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# Pleiotropic activity of hepatocyte growth factor during embryonic mouse testis development

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**Abstract**

The hepatocyte growth factor (HGF) is a pleiotropic cytokine whose action is mediated by c-met, a glycoproteic receptor with tyrosine kinase activity which transduces its multiple biological activities including cell proliferation, motility and differentiation. During embryonic development HGF acts as a morphogenetic factor as previously demonstrated for metanephric and lung development. Recently, culturing male genital ridges, we demonstrated that HGF is able to support in vitro testicular cord formation. In the present paper we report the expression pattern of the HGF gene during embryonic testis development and the multiple roles exerted by this factor during the morphogenesis of this organ. Northern blot analysis reveals a positive signal in urogenital ridges isolated from 11.5 days post coitum (dpc) embryos and in testes isolated from 13.5 and 15.5 dpc male embryos. On the contrary HGF mRNA is undetectable in ovaries isolated from 13.5 and 15.5 dpc embryos. Moreover, we demonstrate that HGF is synthesized and secreted by the male gonad and is biologically active. These data indicate a male specific biological function of HGF during embryonic gonadal development. This hypothesis is supported by the in vitro demonstration that HGF acts as a migratory factor for male mesonephric cells which is a male specific event. In addition we demonstrate that during testicular development, HGF acts as a morphogenetic factor able to reorganize dissociated testicular cells which, under HGF stimulation, form a tridimensional network of cord-like structures. Finally, we demonstrate that HGF induces testicular cell proliferation in this way being responsible for the size increase of the testis. All together the data presented in this paper demonstrate that HGF is expressed during the embryonic development of the testis and clarify the multiple roles exerted by this factor during the morphogenesis of the male gonad. 

*Keywords*: Hepatocyte growth factor; Mouse testis; Testis development; Cord formation

## 1. Introduction

The hepatocyte growth factor/scatter factor (HGF/SF) is a pleiotropic cytokine initially discovered as a factor able to induce liver regeneration (Nakamura et al., 1984, 1989) and subsequently identified as a scatter factor (Stoker et al., 1987; Weidner et al., 1993) able to regulate the cellular activities of normal and transformed cell types (Iyer et al., 1990; Cooper et al., 1984). HGF action is mediated by c-met, a glycoproteic receptor with tyrosine kinase activity codified by the MET protooncogene (Park et al., 1987). C-met specifically binds to HGF transducing its multiple biological activities which includes cell proliferation, motility and differentiation (Birchmeier and Gherardi, 1998). During mouse development HGF regulates embryonic morphogenesis (Sonnenberg et al., 1993) and epithelial-mesenchymal conversion (Tsarfaty et al., 1994; Karp et al., 1994). The morphogenetic role of HGF previously described in epithelial cells isolated from different tissues (Montesano et al., 1991; Soriano et al., 1995; Brinkmann et al., 1995) has also been demonstrated during lung development being, exogenously-added HGF, able to induce branching morphogenesis of the fetal lung (Ohmichi et al., 1998). The crucial role of HGF during the development of different epithelial organs has been pointed out looking at the HGF-null mice embryos who die before birth due to placental defect (Schmidt et al., 1995; Uehara et al., 1995; Birchmeier and Gherardi, 1998).

Seminiferous cord formation is the critical morphogenetic event for testis development. This complex event requires proliferation and differentiation of pre-Sertoli cells present in the 'morphologically indifferent' gonad and their association with the germ cells previously migrated into the differentiating gonad. To obtain a correct cord formation the migration of mesenchymal cells from the

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mesonephros into the developing gonads is also necessary (Merchant-Larios et al., 1993; Buehr et al., 1993; Martineau et al., 1997; Tilmann and Capel, 1999) and the action of specific factors to coordinate the correct association between somatic and germ cell is required. Several growth factors are involved in embryonic testis differentiation: in particular TGFα (Levine et al., 2000), TGFβ (Olaso et al., 1998; Cupp et al., 1999a), NGF and NT3 (Cupp et al., 2000) are expressed in the developing testis and affect embryonic testis growth. Neurotrophins are regulators of paracrine cell–cell interactions in the embryonic testis (Cupp et al., 2000) and p75 neurotrophin receptor is expressed in the cells migrating from mesonephros into the urogenital ridge (UGR, Campagnolo et al., 2001). Also retinoids are regulators of the embryonic development of the testis affecting basement membrane formation of rat seminiferous tubules (Marinos et al., 1995) and inhibiting testis growth and seminiferous cord formation probably increasing the expression of the TGFβ isoforms (Cupp et al., 1999b; Livera et al., 2000). In a recent paper we demonstrated that the HGF/HGFR system is heavily involved in testis development since c-met is expressed early in the developing testis and HGF is able to support testicular cord formation whereas other growth factors presumably involved in fetal testis development, such as TGFβ, do not exert the same morphogenetic effect (Ricci et al., 1999). Conversely, PDGF, in line with the reported ability in influencing lung branching morphogenesis (Souza et al., 1995), is able to support testicular cord formation (Ricci et al., 1999). In the present paper we report that HGF is expressed during embryonic testis development and we describe its multiple functional roles in testicular and mesonephric cells.

