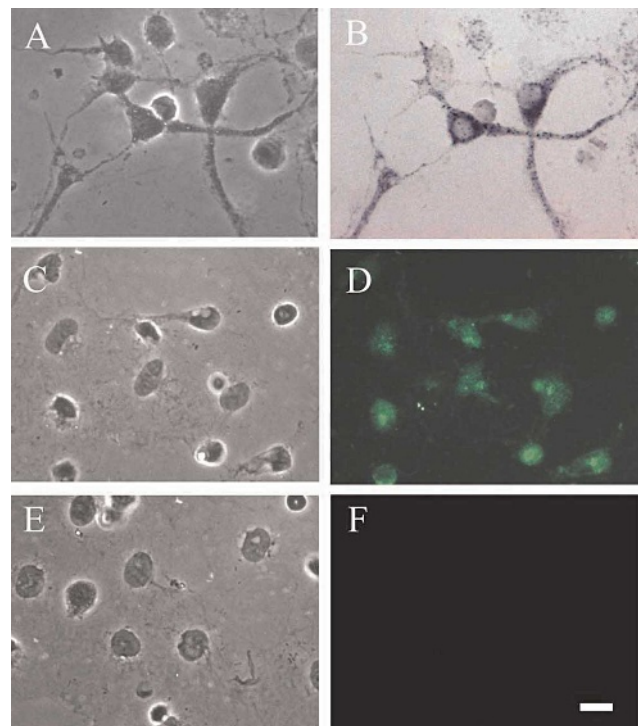


Figure 1. Purity of Leydig cell preparations. Phase-contrast microscopy (A and C) and cell positivity for the enzyme 3 β -hydroxy steroid dehydrogenase (B) and cytochrome P450 side-chain cleavage (P450scc; D). Negative control for P450scc (E and F). Scale bar = 10 μ m. Color figure available online at <http://www.andrologyjournal.org>.

the purity of the cell population obtained was checked by evaluating positivity either for the enzyme 3 β -HSD (Figure 1A and B) or P450scc (Figures 1C through F). Both techniques indicated that our preparations routinely contained more than 90% Leydig cells with peaks of purity at 96%. The presence of c-Met protein was detected by immunolocalization in the purified populations of Leydig cells; Figure 2A presents the phase-contrast microscopy of the purified cells, and Figure 2B shows their positivity for c-Met. C-Met protein is detectable by indirect immunofluorescence of the cells and shows a spotted distribution. In Figure 2D, the negative control using a blocking peptide is presented; Figure 2C shows phase-contrast microscopy of the Leydig cells used as a negative control. All of the negative controls were invariably negative using either isotype serum or the blocking peptide. To evaluate the expression of *c-met* mRNA in Leydig cells, total RNA was extracted from noncultured cells and cells cultured for 24 or 72 hours. RT-PCR analysis was performed as indicated in the "Methods and Materials" section, and RNA extracted from livers of the same rats used for Leydig cell isolation was used as a positive control. We found that Leydig cells express *c-met* mRNA and that

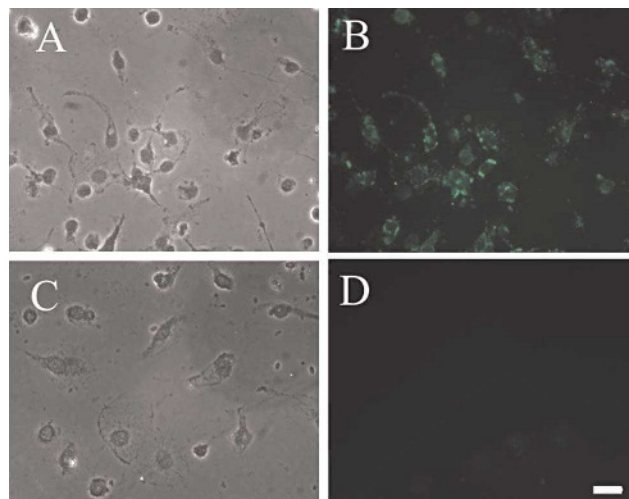


Figure 2. c-Met distribution on rat Leydig cells. (A) Phase-contrast microscopy and (B) indirect immunofluorescence of c-Met on the same cells. Relative phase-contrast microscopy (C) and negative control (D). Scale bar = 20 μ m. Color figure available online at <http://www.andrologyjournal.org>.

the expression is not modified by the incubation time (Figure 3A).

