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Hepatocyte Growth Factor Is a Mouse Fetal Leydig Cell Terminal Differentiation Factor[1]

Giulia Ricci,[2,3] Maria Cristina Guglielmo,[4] Maria Caruso,[4] Francesca Ferranti,[4] Rita Canipari,[4] Michela Galdieri,[3] and Angela Catizone[4]

[3]*Department of Experimental Medicine, Histology and Embryology Laboratory, School of Medicine, Second University of Naples, Naples, Italy*
[4]*Department of Anatomy, Histology, Forensic Medicine and Orthopaedics, School of Medicine, "Sapienza" University of Rome, Rome, Italy*

ABSTRACT

The hepatocyte growth factor (HGF) is a pleiotropic cytokine and a well-known regulator of mouse embryonic organogenesis. In previous papers, we have shown the expression pattern of HGF and its receptor, C-MET, during the different stages of testis prenatal development. We demonstrated that C-MET is expressed in fetal Leydig cells (FLCs) and that HGF stimulates testosterone secretion in organ culture of late fetal testes. In the present study, we analyzed the proliferation rate, apoptotic index, and differentiation of FLCs in testicular organ culture of 17.5 days postcoitum (17.5 dpc) embryos to clarify the physiological role of HGF in late testis organogenesis. Based on our data, we conclude the following: 1) HGF acts as an antiapoptotic factor that is able to reduce the number of apoptotic FLCs and testicular caspase-3 active fragment; 2) HGF does not affect FLC proliferation; 3) HGF significantly increases expression of insulin-like 3 (INSL3), a marker of Leydig cell terminal differentiation, without affecting 3beta-hydroxysteroid dehydrogenase (3betaHSD) expression; 4) HGF significantly decreases the expression of nestin, a marker of Leydig cell progenitors; and 5) HGF significantly increases the number of fully developed FLCs. Taken together, these observations demonstrate that HGF is able to act in vitro as a survival and differentiation factor in FLC population.

differentiation, fetal testis, HGF, Leydig cells

INTRODUCTION

In mammals, the acquisition of male features is established during embryogenesis by androgen-producing fetal Leydig cells (FLCs), which are responsible for testosterone secretion in the prenatal testis. The embryonic origin of FLC precursors is not yet clear. Some evidence suggests that mouse FLC precursors arise from the coelomic epithelium [1–3], whereas another study suggests that Leydig progenitors migrate from the mesonephros into the gonad before 11.0 days postcoitum (dpc), and remain undifferentiated until 12.5 dpc [4]. More recently, DeFalco and colleagues [5] demonstrated that both coelomic epithelium and mesonephric mesenchyme could be considered potential sources of FLC progenitors. It is notable that mesenchymal Leydig stem cells present features that are typical of neural-ectodermal and mesodermal origins [6–11]. These observations gave rise to the hypothesis, which has not been definitively confirmed, that FLCs originate from neural crest migrating precursors [12]. Steroid-producing FLCs appear in the testis approximately 24 h after the differentiation of Sertoli cells [8, 9, 13], and their number declines shortly after birth [8]. The FLC population increases at least 2-fold before birth; however, no mitotic activity has been detected in differentiated FLCs [14–17]. The increase in FLC number has been attributed to the differentiation of a population of progenitor/stem cells that are located in the interstitial and peritubular compartments [10, 17, 18].

Rodent FLC differentiation and testosterone secretion are considered to be independent of pituitary hormones throughout gestation [11, 19–21] because fetal masculinization and increases in plasma androgens occur before the hypothalamic-pituitary-gonadal axis starts to be functional. Confirmation of this hypothesis was obtained using the LURKO mouse animal model. These mice lack the luteinizing hormone receptor and have normal androgen levels and testicular phenotypes at birth; however, these mice develop Leydig cell failure during postnatal Leydig cell development [22, 23].

An increasing amount of evidence suggests that the differentiation and activity of rodent FLCs are regulated by local growth factors, such as Desert hedgehog (DHH), Platelet-Derived Growth Factor (PDGF), Insulin-like Growth Factor-1 (IGF-1), and the transforming growth factor beta (TGF beta) superfamily [18, 21, 24–27], that are secreted by developing testicular cells. However, the network that controls the developmental stages from mesenchymal stem cells to terminally differentiated FLCs is still a topic of debate [8].

Two Sertoli cell-derived signaling molecules, DHH and PDGF, have been implicated in the differentiation of FLCs [9, 13, 28–32]. *Dhh* knockout embryos develop testes with fewer FLCs and abnormal testis cord organization [9, 33]. In male PDGF Receptor alfa (*Pdgfrα*) knockout embryos, Sertoli cell proliferation, mesonephric cell migration, and FLC differentiation are all impaired [3]. These data suggest that both Dhh signaling and PDGF signaling promote the expansion of the Leydig cell precursor population in the mouse fetal testis [3].

Other growth factors are suggested to be involved in Leydig cell differentiation, but they more likely act in the intermediate and terminal differentiation steps of FLC development. In vitro data showed that IGF-1 has a positive effect on rat FLC proliferation, differentiation, and function [34]. In the adult stage, *Igf-1* knockout mice have reduced Leydig cell numbers

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[2]Correspondence: Giulia Ricci, Histology and Embryology Section, Department of Experimental Medicine, Via Costantinopoli, 16, 80138 Napoli, Italy. E-mail: giulia.ricci@unina2.it

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and lower testosterone concentrations, which are partly mediated by an impairment and alteration in androgen biosynthesis and metabolism [35].

Fetal Leydig cells are direct targets for the TGF beta superfamily of growth factors because type I and type II TGF beta and activin receptors and the betaglycan coreceptor have been detected on FLCs in fetal rat and human testis [36–38]. The TGF beta 1, TGF beta 2, and TGF beta 3 isoforms are all produced by fetal rodent testis [36, 39, 40]. In vitro, TGF betas, activins, and inhibins have been reported to regulate Leydig cell number and steroidogenesis [39, 41], and these data indicate that this family of growth factors has a role in the establishment of testis endocrine function. Limited information is available on the actions of the TGF beta superfamily during fetal testis development because knockout mice generally exhibit embryonic or neonatal lethality [42]. TGF beta coreceptor betaglycan knockout mice show fetal testis alterations, such as changes in testis cord development, at the time of sex determination and compromised FLC function [27].

The hepatocyte growth factor (HGF) is a pleiotropic cytokine and a well-known regulator of mouse embryonic morphogenesis [43]. Moreover, the contribution of the C-MET/HGF system in the regulation of the physiology of postnatal testis and ovary is well known [44–49]. In previous papers, we have shown the expression pattern of HGF and its receptor, C-MET, during the different stages of testis prenatal development. We demonstrated that the C-MET/HGF system is able to regulate in vitro cord formation at the time of sex determination [50–52]. We also demonstrated that c-Met is expressed by FLCs in late testis embryonic development. HGF is localized in the connective tissue of the interstitial compartment, but it is not expressed by FLCs. In addition, our in vitro data showed that HGF stimulates testosterone secretion in organ culture of late fetal testis and in primary cell culture of rat pubertal Leydig cells [53, 54]. In the present study, the proliferation rate, apoptotic index, and differentiation of Leydig cells in a testicular organ culture of 17.5-dpc embryos were analyzed to clarify the physiological role of HGF in late testis organogenesis. The present study reports that HGF does not affect the proliferation rate of FLCs but does protect FLCs from apoptosis. Moreover, our ex vivo experiments provide evidence that HGF promotes FLC terminal development in testicular fetal organ culture.