## 2. Results

### 2.1. HGF expression in developing gonads

#### 2.1.1. Northern blot analysis

We studied the temporal expression pattern of HGF mRNA during gonadal development. At 11.5 dpc, male and female gonads are morphologically indistinguishable and the embryos were sexed scoring the presence of the Barr bodies as indicated in Section 4. Intact UGRs (containing both mesonephros and gonadal primordium) from 11.5 dpc mouse embryos have been explanted and total RNA was extracted from male and female UGRs as well as from limb buds and liver. At 13.5 and 15.5 dpc, since the gonads are morphologically distinguishable, testes and mesonephroi were separately isolated and total RNA was extracted. RNA was also extracted from limb bud, liver and ovary of the female embryos of the same age. The presence of one HGF specific transcript was detected by Northern blot in the RNAs extracted from UGR (11.5 dpc), embryonic testes and mesonephroi either at 13.5 dpc or at 15.5 dpc, as shown in Fig. 1. The molecular weight of the detected mRNA species is estimated to be 6 kb since it is coincident with the single mRNA species detectable in the postnatal liver RNA (not shown). HGF was heavily expressed in the testes at the two different ages although the levels decreased with increasing age. As expected (Sonnenberg et al., 1993), HGF expression was detected in the limb bud and the expression levels decreased with age whereas in liver the expression levels were similar between 11.5 and 13.5 dpc as indicated by the densitometric scanning of the bands. In the female embryos the UGR isolated from 11.5 dpc embryos showed one HGF specific transcript whereas in the developing ovary positive signals for HGF were not detected.

#### 2.1.2. Immunolocalization of HGF

The presence of the factor was studied by immunohistochemistry in testes isolated from 13.5 and 15.5 dpc embryos. HGF was present in the tunica albuginea cells, in the interstitial compartment and in the flat peritubular

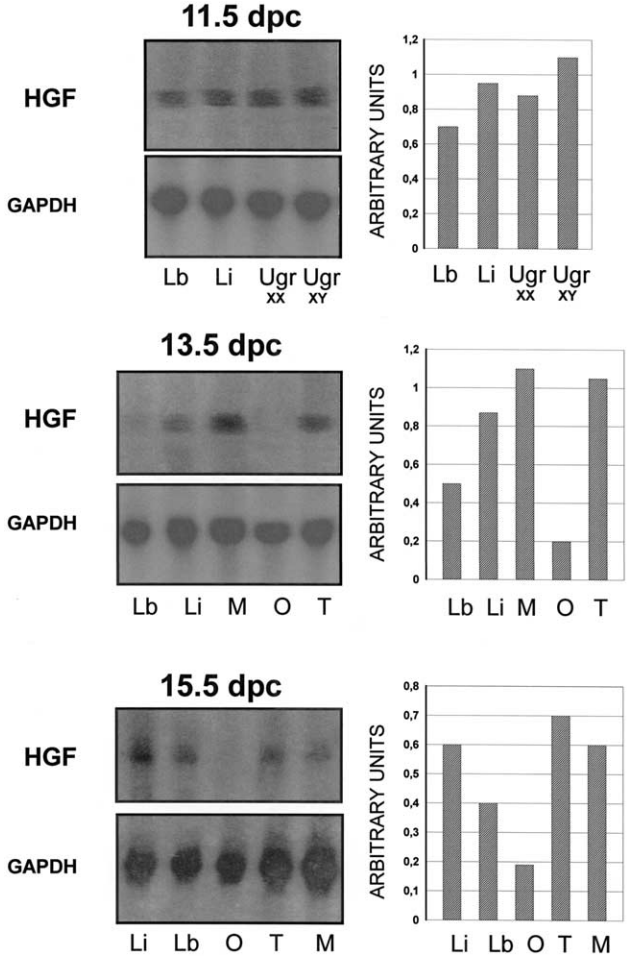



Fig. 1. Expression of HGF mRNA in the developing male gonad. Left: Northern blot analysis was performed on 30 μg of total RNA extracted from the buds of different organs at 11.5, 13.5 and 15.5 dpc. UGR, urogenital ridges; Li, liver; Lb, limb buds; T, testis; O, ovary; M, male mesonephric tissue. X-ray films were exposed for 14 days with intensifying screens. Right: densitometric scanning of the autoradiograms. The results of one representative experiment of the two performed are reported.

