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Expression and Functional Role of Hepatocyte Growth Factor Receptor (C-MET) during Postnatal Rat Testis Development*

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ABSTRACT

The met protooncogene encodes the hepatocyte growth factor receptor (HGFR, c-met). C-met, a tyrosine kinase receptor protein, is widely expressed in different cell types including the male reproductive tract. As we recently demonstrated, both c-met messenger RNA and protein are expressed in prebuberal rat testis. The aim of this work was to detect the expression of c-met during postnatal testis development and to study its functional role. Our findings show that in total rat testis c-met is expressed during postnatal life until the sexual maturation of the animals. To evaluate the receptor expression in the different cell types in the testis, homogeneous cell populations of Sertoli and peritubular myoid cells were isolated from the seminiferous tubules of 10- and 35-day-old animals. c-met gene is expressed in myoid cells at the ages considered and its expression decreases with increasing age. By contrast, in Sertoli cells c-met expression is first detectable at 25 days of life and its expression increases with the increasing age being well evident at 35 days of age. C-met protein was detected by immunocytochemistry and its expression correlates with gene expression. The receptor is functionally active because HGF administration induces morphological changes in myoid cells and in c-met-expressing Sertoli cells. As a consequence of HGF addition, Sertoli cells cultured on reconstituted basement membrane reorganize into cord-like structures that resemble testicular seminiferous cords. The data here reported demonstrate for the first time that in Sertoli cells c-met expression is developmentally regulated being present and functionally active in postpuberal Sertoli cells. Given that c-met expression persists in myoid cells during postnatal testis development and that in Sertoli cells its expression correlates over time with germ cell differentiation and lumen formation, we conclude that the c-met/HGF system is involved in testis development and function. (*Endocrinology* **142:** 1828–1834, 2001)

THE MET protooncogene was initially discovered as a gene able to transform normal fibroblast cell lines (1) and subsequently identified in normal cells isolated from different mammalian tissues (2). This protooncogene encodes the hepatocyte growth factor receptor (HGFR/c-met) (3), a transmembrane glycoprotein with thyrosine kinase activity, which specifically binds to the hepatocyte growth factor/scatter factor (HGF/SF) and transduces its multiple biological effects (4, 5). HGF is a pleiotropic cytokine initially discovered as a mitogenic factor for hepatocytes (6, 7) and subsequently as a scatter factor (8–10), able to regulate the cellular activities of different cell types (11–14).

HGF also has a morphogenic effect on epithelial tissues, resulting in the formation of tubules and gland-like structures in cells derived from kidney and mammary gland (15–17). Its morphogenetic role was recently highlighted by a paper demonstrating that, in organ culture, exogenously added HGF is able to stimulate branching morphogenesis of the fetal lung (18). In a recent paper we demonstrated that the HGF/HGFR system is heavily involved in testis development since c-met is early expressed in the developing testis and HGF is able to support testicular differentiation, whereas other growth factors, presumably involved in fetal testis development, do not exert the same morphogenetic effect (19).

The data available currently in the literature concerning the HGF/c-met system in the reproductive tract during postnatal development of the male gonad do not clarify the role of this factor in the regulation of mammalian spermatogenesis. C-met expression has not been detected in adult mouse testis (2), although it is present in the human testis, prostate and seminal vesicles (20, 21). We recently reported c-met expression in prepubertal rat testis, specifically located in myoid and interstitial cells (22), while other authors have reported the presence of this receptor in postnatal mouse testis (23).

In the present paper, we extend our previous work evaluating c-met expression in rat testis at different times during the testicular development and we present data demonstrating that c-met is expressed in myoid cells at all the postnatal ages considered. By contrast, in Sertoli cells c-met begins to be expressed at puberty and is functionally active as suggested by the fact that HGF administration to cultured Sertoli cells induces the formation of cord-like structures.

Materials and Methods

Animals

Wistar rats were obtained from Charles River Farms (Como, Italy). All animal studies were conducted in accordance with the principles and procedures outlined in the MIH Guide for Care and Use of Laboratory Animals.