HGF Effects on Leydig Cells

c-met Expression—To evaluate the effect of HGF on *c-met* mRNA expression levels, purified cell preparations of Leydig cells were cultured for 24 hours in control medium or in medium supplemented with HGF (100 U/mL) and total RNA was extracted. RT-PCR was performed, and RNA was extracted from the livers of the same rats used for Leydig cell isolation to be used as positive controls. We found that *c-met* mRNA levels were not changed after incubation with HGF (Figure 3B).

Testosterone Production—The potential functional role of HGF signaling in the control of testicular function was explored. To this end, Leydig cells were cultured for 24 hours and the culture media of cells cultured in medium alone or supplemented with HGF were used to evaluate the amount of testosterone secreted. The media were collected and testosterone levels determined by radioimmunoassay; the relative amounts of testosterone secreted in the 7 different experiments are reported in Figure 4A. The results indicated that the amounts of testosterone secreted by the cells cultured in the presence of HGF (100 U/mL) are significantly higher with respect to the control samples. We also present the dose-response curve of testosterone produced in the presence of different doses of HGF (25–300 units/mL) (Figure 4B). The amount of testosterone produced was significantly higher at doses of HGF ranging from 50 to 300 U/mL, whereas the dose

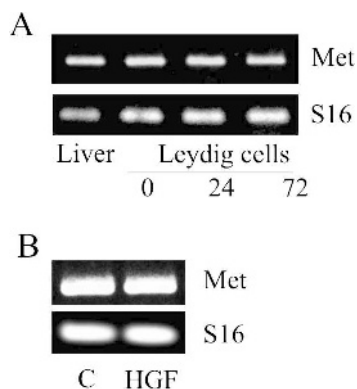


Figure 3. *c-Met* expression in rat Leydig cells. (A) Reverse transcription polymerase chain reaction (RT-PCR) analysis of *c-met* expression in uncultured cells (0) and in cells cultured for 24 hours and 72 hours. RNA extracted from liver was used as a positive control. (B) *c-met* expression in cells cultured for 24 hours in control medium (C) and in medium supplemented with hepatocyte growth factor (HGF). RT-PCR analysis was also conducted using primers for *S-16*.

of 25 U/mL did not produce a significant increase in hormone production.

The effect of HGF on testosterone production was also studied by culturing small explants of testicular tissue prepared as described above (Figure 4C). The explants cultured for 24 hours in medium supplemented with HGF produced significantly higher amounts of testosterone with respect to the control explants.

Apoptosis—Four separate experiments were performed to investigate the role of HGF on Leydig cell apoptosis. Leydig cells were cultured for 24 hours in medium alone or medium supplemented with HGF (100 U/mL). In Figure 5A, the morphologic appearances of the control (C) and HGF-treated Leydig cells (HGF) are shown. Leydig cells undergoing apoptosis are evident for the brown, morphologically abnormal nuclei (arrows). The number of apoptotic cells was not high; however, by counting the apoptotic cells under both culture conditions, we detected an antiapoptotic effect of HGF. As shown in Figure 5B, the number of apoptotic cells was significantly lower when Leydig cells were cultured in the presence of HGF. Different doses of HGF (25–300 units/mL) were also used to evaluate the antiapoptotic effect of HGF (Figure 5C), and the results obtained demonstrate that HGF significantly reduces the number of apoptotic cells starting from the dose of 50 U/mL. Apoptosis of Leydig cells was also evaluated by culturing small explants of testicular tissue. Testicular samples were incubated for 24 hours in fresh medium or medium containing HGF (150 U/mL), and Figure 6A shows the morphology of the cultured tissues. After culture, the number of apoptotic cells present in the interstitial tissue, defined by 500 transverse seminiferous tubules, was evaluated in both samples. As shown

