

HEPATOCYTE GROWTH FACTOR MODULATES RAT LEYDIG CELL FUNCTIONS

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Abstract

The hepatocyte growth factor (HGF) regulates many cellular functions acting through c-met, its specific tyrosine kinase receptor. We have previously reported that in prepubertal 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 paper we extend our study to the interstitial compartment of the testis and we demonstrate that 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 paper that HGF significantly increases the amount of testosterone secreted by the Leydig cells and decreases the number of Leydig cells undergoing apoptosis. The anti-apoptotic effect of HGF is mediated by the caspase-3 activity since the active fragment of the enzyme is decreased in Leydig cells cultured in the presence of HGF. However the treatment with the growth factor does not modify the expression levels of caspase-3 mRNA. All these data indicate that HGF regulates the functional activities of the Leydig cells. Interestingly, the steroidogenetic activity of the cells is increased by HGF also culturing explants of testicular tissues as well as the anti-apoptotic 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

Introduction

Hepatocyte growth factor (HGF) is a cytokine with proliferative and motogenic capacities, 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 protooncogene (Weidner et al. 1993; Hartmann et al. 1994). As we previously demonstrated, HGF receptor is expressed in the postnatal rat testis and is detectable in the interstitial tissue and in the peritubular myoid cells of the seminiferous tubules (Catizone et al. 1999, 2001). In the Sertoli cells c-met expression is developmentally regulated being firstly detectable in cells isolated from pubertal 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 have demonstrated that mitotic and meiotic rat germ cells express c-met and that HGF regulates proliferation and apoptosis of prepubertal rat germ cells (Catizone et al. 2006). HGF is also expressed during embryonic development (Sonnemberg 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 which rarely proliferates in the adult (Saez 1994, De Kretser & Kerr 1994). During postnatal development of the testis, Leydig cells undergo to a series of morphological and functional transformations being present 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 differ in the levels of testosterone synthesis (Haider 2004). Leydig cell proliferation and endocrine functions are regulated by several hormones among them interleukins (Svechnikov et al. 2001; Walch & Morris 2002), transforming growth factors beta (Khan et al. 1992; Dickson et al. 2002), insulin-like factors (Khan et al. 1992; Ge & Hardy 1997) and ghrelin (Barreiro et al. 2004). It is also known that Leydig cells engages the programmed cell death pathway in particular situations such as in response to ethylene dimethanesulfonate (EDS) (Kerr et al. 1985; Morris 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 correct number of Leydig cells. We have recently demonstrated that in the embryonic testes HGF regulates fetal Leydig cells testosterone production (Ricci et al. 2006).

55 Interestingly, HGF increases the amount of testosterone secreted in the culture medium by ‘in vitro’ organ culture of testes isolated from 18.5 dpc embryos but does not modulate the amount of testosterone secreted by testes isolated from 15.5 dpc embryos (Ricci et al. 2006).

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

Materials and Methods

Animals

65 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 and killed by CO₂ asphyxia before testes removal. Usually four 30-32-day-old rats of 100-120 grams of body weight were used for each experiment.

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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, Gibco, Invitrogen s.r.l., 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 min. After dissociation, the enzyme was diluted with culture medium and the seminiferous tubules were removed by sedimentation at gravity (4 min). Tubules were washed again to detach the interstitium and the two supernatants collected and centrifuged (300 x g, 10 min). The pellet was resuspended in MEM containing bovine serum albumin (BSA) 0,1% and DNase 0,01% and the cell suspension was loaded on top of a discontinuous Percoll gradient (20-86% Percoll) and centrifuged at 800 x g for 20 min at 18 °C. After centrifugation, fractions at 1.056 and at 1.068 g/ml were collected, washed with buffer and counted. Isolated Leydig cells were resuspended in MEM culture medium containing 15 mM Hepes, non essential aminoacids, 5 µg/ml gentamicin, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were seeded on Falcon culture plates (Becton Dickinson and Co., Lincoln Park, NJ) at the concentration of 0,5-0,7x10⁶ cells/ml of medium. Viability of Leydig cells was assessed by trypan blue dye exclusion method. Briefly, isolated Leydig cells were mixed with an equal volume of

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0.4% trypan blue (Flow Laboratories, Irvine, Scotland), incubated for 5 min at 37 °C and examined under a microscope. After 24 h of culture Leydig cells were almost totally viable. To assess the effects of HGF *in vitro*, cells were cultured for 26 h at 32 °C in a humidified 5% CO₂-95% air atmosphere in the presence of the growth factor (100 U/ml-30ng/ml) for the last 24 h of culture.