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Materials

Minimum essential medium, DMEM, Hanks' solution, fetal calf serum (FCS), and Random Primers DNA labeling System were purchased from Life Technologies, Inc. (Gaithersburg, MD). Collagenase were obtained from Roche Molecular Biochemicals (Mannheim, Germany). Culture plates were purchased from Corning, Inc. (Corning, NY). Proteinase K and all other reagents were purchased from Sigma (St. Louis, MO). Hybond N+ membrane was purchased from Amersham Pharmacia Biotech (Milan, Italy). Percoll was obtained from Amersham Pharmacia Biotech. Antibody against c-met was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). GFR-Matrigel was obtained from Collaborative Biomedical Products (Pittsburgh, PA).

Cell preparation and culture

Sertoli cells were prepared using 10- to 40-day-old male Wistar rats, as previously described (24). Cells were cultured in MEM for 3 days to allow cellular monolayer formation before cells utilization. When indicated, germ cells associated with the Sertoli cell monolayers were detached according to Galdieri *et al.* (24). Purified myoid cells were prepared according to Palombi *et al.* (25) using the same animals killed for Sertoli cell preparation. In brief, small explants of decapsulated testes were digested for 30 min at 32 C by 0.25% trypsin in PBS to detach the interstitium. The seminiferous tubules were sedimented by gravity and the supernatant was removed and centrifuged at 300× *g* for 5 min to sediment the interstitial cells (fraction called "interstitium"). The seminiferous tubules were treated with collagenase A (0.5 mg/ml) for 30 min at 32 C and then sedimented again by gravity. The supernatant was removed and the tubules washed with PBS and sedimented again. The supernatant was removed, pooled with the first and centrifuged for 2 min at 40× *g*. The pellet was further digested in 0.1% trypsin, 1 mM ethylenediaminetetra-acetic acid (EDTA) in Hanks' solution without Ca^{2+} and Mg^{2+} to obtain a single cell suspension, applied on a discontinuous Percoll gradient and centrifuged for 20 min at 800 × *g* at room temperature. When cells were prepared from 35- to 40-day-old rats, the testes were digested by 0.15% trypsin and incubation time was reduced to 15 min. The fraction corresponding to the myoid cells (density, 1.075 g/ml) was collected, the cells were washed twice with MEM, suspended in MEM and plated at the desired density. The purity of the cells used for the experiments, assessed by the presence of alkaline phosphatase activity (26), was never lower than 94%.

GFR-Matrigel cultures

The bottoms of 24-well tissue culture plates were coated with 150 μl per well of reconstituted basement membrane containing a reduced amount of growth factors (GFR-Matrigel) mixed in a 1:1 ratio with culture medium (MEM). The plates were then incubated for 30 min at 37 C and 5% CO_2 to allow solidification of the gel. Primary Sertoli cells were seeded on top of the gel at a density of 2×10^5 cells/well in 1 ml MEM. Hepatocyte growth factor (recombinant HGF, kindly provided by Dr. M. C. Stella, Torino University) at various concentrations (15–100 U/ml) was added to primary Sertoli cells growing on GFR-Matrigel in MEM.

RNA isolation and Northern blot analysis

Total RNA was extracted from total testes or cultured cells according to the method of Chomczynski and Sacchi (27). The integrity of the RNA was tested through the presence of the ribosomal species in formaldehyde denaturing gels. Northern blot analysis using 30 μg of RNA in each lane was performed on 1% agarose/formaldehyde gels and transferred to Hybond-N+ membrane. Prehybridization, hybridization and washings were performed according to the conditions suggested by the supplier. Rat met complementary DNA (cDNA) was labeled using a random primer labeling kit. Relative differences in hybridization were determined by means of the scanning densitometry of autoradiograms (LKB 2222–020 UltroScan XL; GelScan XL software). C-met expression in total RNA was normalized to the signal for the constitutively expressed glyceraldeyde-3-phosphate dehydrogenase (GAPDH).

Immunocytochemistry

Myoid and Sertoli cells grown as indicated above were fixed in ethanol-acetone (1:1) according to the suggestions of the antibody manufacturer for 10 min at −20 C, treated with 5% BSA for 30 min at room temperature to minimize the nonspecific binding and then exposed to a polyclonal antibody against the carboxy terminus of c-met (1:50 dilution) 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 rabbit antigoat antiserum. The cells were rinsed again with PBS and mounted in buffered glycerol (pH 9). As a negative control, the primary antibody was omitted. Samples were analyzed using a Carl Zeiss axioplan fluorescence microscope.