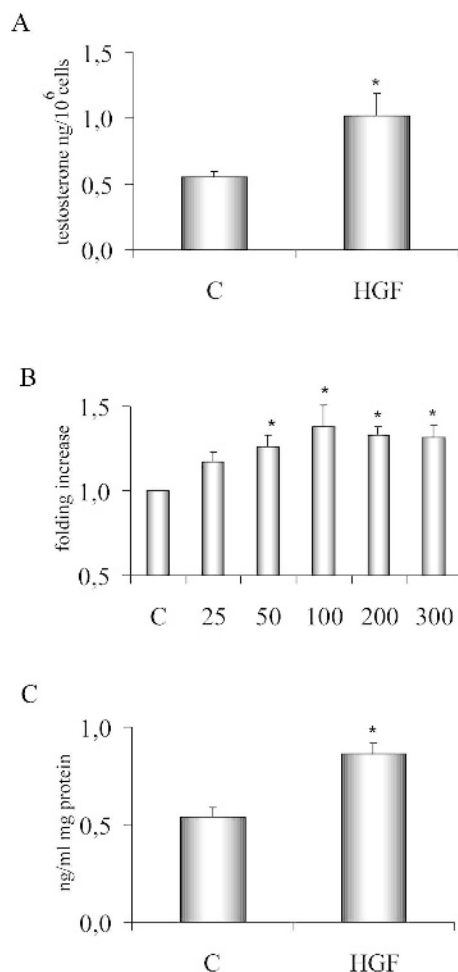


Figure 4. Testosterone production in Leydig cells. (A) Amount of testosterone secreted by the cells cultured in the absence (C) or presence of hepatocyte growth factor (HGF). The $\bar{X} \pm \text{SE}$ of 7 experiments is reported. (*) vs C, $P < .05$. (B) Dose-response curve of testosterone production. The amount of testosterone secreted by control cells is arbitrarily considered as "1." The values of the samples treated with HGF are reported as percentages with respect to the control values. (*) vs C, $P < .05$. (C) Testosterone produced by testicular fragments cultured for 24 hours in control (C) and HGF-supplemented medium (HGF). (*) vs C, $P < .05$.

in Figure 6B, the number of apoptotic cells is strongly reduced in the presence of the growth factor.

Caspase-3 Expression—The expression of caspase-3 mRNA in Leydig cells cultured in control medium and in medium supplemented with HGF (100 U/mL) was evaluated by RT-PCR (Figure 7A), and the results indicated that gene expression is not modified by the HGF treatment. On the contrary, Western blot analysis of the proteins extracted from control and HGF-treated Leydig cells (Figure 7B) showed that HGF significantly reduced the amount of the 17-kD active fragments as shown in Figure 7C. A statistically significant variation in the inactive form of the enzyme was not detected as reported in Figure 7D, which summarizes the amount of