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Evaluation of isolated Leydig cell purity

Purity of Leydig cell preparation was routinely assessed on the basis of positive staining of cells for the enzyme 3 β - *hydroxy steroid dehydrogenase* (HSD) (Payne et al., 1980). Briefly, an aliquot of Leydig cell fraction was taken in a tube containing 0.5 ml of β -nicotinamide adenine dinucleotide (NAD, 9
95 mg/ml, Sigma-Aldrich Co., St. Louis, Mo), 0.2 ml of dehydroepiandrosterone (DHEA, 1mg/ml in methanol) and 0.25 ml of nitroblue tetrazolium (NBT, 2 mg/ml in phosphate buffer pH 7.4, Sigma-Aldrich Co.). The reaction mixture was allowed to stand for 1 h 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
100 cytochrome P450 side chain cleavage (P450 scc). The cells were paraformaldehyde fixed, washed extensively with PBS supplemented with 1% BSA and 0.2% Triton X-100 and left for 30 min in PBS containing 10% goat serum. Cells were then incubated with a polyclonal antibody against the P450 scc (AB1294, 1:200 dilution, Chemicon Int., Temecula, CA, U.S.A.) for 16 h at 4°C. At the end of the incubation period, the cells were washed extensively with PBS and incubated for 45 min at room
105 temperature with a fluorescein isothiocyanate-conjugated goat anti rabbit antiserum (Sigma-Aldrich Co., St. Louis, Mo). 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

110 Fragments of approximately 1 mm³ were isolated from eight testes of 30-32-day-old rats and placed on steel grids previously coated with 2% agar. Grids were then placed in organ culture dishes (Falcon, Becton Dickinson NJ, USA) with 0.8 ml of medium necessary to wet the grid. The chemically defined medium utilized was as indicated in Leydig cell isolation and culture section. HGF (150 U/ml, Sigma-Aldrich Co.) was added to the culture medium when indicated. Samples were cultured for 24 h at 32°C
115 in a humidified atmosphere of 5% CO₂ in air. After culture, the samples were washed twice in PBS, fixed overnight in Bouin's fixative, dehydrated, embedded in paraffin, sectioned at a thickness of 7 μ m and the sections were utilized for 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 the experiments.

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Immunocytochemistry

Leydig cells prepared as indicated above were fixed in methanol according to the suggestions of the antibody manufacturer for 10 min at -20°C, treated with 5% BSA (Sigma-Aldrich Co.) for 30 min at room temperature to minimize the non-specific binding and then exposed to a polyclonal antibody
125 against the carboxy terminus of c-met (sc-162; 1:50 dilution Santa Cruz Biotechnology, Santa Cruz, Ca) for 16 h at 4°C. At the end of the incubation period, the cells were washed extensively with PBS and incubated for 45 min at room temperature with a fluorescein isothiocyanate-conjugated goat anti rabbit antiserum (Sigma-Aldrich Co., St. Louis, Mo). 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
130 rabbit IgG or 10-fold excess by weight of the blocking peptide (sc-162P Santa Cruz Biotechnology). Samples were analyzed using a Zeiss Axioplan fluorescence microscope.

RNA isolation and RT-PCR analysis

Total RNA was extracted from cultured cells utilizing a Perfect RNA, Eucariotic, Mini kit (Eppendorf,
135 Hamburg, Germany). The purity of isolated mRNAs was evaluated spectrophotometrically, using the A260:A280 ratio. To reduce contamination by genomic DNAs, total RNAs were treated with ribonuclease-free deoxyribonuclease I for 15 min as recommended by the manufacturer (Invitrogen, San Giuliano Milanese, Italy). Samples of total RNAs (200 ng) were reverse transcribed with reverse transcriptase (Invitrogen 28025-021) in the presence of oligo(dT) primers (Invitrogen) at 37°C for 50
140 min, and the reaction was terminated by heating at 70 °C for 15 min. Polymerase chain reaction (PCR) was performed utilizing the HotMaster Taq DNA polymerase (Eppendorf 0032002.684) 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 of c-met consisted of a
145 first denaturing cycle at 94°C for 5 min followed by cycles of 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 min. The final incubation was performed at 72 °C for 5 min. The amplification program of S-16 was similar with the exceptions of cycles of amplification (30) and annealing temperature (60 °C). Negative controls contained water instead of cDNA. PCR with no RT gave no

150 product, eliminating the possibility of a 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. RT-PCR analysis conducted utilizing primers against S-16 was utilized to be sure that equal amounts of cDNA were used. A DNA ladder was included in the gels to determine the size of the PCR products.