Statistical analysis

Statistical analysis was performed by Student's *t* test.

Results

C-met gene expression

The expression of c-met gene was analyzed in total testes isolated from rats of different ages (10, 20, 35, and 60 days old) using a specific cDNA. Total RNA was extracted and Northern blot hybridization showed the presence of a c-met specific message in the RNAs isolated from the rats of different ages (Fig. 1A). As a positive control, RNA was extracted from the liver of the 20-day-old animals. One single messenger RNA (mRNA) species was detected, comparable in weight to the liver c-met mRNA (9 kb), and the densito-

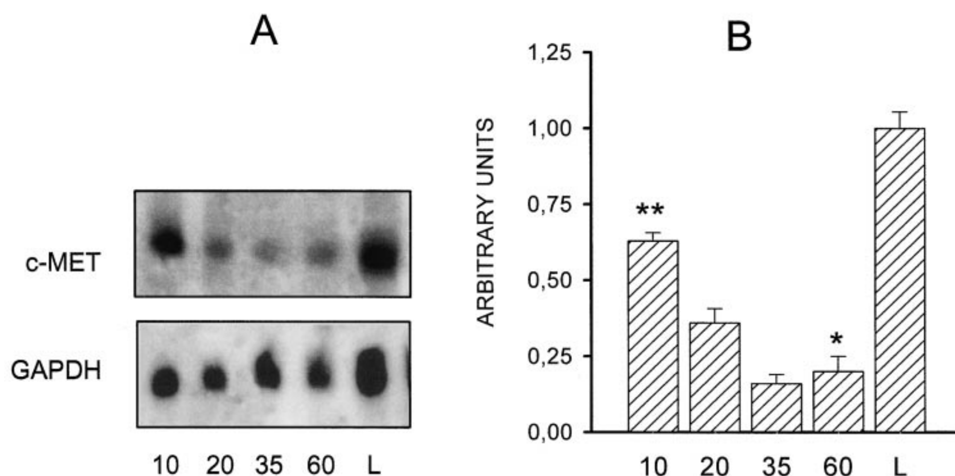

FIG. 1. Expression of c-MET mRNAs in testes isolated from 10-, 20-, 35-, and 60-day-old rats. A, Northern blot analysis was performed on 30 μg of total RNA extracted from liver (L) and total testes. X-ray films were exposed for 14 days with intensifying screens. B, Densitometric scanning of the autoradiograms. The c-met mRNA levels are expressed as arbitrary units considering as '1' the liver c-met mRNA levels. The mean ± SE of at least three experiments are reported. **, $P < 0.01$ *vs.* * values.