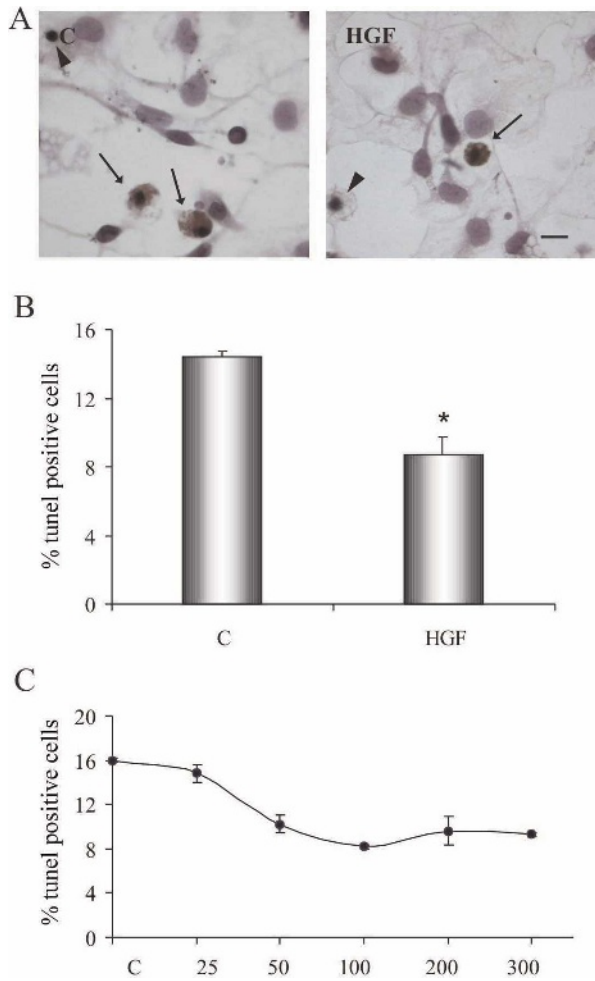


Figure 5. (A) Photomicrographs of Leydig cells cultured for 24 hours in control medium (C) and hepatocyte growth factor (HGF)-supplemented medium (HGF). Arrows indicate the apoptotic cells. Arrowheads indicate pyknotic cells. Scale bar = 10 μ m. (B) Percentage of in situ terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling-positive Leydig cells cultured for 24 hours in control medium (C) and HGF-supplemented medium (HGF). (* vs C, $P < .05$). (C) Apoptosis of Leydig cells cultured in the presence of different doses of HGF (25–300 U/mL). Color figure available online at <http://www.andrologyjournal.org>.

the inactive and active form of caspase-3 obtained in control and HGF-treated cells.

Discussion

The testis is a complex endocrine organ in which different cell types cooperate to ensure male fertility. A huge number of extragonadal and intragonadal hormones and growth factors have been implicated in recent years in the control of testicular function (Saez, 1994; Haider, 2004; Sriraman et al, 2005). We previously demonstrated that HGF is produced by the somatic cells of the testis and is one of the growth factors involved in

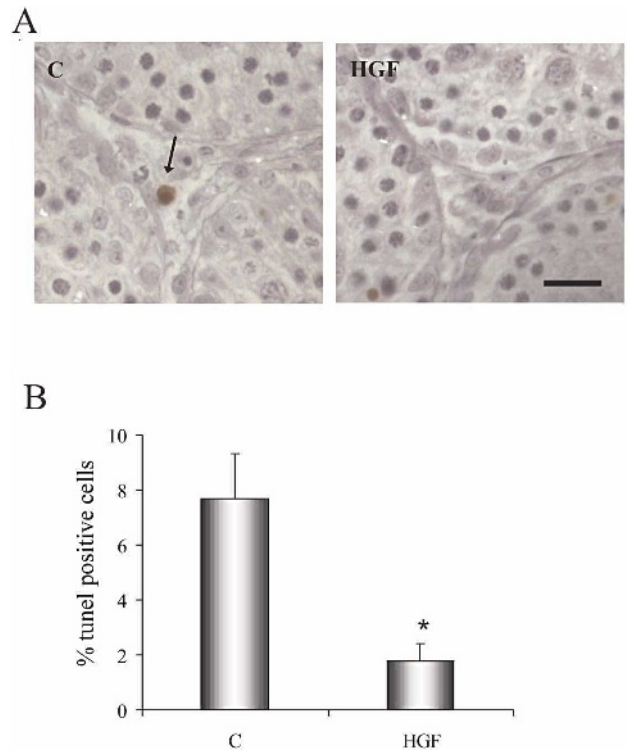


Figure 6. (A) Photomicrographs of histologic sections of rat testis fragments cultured for 24 hours in control (C) and hepatocyte growth factor (HGF)-supplemented medium (HGF). Scale bar = 20 μ m. (B) Percentage of in situ terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling-positive interstitial cells in testes cultured for 24 hours in control medium (C) and HGF-supplemented medium (HGF). (* vs C, $P < .05$). Color figure available online at <http://www.andrologyjournal.org>.

the control of male fertility. As we demonstrated, HGF influences sperm motility (Catizone et al, 2002) and exerts an autocrine role in the regulation of Sertoli cells and peritubular myoid cells (Catizone et al, 1999, 2001, 2005). Recently we demonstrated that HGF modulates survival and proliferation of germ cells during the first spermatogenetic wave of postnatal rat testis development (Catizone et al, 2006). To extend our previous studies, we investigated the presence of the HGF receptor in Leydig cells and the eventual role of HGF on the regulation of the functional parameters of these cells. In pubertal rats, immature and mature Leydig cells are present in the interstitial compartment of the testis, and we found that all of these cells express the HGF receptor, c-Met, without regard to their stage of maturation. We established the presence of the HGF receptor on Leydig cells and investigated the role of HGF on the regulation of the functional activities of these cells. It is well known that Leydig cells are the steroidogenic cells of the testis and that testosterone secretion is regulated by a plethora of endocrine and paracrine signals (Saez, 1994; Sriraman et al, 2005).