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Testosterone evaluation

Leydig cells were cultured for 24 h in presence of different doses of HGF (25-300 units/ml) and the culture medium was utilized for testosterone determination by radioimmunoassay. The 'Access' immunoassay system commercialized by the Beckman Coulter Inc (Fullerton, CA, U.S.A.) was utilized
160 for the determination (Wilson and Foster, 1992).

TUNEL assay

Leydig cells were cultured for 24 h in presence of different doses of HGF (25-300 units/ml). The apoptotic cells were detected by the TUNEL method for apoptotic cell analysis supplied the Q-BIOgene (ApopTag Peroxidase apoptosis kit). As positive controls Leydig cells or testicular sections were treated
165 with DNase I. Negative controls have been done omitting terminal deoxynucleotidyl transferase enzyme in the reaction mixture. 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.

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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 (Sigma, P8340; 1:100 dilution). Lysates were sonicated and the protein content was determined by BCA protein assay (Pierce
175 Biotechnology, Rockford, IL). Equal amounts of protein (40 µg) were resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes. After blocking, the membranes were incubated (16 h at 4 °C) with 0.2 mg/ml rabbit polyclonal anti-human caspase-3 antibody which recognizes full length caspase 3 and proteolytic fragments (Upstate Biotechnology, Lake Placid, NY) and then (60 min, room temperature) with HRP conjugated secondary antibody (1:7000 dilution; Amersham Biosciences UK
180 Limited, Little Chalfont, Buckinghamshire, UK). Peroxidase activity was visualized with the SuperSignal West Pico Trial Kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer's

instructions. The caspase-3 protein content was determined densitometrically. The nitrocellulose membranes were also probed with anti-tubulin monoclonal antibody (Sigma T 5168).

185 *Statistical analysis*

All experimental data were expressed as the mean + SE of at least three separate experiments. Statistical analysis was performed by Student's t-test. Differences were considered significant at $P < 0.05$. ANOVA followed by Duncan's test for multigroup comparison was also employed to evaluate the significance of differences.

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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 the purity of the cell population obtained was checked evaluating the positivity either for the enzyme 3β -HSD (Fig 1A, B) or for the cytochrome P450 side-chain cleavage enzyme (P450_{scc}) (Fig. 1C, D, E, F). Both the techniques indicate that our preparations contained routinely more than 90% Leydig cells with peaks of purity of 96%. The presence of c-met protein was detected by immunolocalization on these purified populations of Leydig cells and in Fig. 2A the phase contrast microscopy of the purified cells and their positivity for c-met (Fig. 2B) is presented. C-met protein is detectable by indirect immunofluorescence on the cells and shows a spotted distribution. In Fig. 2D the negative control obtained utilizing a blocking peptide is presented and in Fig. 2C the phase contrast microscopy of the Leydig cells used as a negative control is shown. All the controls performed were invariably negative either using isotype serum or the blocking peptide. To evaluate the expression of c-met mRNA in Leydig cells total RNA was extracted from non cultured cells and cells cultured for 24 or 72 h. The RT-PCR analysis was performed as indicated in the methods section and RNA extracted from livers of the same rats used for Leydig cell isolation was utilized as a positive control. We found that Leydig cells express c-met mRNA and the expression is not modified by the culture time (Fig. 3A).

210 **HGF effects on Leydig cells**

C-met expression

In order to evaluate the effect of HGF on c-met mRNA expression levels, purified cell preparations of Leydig cells were cultured for 24 h in control medium or in medium supplemented with HGF (100

U/ml) and total RNA extracted. RT-PCR analysis was performed and RNA extracted from the liver of
215 the same rats utilized for Leydig cell isolation was used as a positive control. We found that c-met
mRNA levels were not changed after incubation with HGF (Fig. 3B).