MATERIALS AND METHODS

Animals

CD-1 mouse embryos were used for all experiments. Pregnant mice were housed at the DAHFMO facility of the ''Sapienza'' University of Rome with the approval of the Italian Ministero del Lavoro, della Salute e delle Politiche Sociali. All animal studies were conducted in accordance with the principles and procedures outlined in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, and mice were killed by CO_2 asphyxiation. For the determination of embryonic age, the morning after the vaginal plug formed was considered Day 0.5 of embryonic development.

Whole-Mount In Situ Hybridization

In situ hybridization was performed on fetal testes as previously described [50]. Briefly, fetal testes were fixed overnight by immersion in 4% paraformaldehyde (PFA) in PBS (pH 7.4) at 4°C.

Antisense and sense riboprobes were generated to the mouse *c-Met* cDNA and subcloned in Bluescript II Sk (kindly provided by Prof. Carola Ponzetto) by in vitro transcription using T3 and T7 RNA polymerases in the presence of digoxigenin-labeled UTP following the manufacturer's instructions (Dig RNA labeling kit; Roche). Detection of *c-Met* mRNA by whole-mount in situ hybridization was carried out according to Wilkinson and Nieto [55]. The hybrids bound to alkaline phosphatase-conjugated antidigoxigenin antibody were visualized by a colorimetric reaction mixture containing 1% Tween-20 and 2 mM Levamisole in BM purple Alkaline Phosphatase substrate (Roche). The samples were viewed and photographed using a Stereomicroscope (Nikon SMZ800).

Immunolocalization of c-Met and Insulin-Like 3 in Fetal Testis Serial Sections

Testes isolated from 17.5-dpc embryos were fixed overnight in Bouin solution. Samples were then dehydrated, embedded in paraffin, and sectioned at a thickness of 2.5 μm (serial sections). Sections were dewaxed, hydrated, and rinsed with PBS. Endogenous peroxidases were blocked by incubation with 3% hydrogen peroxide in PBS for 20 min at room temperature. Sections were incubated overnight with primary antibodies at 4°C. The primary antibodies used were against C-MET (rabbit polyclonal SP-260; 1:20 dilution; Santa Cruz Biotechnology) and against Insulin-like 3 (INSL3; rabbit polyclonal M-122 sc134587; 1:50 dilution; Santa Cruz Biotechnology). Immunolocalization experiments were performed according to the manufacturer's instructions (Histostain-Plus kit; Zymed Laboratories). The avidin-biotin immunoperoxidase system with 3,3-diaminobenzedine as chromogen was used to visualize bound antibodies. The preparations were counterstained with hemalum, dehydrated, mounted with Eukitt (FLUKA; Sigma-Aldrich), and analyzed using a Zeiss Axioscope.

In order to validate the anti-INSL3 specificity, INSL3 peptide was preincubated with the antibody. This preincubation prevented anti-INSL3 binding to our samples.

Negative controls were processed in the absence of the primary antibody and using the primary antibody preimmune isotype immunoglobulins: both negative controls did not give detectable background.

Organ Culture

Testes isolated from 17.5-dpc male embryos were cultured for 24 h on steel grids previously coated with 2% agar and placed in organ culture dishes (Falcon). The medium used contained Dulbecco modified Eagle medium (Sigma-Aldrich) supplemented with glutamine (2 mM), Hepes (15 mM), nonessential amino acids, penicillin (100 IU/ml), and streptomycin (100 mg/ml). Hepatocyte growth factor (100 U/ml; Sigma-Aldrich) was added to the culture medium where indicated, and it was administered for the entire period of culture. Testes were cultured at 37°C in a humidified atmosphere of 5% CO_2. After 24 h of culture, testes were directly processed for Western blot analysis, quantitative real-time PCR, or in situ caspase activity detection (see below). Alternatively, testicular samples were fixed overnight in 4% paraformaldehyde in PBS (pH 7.4) at 4°C and used for whole-mount immunofluorescence or terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) experiments.

Testosterone Assay

Testosterone secretion quantification was used to establish the most efficient dose of the factor in order to choose HGF concentration for all of the other experiments. Conditioned culture medium from 17.5-dpc testes, cultured for 24 h without (control) or with different doses of HGF (50–200 U/ml), was used for testosterone determination. To this aim, radioimmunoassay was performed using the Access immunoassay system commercialized by Beckman Coulter Inc. A total of 100 U/ml HGF was the most effective dose, and this concentration was chosen for all of the other experiments.

Whole-Mount Caspase Activity Assay

To detect activated caspases in situ in the living fetal testis, we used a CaspGLOW In Situ Caspase Staining Kit (Biovision). This assay uses caspase inhibitors that are conjugated to rhodamine as fluorescent in situ markers. The caspase inhibitors are cell permeable and nontoxic, and they irreversibly bind to activated caspases in apoptotic cells. Using these rhodamine labels, activated caspases in apoptotic cells can be visualized directly using fluorescence microscopy. After 24 h of culture, fetal testes were treated with RED-Z-VAD-FMK at 37°C for 45 min following the manufacturer's instructions. Samples were then fixed with PFA 4% and used for 3βHSD whole-mount immunofluorescence. Caspase inhibitors without rhodamine labels were used as negative controls.

Whole-Mount Immunofluorescence

Testicular organ cultures of 17.5-dpc embryos fixed overnight in 4% PFA were washed in PBS, permeabilized in PBS/1% bovine serum albumin (BSA)/0.2% Triton, and blocked in 1 M glycine (pH 8) for 1 h and in PBS/1% BSA/5% donkey serum for 4 h. Samples were then incubated overnight with anti-

3βHSD (raised in rabbit; 1:2000; kindly provided by Dr. Ian Mason), anti-Phospho-Histone H3 (for M-phase cell detection; mouse monoclonal 9706; 1:100 dilution; Cell Signaling Technology), or anti-INSL3 (1:50 dilution). Samples were then washed three times with PBS/1% BSA/0.1% Triton and incubated for 1 h and 30 min with donkey anti-mouse fluorescein isothiocyanate (FITC; 715-095-150; 1:200 dilution; Jackson ImmunoResearch Laboratories) for Phospho-Histone H3 detection. For detection of 3βHSD or INSL3, samples were incubated with donkey anti-rabbit Cy3 (711-165-152; 1:400 dilution; Jackson ImmunoResearch Laboratories) or donkey anti-rabbit FITC (711-095-152; 1:200 dilution; Jackson ImmunoResearch Laboratories). All samples were then labeled with TO-PRO3 iodide nuclear fluorescent dye (Molecular Probes-Life Technologies) and mounted in buffered glycerol (pH 9.5). All treatments were performed using continuous rotation.

Negative controls were processed in the absence of the primary antibody and using the primary antibody preimmune isotype immunoglobulins: both negative controls did not give detectable background.

Confocal Microscopy and Image Analysis

Whole-mount immunofluorescence experiments and caspase activity assays were analyzed using a confocal microscope (Leica IRE SP2-Laser Scanning TCS SP2) equipped with Ar/ArKr and He/Ne lasers. Images of the optical sections were acquired using the Leica confocal software. The laser line was at 488 nm for FITC excitation and at 543 nm for Cy3 and rhodamine excitation. Images were scanned under a 20× objective. To analyze the whole testis, each sample was optically divided into four quadrants. Each quadrant was analyzed using a spatial series through the z axis. Each spatial series was composed of approximately 12 optical sections with a step size of 5 μm. Color channels were merged, and colocalization was analyzed using the Leica confocal software.