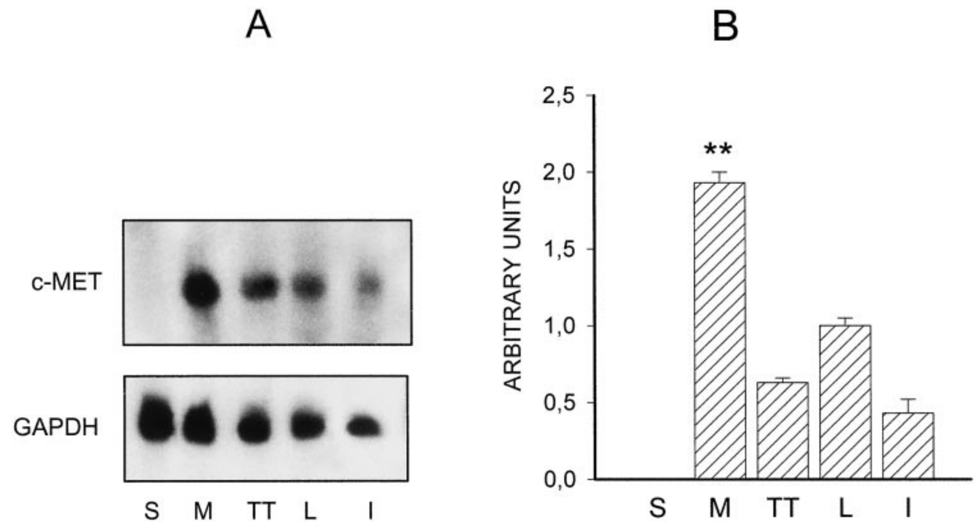

FIG. 2. Expression of c-met mRNA in different cell populations isolated from testes of 10-day-old rats. A, Northern blot analysis was performed on 30 μg of total RNA extracted from Sertoli cells (S), myoid cells (M), interstitial cells (I) and total testes (TT). L, RNA from prepubertal rat liver. B, Densitometric scanning of the autoradiograms. The c-met mRNA levels are expressed as arbitrary units considering as '1' the liver c-met mRNA levels. The mean ± SE of at least three experiments are reported. **, $P < 0.01$ *vs.* TT and I values.

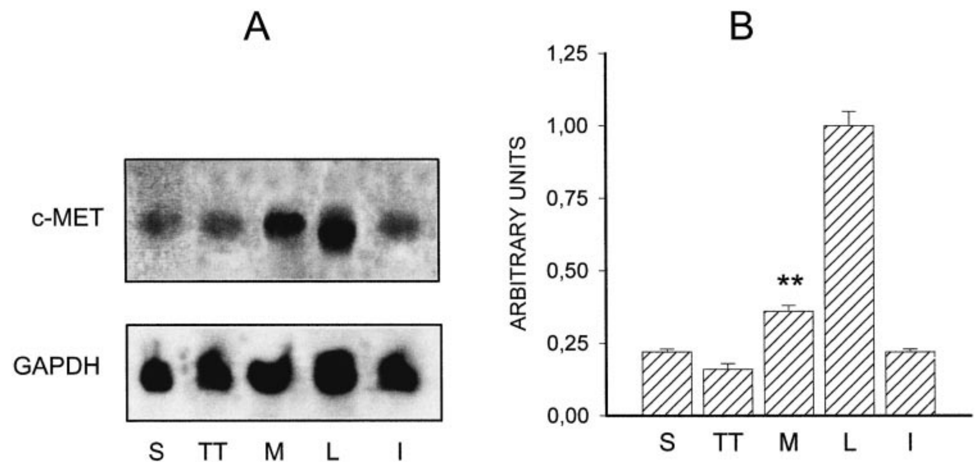

FIG. 3. Expression of c-met mRNA in different cell populations isolated from testes of 35-day-old rats. A, Northern blot analysis was performed on 30 μg of total RNA extracted from Sertoli cells (S), myoid cells (M), interstitial cells (I), and total testes (TT). L, RNA from prepubertal rat liver. B, Densitometric scanning of the autoradiograms. The c-met mRNA levels are expressed as arbitrary units considering as '1' the liver c-met mRNA levels. The mean ± SE of at least three experiments are reported. **, $P < 0.05$ *vs.* TT values and not significant (n.s.) *vs.* I and S values.

metric scanning of the band showed that the expression levels were higher in the 10-day-old rats and decreased with increasing age of the donor animals (Fig. 1B).

To understand in which cell type(s) of the testis c-met RNA is expressed, homogeneous cell populations of Sertoli and myoid cells were isolated from 10-day-old animals. Total RNA was extracted from these cell populations and from a mixed cell population of interstitial cells (see *Materials and Methods*). Northern blot analysis of c-met RNA isolated from the cell populations (Fig. 2A) as well as the densitometric scanning of the bands (Fig. 2B) are reported. C-met was highly expressed in myoid cells, the expression levels appearing to be almost twice as high as those present in the liver used as a positive control. A relevant expression of c-met was also detected in the mixed cell population isolated from the interstitial tissue. By contrast, Sertoli cells did not express positive signals. Myoid cells are c-met positive even in the 20-day-old rats, whereas Sertoli cells are negative as we previously observed (22).