Testosterone production

The potential functional role of HGF signalling in the control of testicular function was explored. To this
220 end, Leydig cells were cultured for 24 h and the culture media of cells cultured in medium alone or
supplemented with HGF were utilized to evaluate the amount of testosterone secreted. The media were
collected, testosterone determined by radioimmunoassay and the relative amount of testosterone secreted
in the seven different experiments performed is reported in Fig. 4A. The results obtained indicate that
the amount of testosterone secreted by the cells cultured in the presence of HGF (100 U/ml) is
225 significantly higher respect to the control samples. We also present the dose-response curve of
testosterone produced in presence of different doses of HGF (25 to 300 units/ml) (Fig. 4B). The amount
of testosterone produced was significantly higher at doses of HGF ranging from 50 to 300 U/ml whereas
the dose of 25 U/ml did not give significant increase of the hormone production.

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

Apoptosis

Four separate experiments were performed to investigate the role of HGF on Leydig cell apoptosis.
235 Leydig cells were cultured for 24 h in medium alone or supplemented with HGF (100 U/ml). In Fig. 5A
the morphological appearance of the control (C) and the HGF-treated Leydig cells (HGF) is shown.
Leydig cells undergoing apoptosis are evident for the brown, morphologically abnormal nucleus
(arrows). The number of apoptotic cells was not high, however counting the apoptotic cells in both the
culture conditions we have detected an anti-apoptotic effect of HGF. As shown in Fig. 5B the number of
240 apoptotic cells is significantly lower when Leydig cells are cultured in the presence of HGF. Different
doses of HGF (25-300 units/ml) were also utilized to evaluate the anti-apoptotic effect of HGF (Fig. 5C)
and the results obtained demonstrate that HGF significantly reduces the number of the apoptotic cells
starting from the dose of 50 U/ml. Apoptosis of Leydig cells was also evaluated culturing small explants
of testicular tissue. Testicular samples were incubated for 24 h in fresh medium or medium containing
245 HGF (150 U/ml) and Fig. 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 in Fig. 6B, the number of apoptotic cells is strongly reduced in the presence of the growth factor.

250 *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 (Fig. 7A) and the results obtained indicate that the gene expression is not modified by the HGF treatment. On the contrary, the western blot analysis of the proteins extracted from control and HGF-treated Leydig cells (Fig. 7B) showed that
255 HGF significantly reduces the amount of the 17 KDa active fragments as reported in Fig. 7C in which the results obtained in all the experiments performed are shown. A statistically significant variation of the inactive form of the enzyme was not detected as reported in Fig. 7D which summarized the amount of the inactive and active form of caspase-3 obtained in control and HGF-treated cells.

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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 the
265 last years in the control of testicular function (Saez 1994; Haider 2004; Sriraman et al. 2005). We have previously demonstrated that HGF is produced by the somatic cells of the testis and is one of the growth factors acting in 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 have demonstrated that HGF modulates survival
270 and proliferation of germ cells during the first spermatogenetic wave of postnatal rat testis development (Catizone et al. 2006). To extend our previous studies, in the present paper we have investigated the presence of the HGF receptor in the 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 these cells express the HGF receptor, c-met,
275 without relation to their stage of maturation. Established the presence of the HGF receptor on Leydig cells we have 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). Therefore we have first of all investigated on the effect of HGF on this relevant function of Leydig cells and we demonstrate that HGF acts on Leydig cells increasing their ability to secrete testosterone. Interestingly, an increase of testosterone production is obtained also culturing small explants of testicular tissue that is culturing Leydig cells in a more physiological condition of culture in which the relationships between the testicular cells are maintained. In our opinion these combined data indicate that HGF influences the testosterone secretion also *in vivo*, in the intact animals.

Considering the relevance of the apoptotic process in the regulation of the number of the Leydig cells we have then evaluated the role of HGF in this process and our experiments clearly demonstrate 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 the Leydig cells however the finding that testosterone is highly secreted in this culture condition allows to conclude that, besides other interstitial cells, also Leydig cells are protected against apoptosis by HGF. Caspases are enzymes playing 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. (Nunez et al. 1998). Among the caspases, caspase-3, one of the effector caspases, appears to be a key protease in the apoptotic pathway (Porter & 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 the 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 have 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 at the moment unknown which are the cells of the interstitial compartment secreting HGF. Further studies are necessary to clarify this point.