Western Blot Analysis

Cultured testes were homogenized in RIPA buffer, and the protein contents were determined using the BCA method (Sigma-Aldrich). Proteins were resuspended in boiling Laemmli buffer under nonreducing conditions (80 μg per well). Proteins were separated on 12% or 7% SDS-PAGE gels depending on the predicted molecular weight of the investigated proteins. Gels were then electrotransferred to nitrocellulose membranes (Protran; Whatman-GE-Healthcare). Nonspecific binding was blocked by incubation with 5% nonfat milk in TBS buffer (20 mM Tris [pH 7.6] and 150 mM NaCl). After the blocking step, membranes were incubated in 5% nonfat milk in TBS for 1 h at room temperature with the antibodies listed below: anti-3βHSD (raised in rabbit 1:2000; kindly provided by Dr. Ian Mason), anti-nestin (mouse monoclonal MA1-91657; 1:500 dilution; Thermo-Scientific), anti-caspase-3 (rabbit polyclonal 06–735; 1:500 dilution; Upstate Biotechnology), anti-β-actin (mouse monoclonal A5441; 1:500 dilution; Sigma-Aldrich;), and anti-α-tubulin (mouse monoclonal T5168; 1:1000 dilution; Sigma-Aldrich). The membranes were then incubated with the appropriate alkaline phosphatase- or HRP-conjugated secondary antibody (goat anti-rabbit 62-6122, 1:2500 dilution, Zymed Laboratories; rabbit anti-mouse A4312, 1:3000 dilution, Sigma-Aldrich; donkey anti-rabbit NA9340V, 1:3000 dilution, GE Healthcare) for 1 h at room temperature. Immunocomplexes were detected using a Western blot chemiluminescent reagent (CDP-star; Perkin Elmer; or ECL Western blotting detection reagents; GE Healthcare) following the manufacturer's instructions.

TABLE 1. PCR primer sequences and relative product length.

Gene	Primer*	Product length
INSL3	F: 5′-CACGCAGCCTGTGGAGAC-3′	125 bp
	R: 5′-GAGAAGCCTGGAGAGGAAGC-3′	
3βHSD	F: 5′-CTCAGTTCTTAGGCTTCAGCAATTAC-3′	99 bp
	R: 5′-CCAAAGGCAAGATATGATTTAGGA-3′	
Nestin	F: 5′-TCTGCTGGAGGCTGAGAAC-3′	155 bp
	R: 5′-AGGATGTTGGGCTGAGGAC-3′	

* F, forward primer; R, reverse primer.

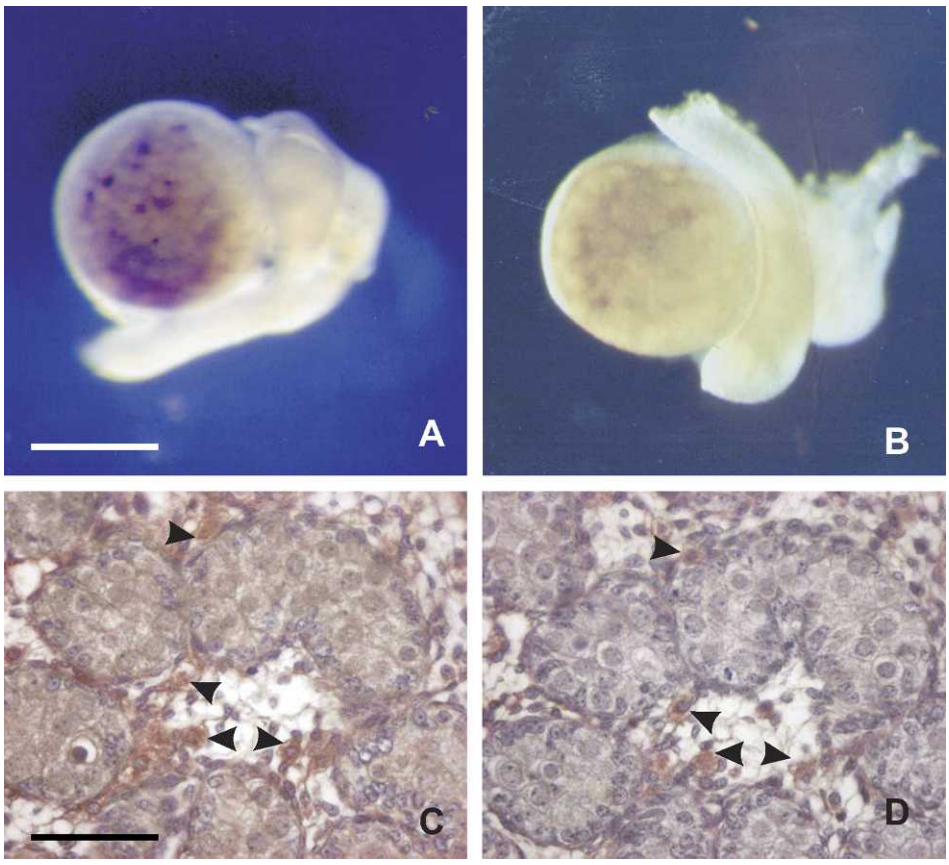

FIG. 1. **A**) Whole-mount in situ hybridization of the c-Met antisense probe in 17.5-dpc testis and mesonephric derivatives. **B**) Hybridizations of the sense probe at the same developmental stage as shown in **A**. Bar = 300 μm. **C** and **D**) Localization of c-Met and INSL3 proteins in serial sections of a 17.5-dpc testis. **C**) Immunolocalization of c-Met protein in a 17.5-dpc testis. **D**) Immunolocalization of INSL3 protein in a 17.5-dpc testis. Arrowheads indicate the most representative cells that are positive for c-Met and INSL3. Bar = 60 μm.