C-met expression was also analyzed in cell populations isolated from testes of 35-day-old animals. Experiments of Northern blotting were performed and the results obtained showed that even in 35-day-old animals both myoid cells and interstitial cells, are positive for c-met expression. It is noteworthy that, contrary to what was observed in 10- and 20-day-old animals, at 35 days c-met expression in myoid cells is lower than that in the liver used as a positive control. Sertoli cells isolated from 35-day-old rats, unlike those from younger rats, express c-met gene although at lower levels than myoid cells (Fig. 3). To better characterize the temporal appearance of c-met expression in Sertoli cells, RNA was extracted from Sertoli cells isolated from 25- and 30-day-old animals and the expression of the receptor tested by means of Northern blotting. As shown in Fig. 4, in this cell type c-met expression is already detectable at 25 days of age, although at low levels, its expression increasing at 35 days.

The presence of c-met protein was detected by immunolocalization on purified cell populations of myoid cells isolated from animals of 10-, 20-, and 35-day-old as well as on Sertoli cells isolated from 35-day-old rats. On both myoid cells and Sertoli cells, c-met protein was present and its distribution was spotted (data not shown).

HGF effects on myoid and Sertoli cells

The morphology of myoid cells isolated from 10-day-old animals and cultured in the presence of HGF (50–100

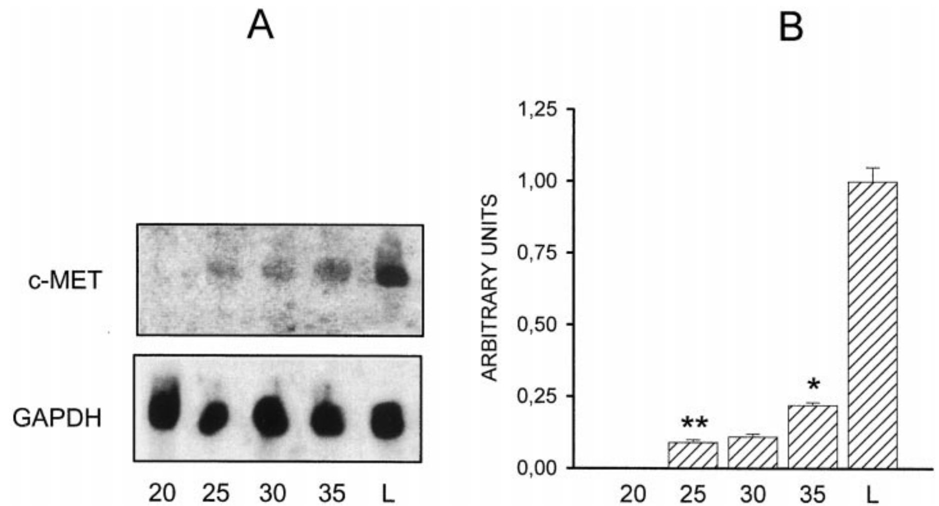

FIG. 4. Expression of c-met mRNA in Sertoli cells isolated from testes of rats of different ages. A, Northern blot analysis was performed on 30 μg of total RNA extracted from Sertoli cells isolated from 20-, 25-, 30-, 35-day-old rats. L, RNA from prepubertal rat liver. B, Densitometric scanning of the autoradiograms. The c-met mRNA levels are expressed as arbitrary units considering as '1' the liver c-met mRNA levels. The mean ± SE of at least three experiments are reported. **, $P < 0.05$ *vs.* * values.