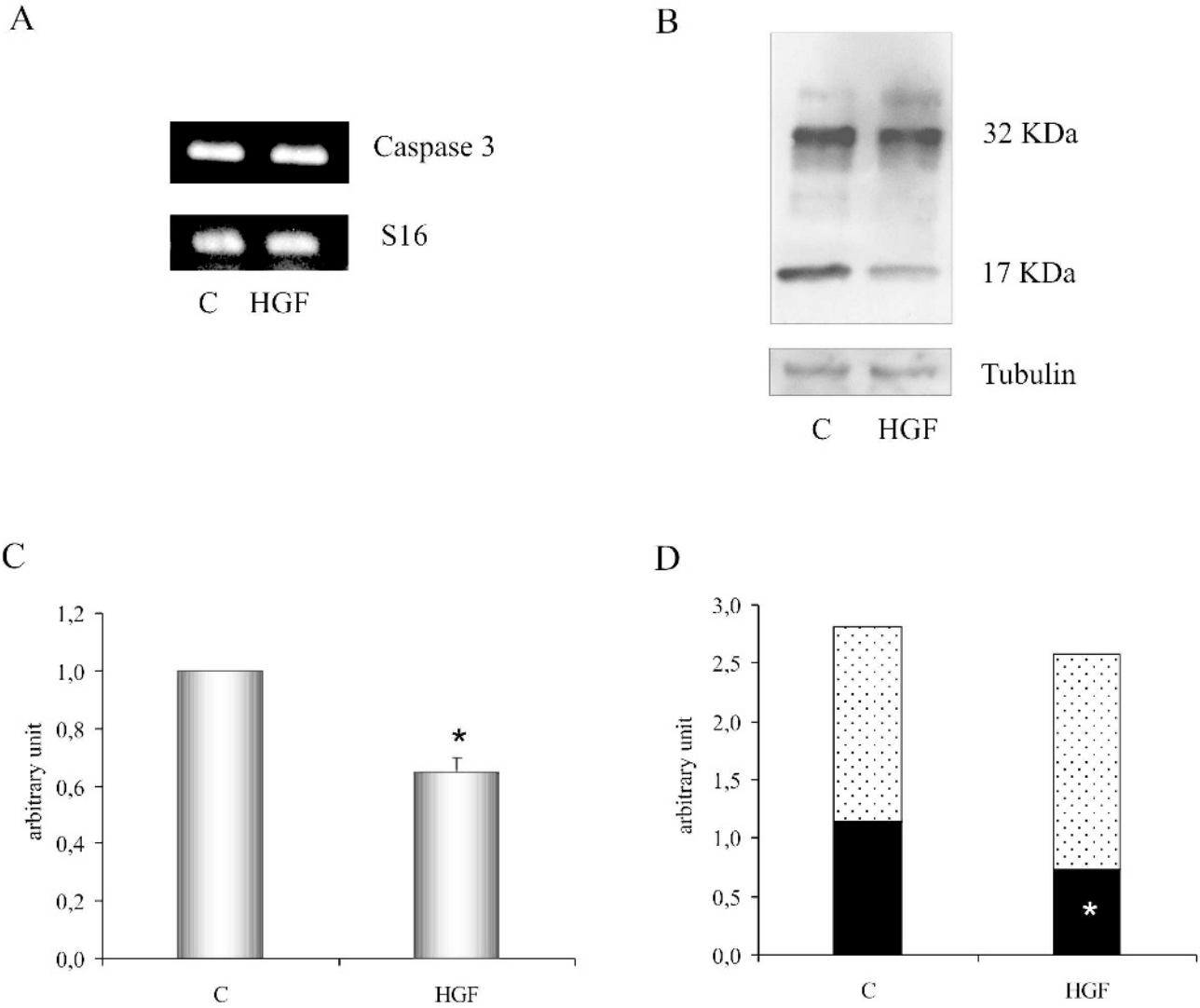


Figure 7. (A) Reverse transcription polymerase chain reaction analysis of caspase-3 mRNA expression in Leydig cells cultured for 24 hours in control medium (C) and medium supplemented with hepatocyte growth factor (HGF). (B) Western blot analysis of proteins extracted from control and HGF-treated Leydig cells. Proteins were detected with an anti-caspase-3 polyclonal antibody and anti-tubulin monoclonal antibody. (C) Densitometric scanning of the active fragment (17 kd) of caspase-3. The values of the samples treated with HGF are reported as percentages with respect to the control values arbitrarily considered as "1." (D) Values of the densitometric scanning of caspase-3 active fragment (dark columns) and precursor (dotted columns) in control and HGF-treated cells. In C and D, the $\bar{X} \pm SE$ of the 3 experiments performed is reported and (*) vs C, $P < .05$.

Therefore, we first investigated the effect of HGF on this relevant function of Leydig cells and then demonstrated that HGF acts on Leydig cells, increasing their ability to secrete testosterone. Interestingly, an increase in testosterone production is also obtained by culturing small explants of testicular tissue so that Leydig cells are in a more physiologic condition in which the relationships between the testicular cells are maintained. In our opinion, these combined data indicate that HGF also influences testosterone secretion in vivo in intact animals.

Considering the relevance of the apoptotic process in the regulation of the number of Leydig cells, we then

evaluated the role of HGF in this process and our experiments clearly demonstrated that HGF prevents apoptosis of cultured Leydig cells. Culturing explants of testicular tissue, we also found that HGF strongly reduces the number of apoptotic interstitial cells. We cannot ascribe the effect exclusively to Leydig cells; however, the finding that testosterone is highly secreted under this culture condition allows us to conclude that, besides other interstitial cells, Leydig cells are protected against apoptosis by HGF. Caspases are enzymes that play a critical role in the execution of apoptosis in a number of different cell types (Villa et al, 1997). Most of them are synthesized as inactive proenzymes that are