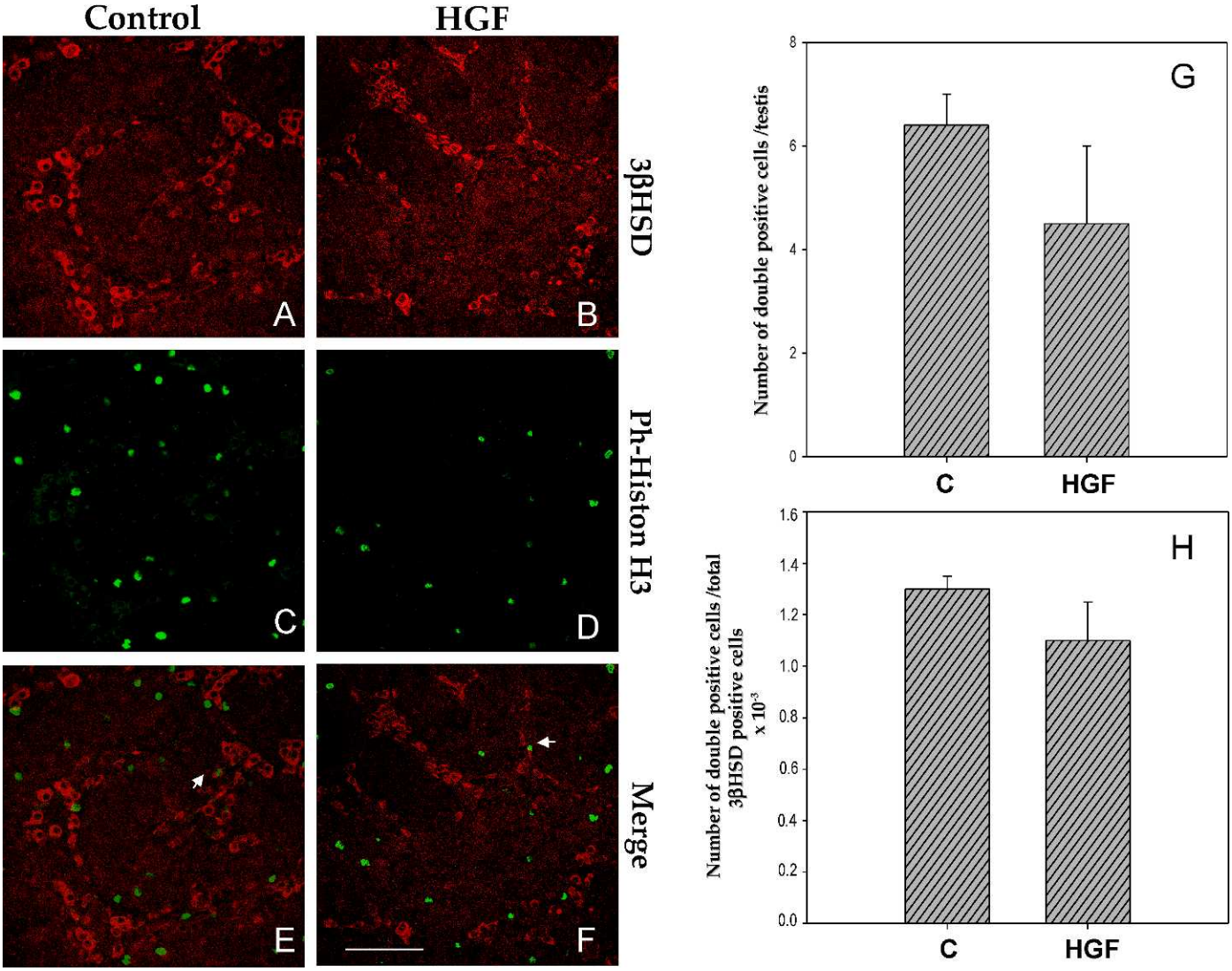

FIG. 2. Confocal analysis of single optical slices through 17.5-dpc embryos of testicular organ culture from control (**A**, **C**, and **E**) or HGF (**B**, **D**, and **F**) treatment. The organs were stained for 3βHSD (red; **A** and **B**) and Phospho-Histone H3 (green; **C** and **D**). In **E** and **F**, the merged image of both stains is reported. White arrows indicate cells positive for both stains. Bar = 40 μm. **G**) The mean number of cells positive for both stains per cultured testis ± SEM is shown. **H**) The mean number of cells positive for both stains normalized versus the total number of 3βHSD-positive cells ± SEM is shown.

TUNEL Assay

Cultured testes were fixed overnight in 4% PFA in PBS (pH 7.4) at 4°C, dehydrated, and embedded in paraffin. Samples were sectioned at a thickness of 5 μm. For apoptotic cell analyses, TUNEL assays were performed on sections using the Mebstain Apoptosis Kit II (MBL International Corp.) according to the manufacturer's instructions. This assay allowed labeling of 3′ DNA nick ends (generated during the apoptotic process) with biotinylated dUTP by terminal deoxynucleotidyl transferase. The labeled nick ends were then stained using FITC-conjugated avidin to allow specific and sensitive staining of apoptotic cells. Proteinase K digestion was needed before DNA nick end labeling. All positive controls sections were treated with DNase I. For negative controls, terminal deoxynucleotidyl transferase enzyme was omitted from reaction mixtures. Labeled sections were visualized and photographed using a Zeiss Axioscope.

RNA Extraction and RT

Total RNA from 17.5-dpc testes cultured with or without HGF (100 U/ml) was isolated using a silica gel-based membrane spin column (RNeasy Kit; Qiagen S.p.A.). The purity and integrity of the RNA were checked spectroscopically and by gel electrophoresis. Total RNA (1 μg) was reverse transcribed in a final volume of 20 μl using the M-MLV Reverse Transcriptase kit according to the instructions of manufacturer (Invitrogen).

Quantitative Real-Time PCR

To determine the presence of specific transcripts, several relative, quantitative real-time PCR reactions were performed. The reactions were carried out using a RealMasterMix SYBR ROX according to the manufacturer's instruction (5 Prime; Eppendorf). The β-actin was chosen as the most suitable housekeeping gene and was used as an internal control using primers designed by Eppendorf S.R.L. Primers used in quantitative real-time PCR reactions of the selected genes (Table 1) were designed using the program Oligo Analyzer. Each primer pair was previously tested for specific amplification.

Reactions were set up in a total volume of 25 μl using 12.75 μl of cDNA (diluted 1:50), 11.25 μl of RealMasterMix SYBR ROX (5 Prime; Eppendorf), and 1 μM each gene-specific primer and were performed in the 7500 Fast Real-Time PCR System (Applied Biosystems). The cycling conditions were the following: 95°C for 10 min; 45 cycles of 95°C for 15 sec, 60°C for 15 sec, 72°C for 30 sec, and a final elongation step carried out at 72°C for 10 min. Specificity of the PCR products was confirmed by analysis of the dissociation curve. Analysis of each reaction was conducted with Sequence Detector Software (version 1.4; Applied Biosystems), and then the results were obtained using the ddCt method.

Additionally, the expected size of each amplicon and the absence of nonspecific products were confirmed by analysis of real-time PCR products in