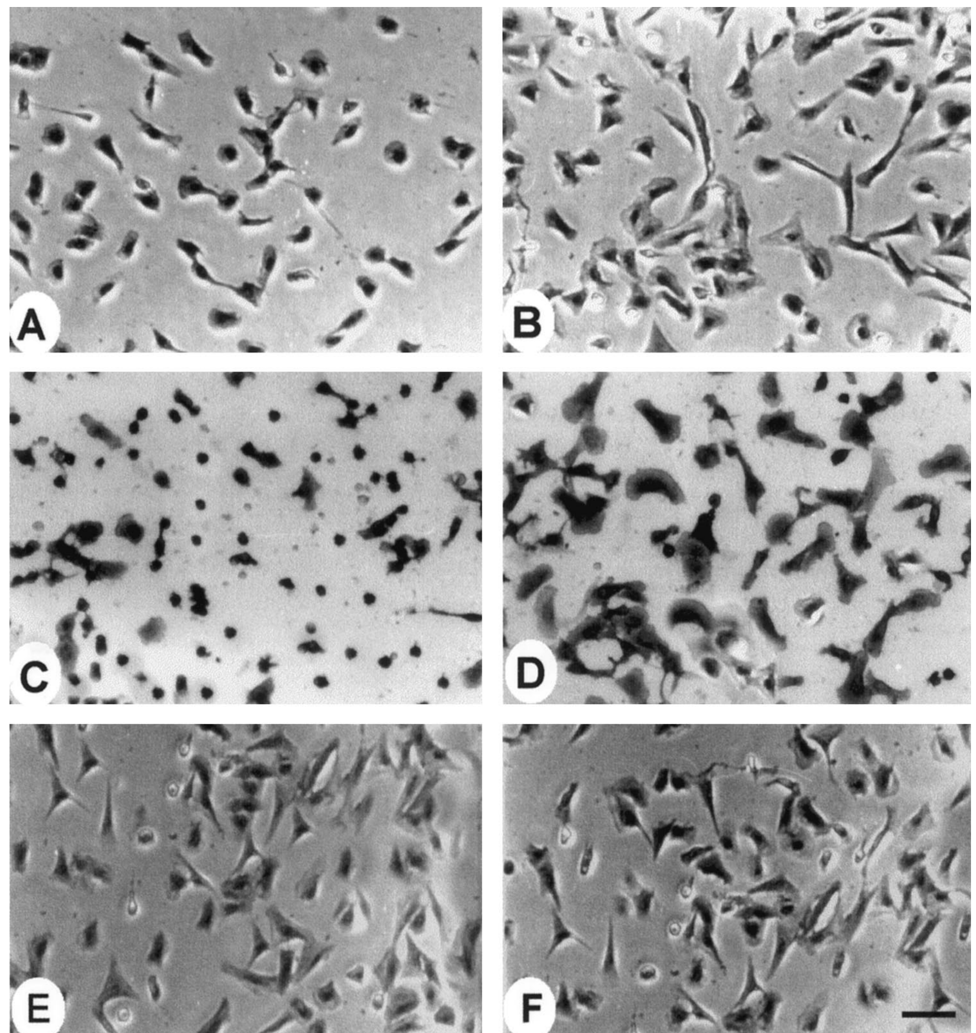

FIG. 5. Bright-field microscopy of myoid cells isolated from 10-, 20-, and 35-day-old rats, cultured for 24 h in the absence (A, C, E) and in the presence (B, D, F) of HGF (100 U/ml). Cells were fast-blue stained to detect alkaline-phosphatase activity that is a marker for myoid cells. *Bar*, 45 μm.

U/ml) undergoes a change from a round, compact (Fig. 5A) to an elongated, enlarged shape (Fig. 5B). Similar behavior was shown by the myoid cells isolated from 20-day-old rats and cultured in the same conditions (Fig. 5, C and D), as we previously demonstrated (22). The maximal effect was evident after 16 h of treatment with HGF, with some of the cells acquiring a fibroblast-like morphology. HGF addition to the myoid cells isolated from 35-day-old animals did not produce evident morphological effects (Fig. 5, E and F).

Sertoli cells isolated from 35-day-old animals and plated at 0.5×10^5 cells/cm^2 were cultured in MEM in the presence or in the absence of different doses of HGF ranging from 15 to 100 U/ml. After 20–24 h of culture, HGF induced evident changes in the general aspect of the culture: untreated cells formed an almost continuous monolayer (Fig. 6 B), whereas treated cells were organized in clusters of different sizes (Fig. 6A). HGF administration did not induce mitosis or detachment of the Sertoli cells because the number of the cells did not change after treatment (data not shown). Sertoli cells isolated from 35-day-old rats were also plated on GFR-Matrigel. Both control and HGF-treated cells adhered very rapidly to the reconstituted basement membrane and then migrate during the subsequent hours of culture. After 20–24 h of culture, Sertoli cells maintained in medium alone formed aggregates of different sizes which maintained their morphology and remained attached to the GFR-Matrigel for at least 7 days (Fig. 6D). Sertoli cells cultured in the presence of HGF showed a higher degree of reorganization and formed cord-like structures (Fig. 6C). The number of the aggregates present in the cultures was inversely proportional to the amount of supplemented HGF and the cells treated with the higher doses (50–100 U/ml) almost totally reorganized in cords that formed anastomoses with neighboring cords during the next 24 h of culture. The described effect was evident in all the three experiments performed. Sertoli cells isolated from 20-day-old rats were also used for similar experiments. When cultured on either plastic or GFR-Matrigel, the morphology of the cells was not altered by the presence of HGF. As shown in Fig. 6F, on GFR Matrigel Sertoli cells formed aggregates of irregular shape and size, morphologically similar to the aggegates formed in the presence of HGF (Fig. 6E).