In conclusion, we demonstrate 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 the Leydig cells. Moreover HGF decreases the number of Leydig cells undergoing apoptosis and the anti-apoptotic effect of HGF is mediated by the caspase-3

310 activity since the active fragment of the enzyme is decreased in Leydig cells cultured in the presence of HGF. On the contrary the treatment with the growth factor does not modify the expression levels of c-met and caspase-3 mRNA. All these data indicate that HGF regulates the functional activities of the Leydig cells, the steroidogenetic cells of the interstitial compartment of the testis.

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Figure legends

Fig. 1. Purity of Leydig cell preparations. Phase contrast microscopy (A, C) and their positivity for the enzyme 3β -HSD (B) and P450_{scc} (D) are shown. In E and F the negative control for P450_{scc} is presented. Bar=10 μ m.

Fig. 2. C-met distribution on rat Leydig cells. A: phase contrast microscopy and (B) indirect immunofluorescence of c-met on the same cells. D: negative control and relative phase contrast microscopy (C). Bar=20 μ m.

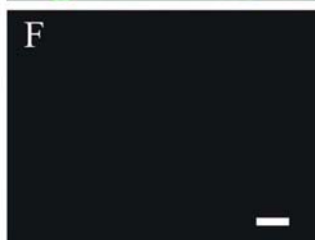
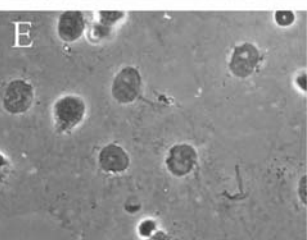
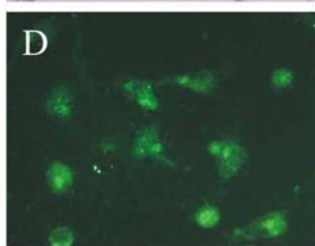
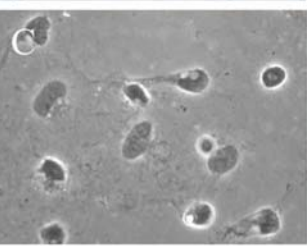
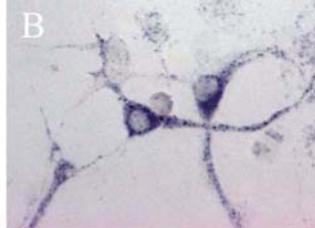
Fig. 3. C-met expression in rat Leydig cells. A: RT-PCR analysis of c-met expression in uncultured cells (0) and in cells cultured for 24 h and 72 h. RNA extracted from liver was used as positive control. B: c-met expression in cells cultured for 24 h in control medium (C) and in medium supplemented with HGF (HGF). RT-PCR analysis was also conducted utilizing primers against S-16.

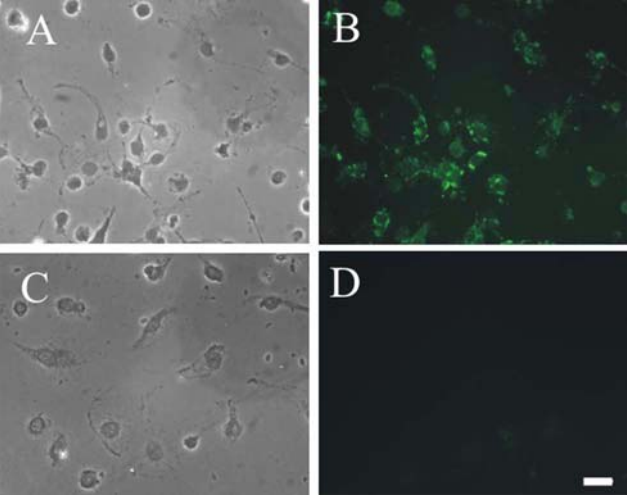
Fig. 4. Testosterone production of Leydig cells. A: amount of testosterone secreted by the cells cultured in the absence (C) or in the presence of HGF (HGF). The mean \pm S.E. of seven experiments is reported. (*) vs. C, $P < 0.05$. B: dose-response curve of testosterone production. The amount of testosterone secreted by control cells is arbitrarily considered as '1'. The values of the samples treated with HGF are reported as percentage respect to the control values. (*) vs. C, $P < 0.05$. C: testosterone produced by testicular fragments cultured for 24 hours in control (C) and HGF-supplemented medium (HGF). (*) vs. C, $P < 0.05$.