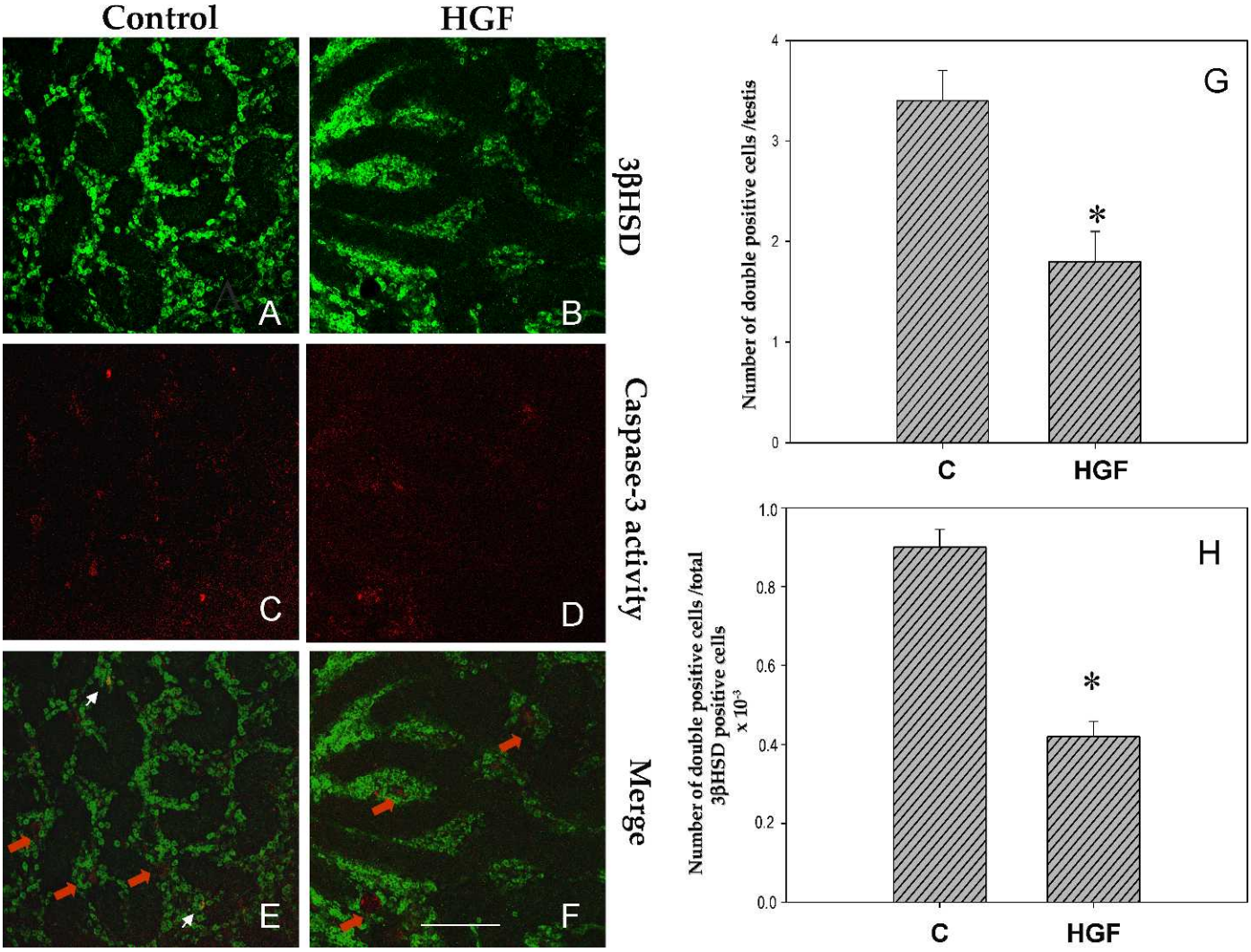

FIG. 3. Confocal analysis of single optical slices through 17.5-dpc embryos of testicular organ culture from control (**A**, **C**, and **E**) or HGF (**B**, **D**, and **F**) treatment. The organs were stained for 3βHSD (green; **A** and **B**) and caspase-3 activity (red; **C** and **D**). In **E** and **F**, the merged image of both stains is reported. Red arrows indicate the caspase-3 active areas that are 3βHSD negative. The white arrow indicates a cell positive for both stains. Bar = 80 μm. **G**) The mean number of cells positive for both stains per cultured testis ± SEM is shown. *Versus control, $P < 0.01$. **H**) The mean number of cells positive for both stains normalized versus the total number of 3βHSD-positive cells ± SEM is shown. *Versus control, $P < 0.01$.

2% agarose gels stained with ethidium bromide and visualized under ultraviolet light.

Statistical Analysis

All experimental data were expressed as the mean ± SEM of at least three separate experiments, and in each experiment each experimental condition was carried out at least in quadruplicate.

Student *t*-test and ANOVA followed by Duncan test for multigroup comparison were carried out, where appropriate, to evaluate the significance of differences. Differences were considered significant at a P value <0.05.

RESULTS

c-Met Expression Studies

Testes isolated from 17.5-dpc embryos were used to determine the C-MET pattern of expression by in situ hybridization and immunohistochemical analysis. In situ hybridization results showed that *c-Met* mRNA is present in the interstitial compartment at 17.5 dpc (Fig. 1, A and B). Immunohistochemistry of serial sections showed that C-MET protein is localized in many peritubular and interstitial cells. Immunostaining against C-MET and INSL3 on serial sections showed that all fully developed Leydig cells, which are cells positive for INSL3, are also C-MET positive (Fig. 1, C and D, arrowheads).

Organ Culture Experiments

To understand the role of the HGF/c-Met system in fetal testis physiology, we performed organ culture experiments followed by testosterone secretion, proliferation, and apoptosis and differentiation assays. We first checked the capability of HGF to trigger testosterone secretion in 17.5-dpc, 24-h testicular organ culture. We found the following testosterone concentrations in the differently treated media, measured as nanograms of secreted testosterone per testis ± SEM: control, 1.8 ± 0.21; HGF 50 U/ml, 2.05 ± 0.23; HGF 100 U/ml, 2.81 ± 0.17; and HGF 200 U/ml, 2.59 ± 0.24. Hepatocyte growth factor 100 U/ml was the most effective dose, and for this reason we chose this HGF concentration in the organ culture experiments (see the following paragraphs). Hepatocyte growth factor 50 U/ml did not exert a significant increase in

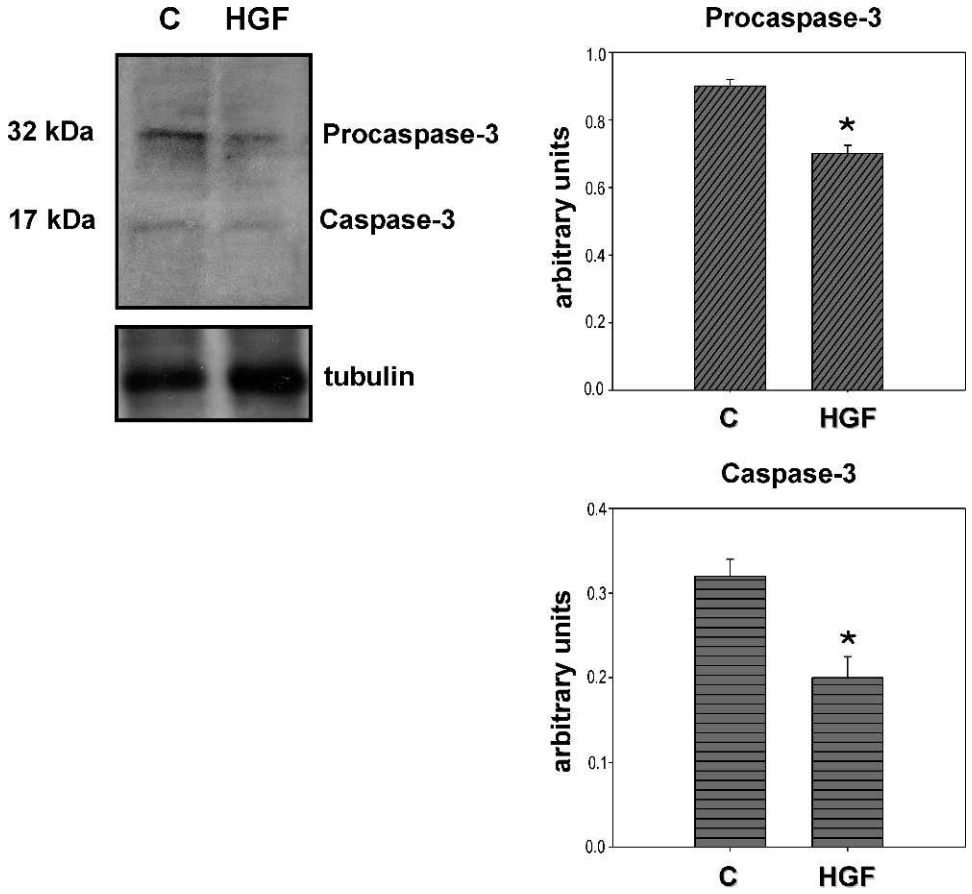

FIG. 4. Western blot analysis of caspase-3 in testicular organ culture of 17.5-dpc embryos. Two bands of 32 and 17 kDa were detected by immunoblot analysis, which correspond to caspase-3 and active fragments of caspase-3, respectively. The densitometric analyses of the bands observed in three different experiments are reported in the graphs in the right panel. *Versus control, $P < 0.01$.

testosterone secretion. These data overlap with the results previously presented by our group [53] that found an increase of approximately 50% in the 100 U/ml HGF-treated samples over the control samples.