Discussion

We recently reported that the gene encoding the HGF receptor, c-met, is expressed in the seminiferous tubules of prepubertal rat testes (22) and in total testes isolated from rats of different ages (28). In the present work, we extend our previous study reporting that in myoid cells c-met is expressed throughout postnatal testicular development, whereas in Sertoli cells c-met begins to be expressed at puberty. Purified myoid cell populations were prepared from animals at different postnatal ages; a c-met specific message was detected at all the ages considered although at different levels. In our myoid cell preparations obtained from 35-day-old rats, a lower expression of c-met was in fact detected. However, met protein appears to be present in myoid cells

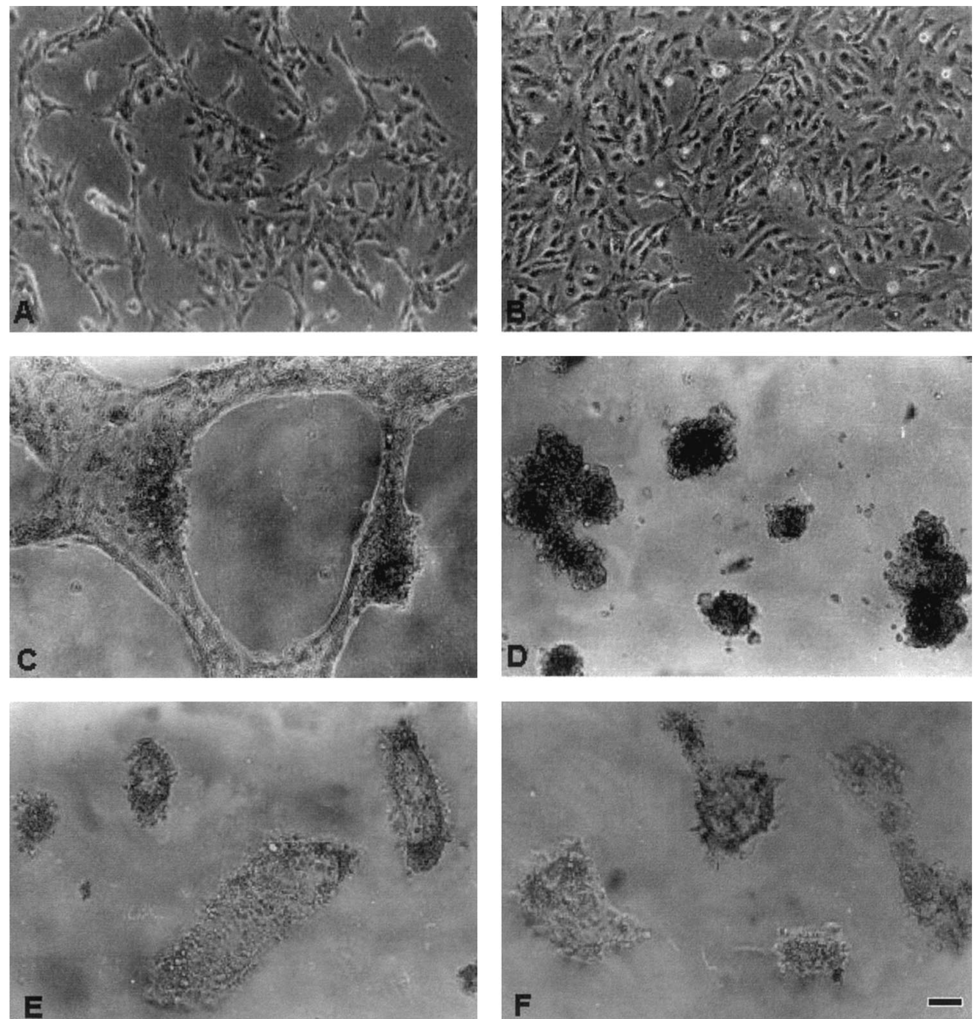

FIG. 6. Phase-contrast microscopy of Sertoli cells isolated from 35-day-old (A–D) and 20-day-old (E, F) rats and cultured for 20 h in the presence (A, C, E) and in the absence (B, D, F) of HGF (50 U/ml). In C, D, E, and F, Sertoli cells were plated on GFR-Matrigel. In A and B Sertoli cells were plated on plastics. The figure reports the results obtained in one of the three experiments performed. *Bar*, 77 μm.

at all the ages considered, localized in a spotted way as shown by the immunocytochemistry experiments. In addition, we demonstrate that myoid cells isolated from prepubertal rats change their morphology following HGF administration shifting from a compact to an enlarged shape, whereas myoid cells isolated from older animals do not show evident morphological changes after HGF treatment. This finding may be due either to the lower expression of c-met detected in the cells isolated from older animals or to a different physiological status of the cells whose responsiveness to HGF may decrease during the postnatal differentiation of the testis. This decline of c-met expression in myoid cells is paralleled by the detection of the expression of this gene in Sertoli cells in which c-met is first detectable by Northern blot at 25 days of age and more markedly expressed in cells isolated from mature animals. HGF has a functional role on c-met-expressing Sertoli cells as suggested by the behavior of cells cultured in the presence of this growth factor that form cell clusters only when HGF is present in the culture medium. To better characterize the HGF effect on Sertoli cells, a reconstituted basement membrane was used given that the extracellular matrix is a dynamic structure able to modulate signal transduction pathways triggered by growth factors interacting with cell surface receptors. Sertoli cells were cultured on GFR-Matrigel to minimize the effect of the growth factors contained in the reconstituted basement membrane since Matrigel is known to induce the formation of