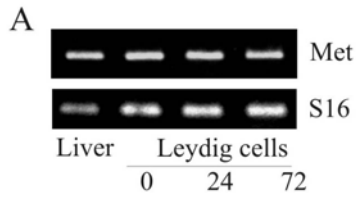
Fig. 5. A: Photomicrographs of Leydig cells cultured for 24 hours in control medium (C) and HGF-supplemented medium (HGF). Arrows indicate the apoptotic cells. Arrowheads indicate picnotic cells. Bar=10 μ m. B: percentage of Tunel-positive Leydig cells cultured for 24 hours in control medium (C) and HGF-supplemented medium (HGF). (*) vs. C, $P < 0.05$. C: apoptosis of Leydig cells cultured in presence of different doses of HGF (25-300 U/ml).

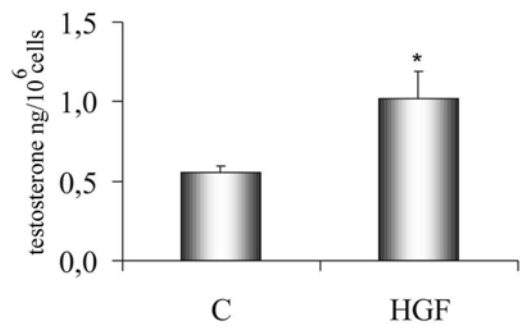
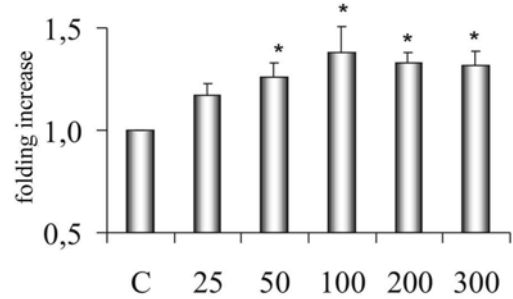
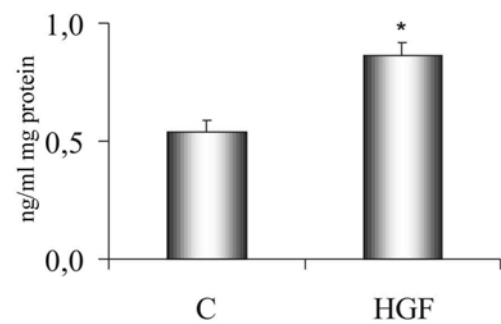
Fig.6. A: Photomicrographs of histological sections of rat testis fragments cultured for 24 hours in control (C) and HGF-supplemented medium (HGF). Bar=20 μ m. B: percentage of Tunel-positive interstitial cells in testes cultured for 24 hours in control medium (C) and HGF-supplemented medium (HGF). (*) vs. C, $P < 0.05$.

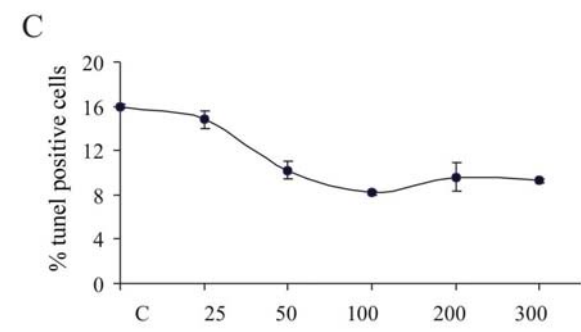
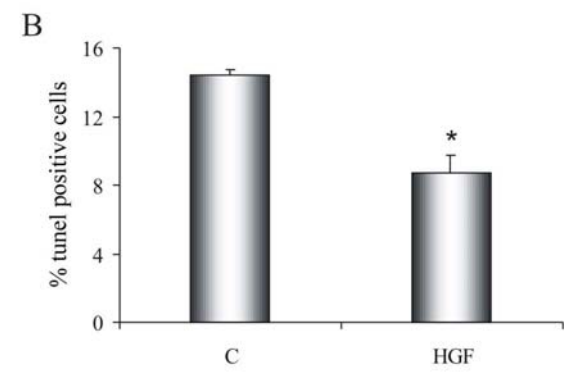
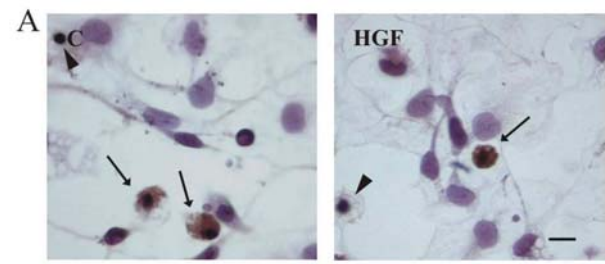
Fig.7. A: RT-PCR analysis of caspase-3 mRNA expression in Leydig cells cultured for 24 h in control medium (C) and in medium supplemented with HGF (HGF). B: Western blot analysis of proteins extracted from control and HGF-treated Leydig cells. Proteins were probed with anti-caspase-3 polyclonal antibody and anti-tubulin monoclonal antibody. C: densitometric scanning of the active fragment (17 KDa) of caspase-3. The values of the samples treated with HGF are reported as percentage 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 mean \pm S.E. of the three experiments performed is reported and (*) vs. C, $P < 0.05$.