Proliferation Data

After 24 h of culture, 17.5-dpc testes in the presence or in the absence of HGF were double labeled with 3βHSD and Phospho-Histone-H3, markers of the Leydig cell lineage and the M phase of the cell cycle, respectively. Using a Leica confocal microscope, we scanned the entire testis by performing optical spatial series. In each of three different experiments, we analyzed 12 fetal testes of control and HGF-treated organs, respectively; approximately 500 optical sections of each of these samples were analyzed. Double-labeled cells of control and HGF-treated testes were counted. The main FLC population is mitotically inactive, although we found that a few 3βHSD-positive cells are also positive for Phospho-Histone-H3 (Fig. 2, A–F). In our ex vivo system, HGF does not promote proliferation of FLCs because the difference between the control and HGF-treated samples is not significant (Fig. 2, G and H).

Apoptotic Indexes

We checked the apoptotic profile of 17.5-dpc testicular organ cultures of control and HGF-treated samples by caspase in situ activity assays (CaspGLOW; BioVision Inc.), by Western blot analyses, and by TUNEL assays. We tested caspase activity using in situ assays and found a detectable signal of caspase activity in the interstitial compartment of cultured fetal testes in control and HGF-treated samples (Fig. 3, C and D). Cultured testes were stained with CaspGLOW and also immunostained with 3βHSD. In each of three different experiments, we analyzed 15 fetal testes each from control and HGF-treated organs. Approximately 600 optical sections were analyzed in both treatments. Double staining showed that there were only a few apoptotic FLCs (Fig. 3, A–F). However, we counted apoptotic FLCs and found that HGF was able to significantly decrease Leydig cell apoptosis (Fig. 3, G and H).

Western blot analyses of testicular organ culture showed that HGF-treated samples presented a significantly lower amount of caspase-3 inactive precursor and of caspase-3 active fragment (Fig. 4). TUNEL assays were also performed on cultured and paraffin-embedded testes. In three different experiments of at least 80 sections for the control or HGF-treated organs, apoptotic cell nuclei appeared FITC labeled in TUNEL assays (Fig. 5, A and B). The apoptotic nuclei of tubular and interstitial compartments were counted, and we found that both compartments presented a significantly lower amount of apoptotic cells after HGF was added (Fig. 5, C and D).

FLC Differentiation Experiments

In this study, we also performed experiments to characterize the effect of HGF on FLC differentiation. Using the same organ culture conditions described above, we performed RNA

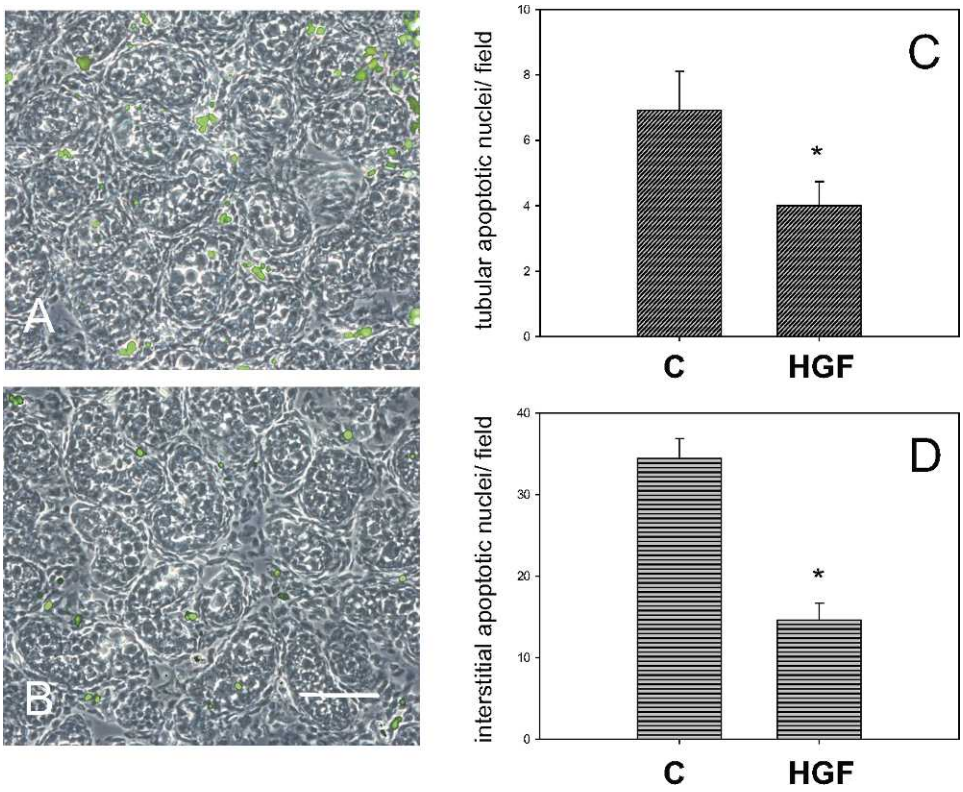

FIG. 5. TUNEL assays of apoptotic cells in testicular organ culture of 17.5-dpc embryos. **A** and **B**) Apoptotic nuclei are labeled in green. A merge of the images from phase contrast microscopy and TUNEL fluorescence is shown in **A** (control samples) and **B** (HGF-treated samples). Bar = 73 μm. **C**) Graph of the mean number ± SEM of tubular apoptotic nuclei. **D**) Graph of the mean number ± SEM of interstitial apoptotic nuclei. *Versus control, $P < 0.01$.

extraction followed by quantitative real-time PCR of cultured samples. Interestingly, we found that HGF treatment was able to induce a significant increase in INSL3 mRNA, which is a marker of differentiated Leydig cells (Fig. 6). The quantitative

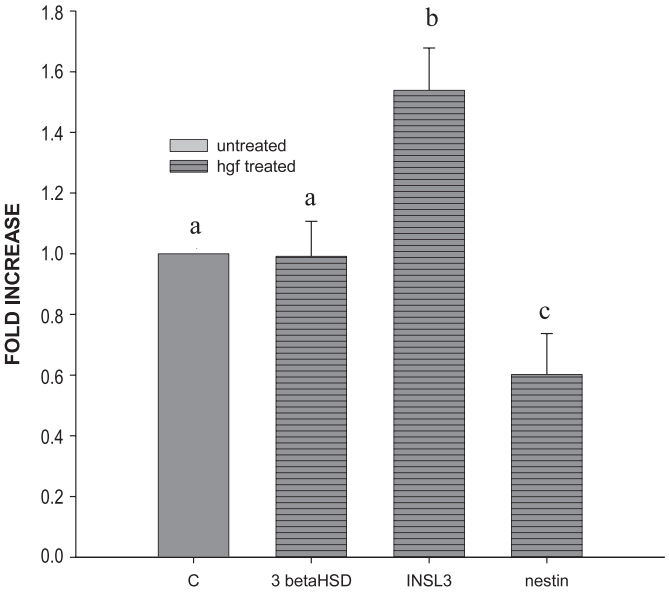

FIG. 6. Relative fold induction of the 3βHSD, INSL3, and nestin genes in testicular organ culture of 17.5-dpc embryos stimulated with HGF. Relative fold induction was calculated using the DDC*t* method. The mean of each triplicate is plotted and the error bars represent SEM. a versus b, $P < 0.05$; a versus c, $P < 0.05$; and b versus c, $P < 0.01$. Same letters indicate no significant differences.

real-time PCR data were confirmed and further verified by confocal analyses of INSL3 immunolocalization in 17.5-dpc testicular organ culture (Fig. 7, A–F). To quantify the number of INSL3-positive cells, three independent experiments were performed, and we analyzed 12 control and 12 HGF-treated fetal testes. We counted INSL3-positive cells by analyzing approximately 500 optical sections of each of the treatments. We found that fully developed Leydig cells are significantly increased in number (an approximately 45% increase in cell number) after HGF treatment (Fig. 7G).