cord-like structures (29). If plated on GFR-Matrigel, Sertoli cells form aggregates without a clear morphological structure. On the contrary, when HGF was supplemented to the culture medium, cells reorganized into cord-like structures resembling testicular seminiferous cords, thus suggesting that HGF exerts a tubulogenetic role in the testis. Our finding is in line with the reported tubulogenetic activity of HGF able to induce tubulogenesis in different cell types derived from kidney, mammary gland, mouse Sertoli cells and in an immortalized Sertoli cell line (SF7) (15–17, 23). As we have previously demonstrated, HGF is also able to induce tubule formation during embryonic differentiation of the testis (19).

Moreover it is interesting to consider that c-met expression in Sertoli cells correlates with the period of time in which lumen formation occurs in the tubules of the postnatal testis because the lumen is present in the totality of the rat testis tubules only 30 days after birth (30). During lumen formation, the cells of the tubules have to rearrange their relative positions to allow the formation of the lumen and therefore have to disarrange their cellular contacts, move and establish new contacts and new junctions with the neighboring cells. In view of its scattering activity, it is conceivable that HGF plays a key role in these cellular movements and/or rearrangements. It has been reported that, following HGF addition, epithelial cells scatter as a consequence of decreased cell-cell adhesion and disassembly of junctional components (31). Moreover, HGF down regulates the expression of the gap junctional proteins, connexins, thereby increasing their protein degradation (32, 33).

In conclusion, the data we report in the present paper demonstrate the presence of the HGF receptor during testicular development and its functional activity. We show that functionally active c-met is present both in myoid and Sertoli cells, that c-met expression in Sertoli cells is developmentally regulated, and that HGF is able to induce the formation of cord-like structures. C-met expression in Sertoli cells is temporally concomitant with lumen formation in rat seminiferous tubules, and this temporal correlation suggests that HGF is involved in this essential morphogenetic event, possibly modulating Sertoli cell positional rearrangement during tubule formation. Because c-met is functionally active, we demonstrate that HGF is one of the growth factors regulating myoid and Sertoli cell functional activities during testis development and, considering the fundamental role exerted by these cells in the formation of the microenvironment necessary for germ cell differentiation, we conclude that HGF indirectly modulates mammalian spermatogenesis.

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