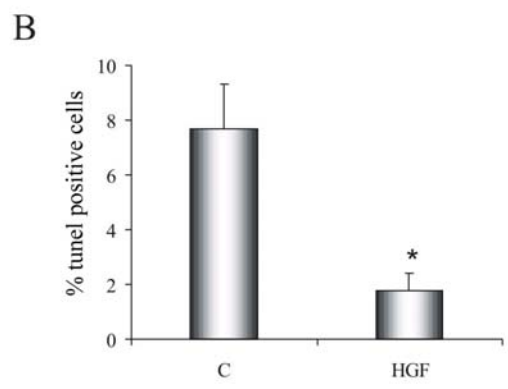
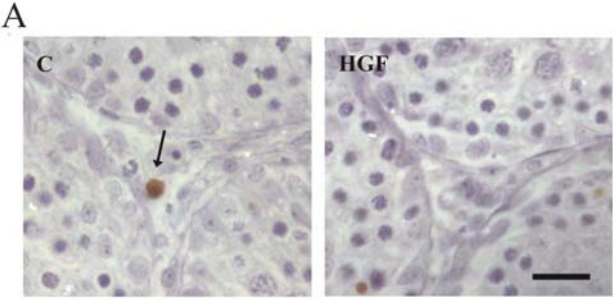


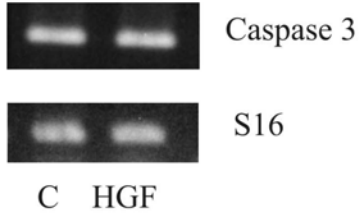
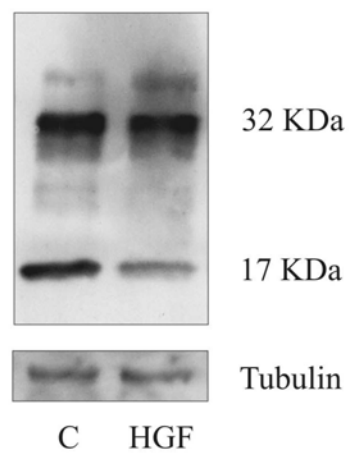
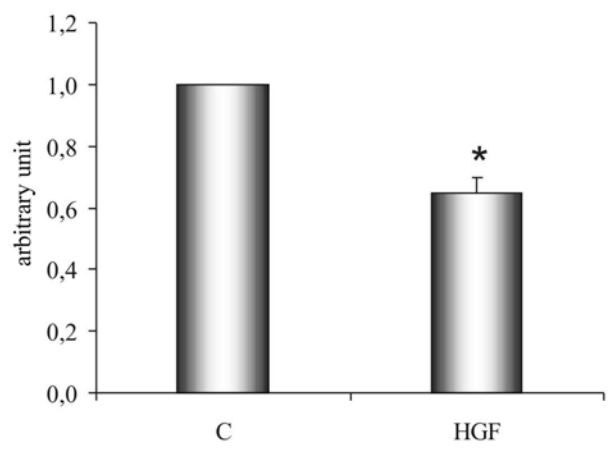




A**B****C**





A**B****C****D**