processed to active forms in cells undergoing apoptosis (Nuñez et al, 1998). Among the caspases, caspase-3, one of the effector caspases, appears to be a key protease in the apoptotic pathway (Porter and Janicke, 1999): activated caspase-3 targets DNA fragmentation factor, which is integrally involved in degrading DNA (Liu et al, 1997; Nagata, 1997). It has been reported that caspase-3 is associated with testicular germ cell apoptosis (Kim et al, 2001) and with Leydig cell apoptosis induced by EDS (Kim et al, 2000). Therefore, we studied the effect of HGF on caspase-3 gene expression, and we found that treatment with the growth factor does not modify the expression levels of caspase-3 mRNA. On the contrary, HGF appears to affect the activation of the enzyme because we found by Western blot that the amount of the active fragment of the enzyme was significantly reduced when Leydig cells were cultured in the presence of HGF. We reported that in the rat testis peritubular myoid cells secrete HGF (Catizone et al, 1999, 2001, 2005); therefore, myoid cells could be the source of the factor. However, it is unknown now which of the cells of the interstitial compartment secrete HGF. Further studies are necessary to clarify this point.

In conclusion, we demonstrated that rat Leydig cells express the receptor of HGF, c-Met, and that the receptor is functionally active because HGF influences the steroidogenic pathway and significantly increases the amount of testosterone secreted by Leydig cells. Moreover, HGF decreases the number of Leydig cells undergoing apoptosis, and the antiapoptotic effect of HGF is mediated by caspase-3 activity because amounts of the active fragment of the enzyme are decreased in Leydig cells cultured in the presence of HGF. On the contrary, treatment with the growth factor does not modify the expression levels of *c-met* and caspase-3 mRNA. All of these data indicate that HGF regulates the functional activities of Leydig cells, the steroidogenic cells of the interstitial compartment of the testis.

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