Furthermore, quantitative real-time PCR experiments and Western blot analysis provided evidence that nestin, a marker of progenitor cells of Leydig cell lineage [10, 56], appeared to be downregulated after HGF treatment (Figs. 6 and 8). Conversely, 3βHSD, which is a marker of all FLCs, does not appear to be regulated by HGF (Figs. 6 and 8). According to these data, even the number of 3βHSD-positive cells per testis detected by immunofluorescence (Figs. 2 and 3) does not change significantly after HGF administration (control, 4021 ± 82; HGF 100 U/ml, 4088 ± 103).

DISCUSSION

The testicular fetal microenvironment can be considered a complex morphogenetic field in which different signals cooperate together to coordinate the development and maturation of testicular cells. The growth factors that promote FLC differentiation and function are only partially known. In this study, we provide evidence that the HGF is one of these growth factors. The C-MET/HGF system has a well-established morphogenetic role in the embryonic development of mouse male gonads [43, 50–53]. In addition, an increasing amount of evidence demonstrates the implication of the C-MET/HGF

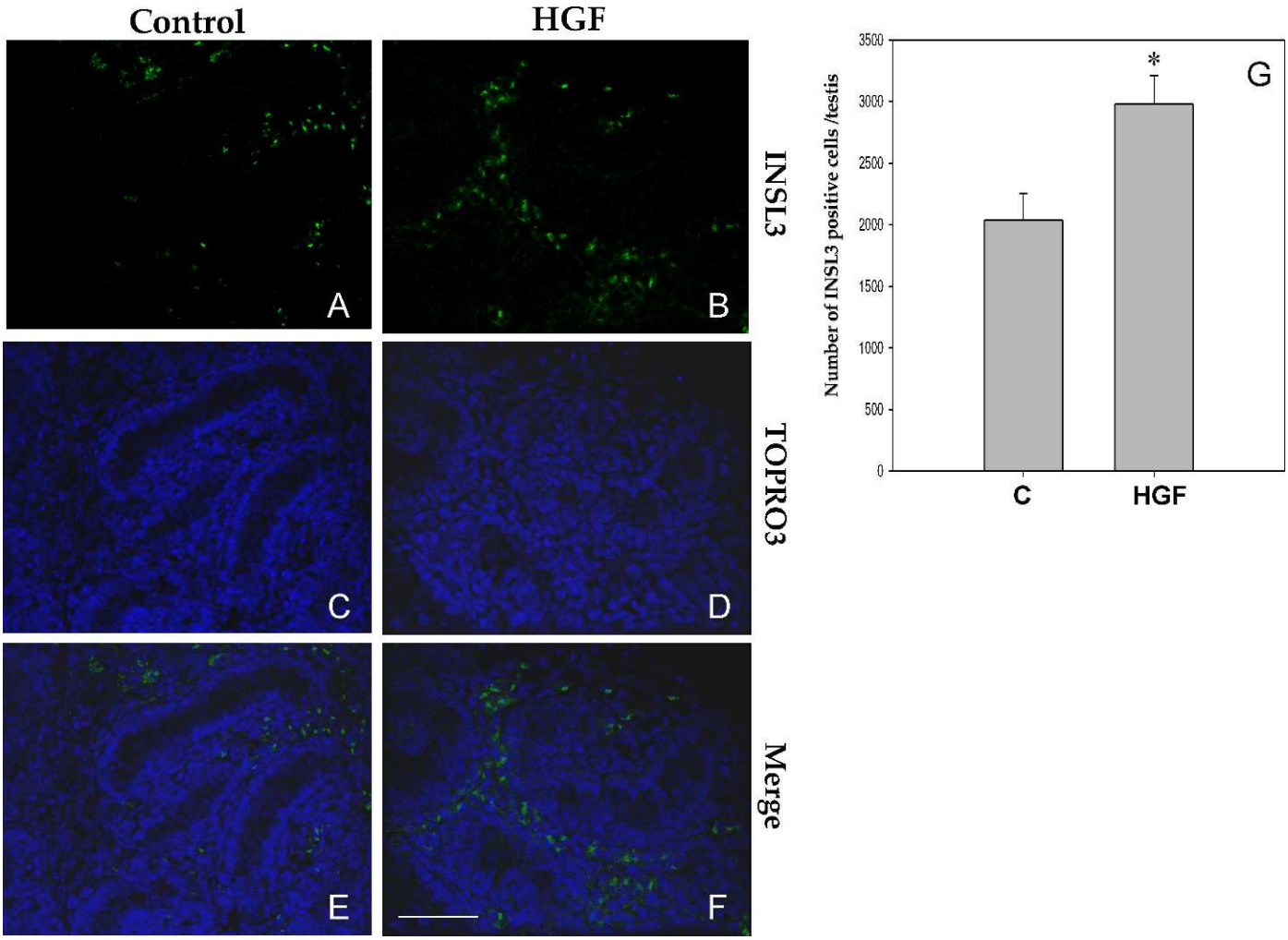

FIG. 7. Confocal analysis of single optical slices through 17.5-dpc embryos of testicular organ culture from 17.5-dpc embryo in control (**A**, **C**, and **E**) or HGF (**B**, **D**, and **F**) treatments. The organs were stained for INSL3 (green; **A** and **B**) or TOPRO3 nuclei staining (blue; **C** and **D**). In **E** and **F**, the merged image of both stains is reported. Bar = 80 μm. **G**) The mean number of INSL3-positive cells per cultured testis ± SEM is shown in the graph presented in the right panel. *Versus control, $P < 0.01$.

system in the physiology of postnatal male and female reproductive organs as well as in the regulation of their differentiation [43–45]. The present study extends previous findings by our group focusing on the role of C-MET/HGF system in late embryonic development and in Leydig cells that are the main targets of HGF signals at this developmental stage [53]. We first observed that *c-Met* mRNA and protein appear clearly localized in the interstitial cells after 17.5 dpc; however, *c-Met* mRNA appears localized in the tubular compartment at earlier developmental stages [50, 53]. Double immunofluorescence with 3βHSD and Phospho-Histone H3 in cultured testes showed that mitotically active FLCs are quite rare and that HGF does not affect the proliferation rate of the FLC lineage. This result is in agreement with previously reported data [17, 39, 57] that showed that FLCs differentiate from mitotically inactive precursors.

Western blot analysis showed that HGF is able to significantly reduce the expression of caspase-3 inactive precursor and of the active caspase-3 fragment. Confocal analysis of caspase activity merged with 3βHSD immunolocalization showed that HGF acts as a survival factor on this cell lineage, although the number of apoptotic FLCs is small, even in the control samples. We hypothesized that HGF has both direct and indirect protective effects against apoptosis in other testicular cell lineages because it seemed unlikely that the small number of FLCs protected by HGF against the apoptotic process could account for the result obtained by caspase-3 Western blot analysis. The results from TUNEL assays confirmed this hypothesis: HGF treatment significantly decreased the number of apoptotic cells in both the interstitial and tubular compartments of cultured testes. The antiapoptotic effect of HGF on tubular cells seems to be indirect because C-MET protein was not detectable in seminiferous cords at this developmental stage. It is interesting that caspase activity localization and TUNEL assay gave similar results: the greatest caspase activity as well as the most TUNEL-positive cells were recorded in the interstitial compartment in our culture conditions. However, we found few apoptotic Leydig cells in the caspase activity assays, and these data suggest that HGF can also protect other interstitial cell types from apoptosis. Taken together, these data lead us to the intriguing hypothesis that HGF treatment modifies the fetal testicular microenvironment and promotes the production of survival factors to protect testicular cells against apoptosis. Apoptotic process is physiologically relevant for the regulation of Leydig cell number and Leydig cell selection even in the postnatal testis. It is interesting to notice that our group observed HGF antiapoptotic effects even on rat pubertal Leydig cells [54]. Together, these

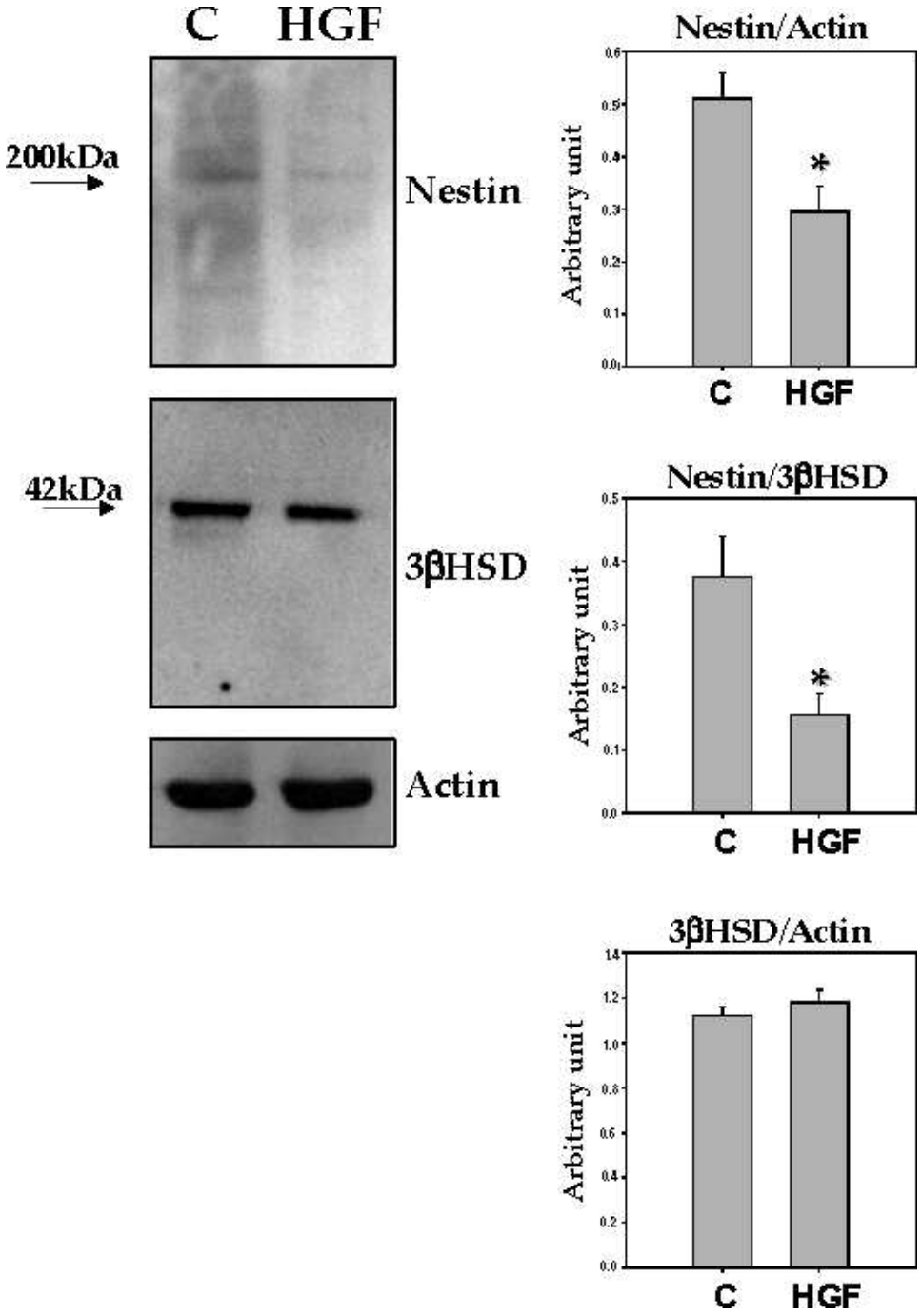

FIG. 8. Western blot analysis of 3βHSD and nestin in testicular organ cultures of 17.5-dpc embryos. In the left panels, immunoblot analyses on the same filter of nestin and 3βHSD and actin are shown. As expected, anti-nestin detected one band of 200 kDa and anti-3βHSD detected one band of 42 kDa. The densitometric analysis of the bands from three different experiments is reported in the graphs in the right panels. Nestin densitometric analyses were normalized versus 3βHSD densitometric values as well as versus actin densitometric values, whereas 3βHSD signal was normalized versus actin only. *Versus control, $P < 0.05$.

data suggest that HGF may represent a key regulator of Leydig cell survival both in the prenatal and postnatal testicular microenvironment.

The confocal analysis of Leydig cell differentiation allowed us to observe that HGF treatment increases the number of fully developed FLCs. This observation was confirmed by quantitative real-time PCR of *Insl3* mRNA. These data might also explain the reported increase in testosterone secretion that we observed in our organ culture conditions, because it seems unlikely that it could be attributed solely to the antiapoptotic effect exerted by HGF. Moreover, in the reported quantitative real-time PCR analysis, we observed that 3βHSD expression, a marker of all Leydig cells, is not regulated by HGF. This observation suggests that HGF is not able to recruit newly formed FLCs from the progenitor pool and does not affect significantly the FLC whole population. Furthermore, quantitative real-time PCR and Western blot analysis allowed us to observe that nestin, a marker of Leydig cell precursors, is downregulated after HGF treatment. This last observation suggests that HGF is unable to maintain or expand the FLC

progenitor pool. Taken together, these data indicate that HGF is specifically involved in FLC terminal differentiation. Based on these data, HGF may be considered a stromal testicular factor responsible for FLC terminal development, whereas DHH and PDGF, which are Sertoli cell-derived factors, can be considered responsible for the expansion of the Leydig cell precursor population in the mouse fetal testis [9, 13, 28–32]. This is probably the reason why c-Met appears to be expressed by FLCs only in the late period of pregnancy [53], whereas DHH and PDGF receptors are expressed by FLCs earlier in testicular development [3]. To our knowledge, HGF is the first stromal-derived factor described to affect FLC differentiation and survival. This finding confirms the role of HGF in the epithelium-mesenchyme exchange even in the fetal testicular interstitial compartment, and it indicates that HGF acts on FLCs in a paracrine fashion. It will be of great interest to study how the C-MET/HGF system interacts with other signaling pathways in the fetal testicular microenvironment. Such future studies will increase our knowledge about the network of growth factors that promote morphological and cellular development in the testis.

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