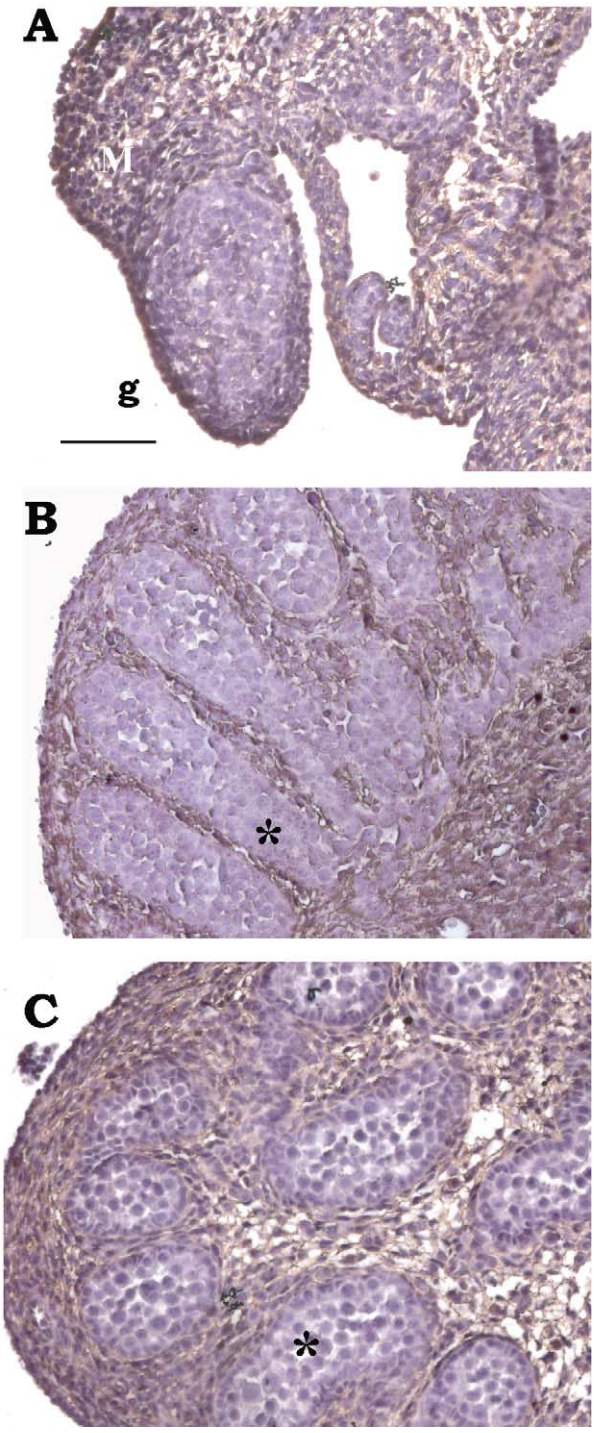
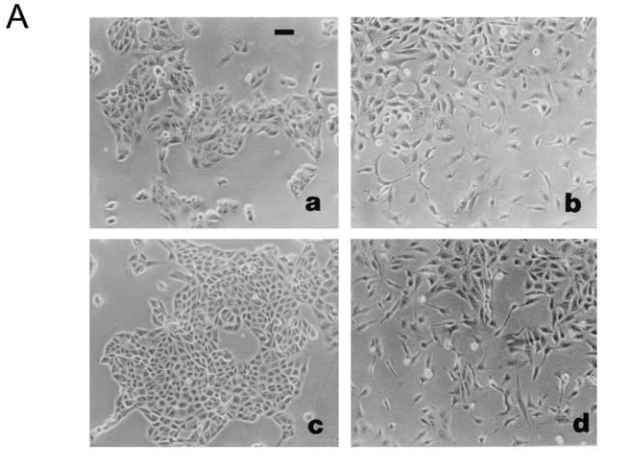



Fig. 2. Immunohistochemical localization of HGF in the developing testis. (A) Section of urogenital ridge and mesonephric tissue isolated from 11.5 dpc embryo (g = gonad; M = mesonephic tissue). (B) Section of testis isolated from 13.5 dpc embryo (* = testicular cord). (C) Section of testis isolated from 15.5 dpc embryo (* = testicular cord). Bar: 66 μm.

cells in testes isolated from both 13.5 and 15.5 dpc embryos (Fig. 2B, C). In the undifferentiated male UGRs isolated from 11.5 dpc embryos, the positivity for HGF was present in the coelomic epithelium surrounding the genital ridge and in single or small aggregates of cells located inside the genital ridge (Fig. 2A). At this age, the mesonephric mesenchyme appears to heavily express HGF.

### 2.1.3. Scatter activity of gonadal culture medium

Conditioned media obtained from 13.5 dpc testis cultures were added to cultured MDCK cells. Fig. 3A shows the appearance of untreated MDCK cells (a) and the scattered appearance of the same cells after a 16 h incubation in medium supplemented with HGF (b), in conditioned medium from male (d) embryonic gonads. The scatter effect was obtained with media conditioned for 4 days and lasted for at least 2 days. The effect was obtained both on preformed clusters of MDCK cells and on single cells which, after the addition of gonadal culture medium, lost the ability to form clusters. Gonadal culture medium was diluted several times and each diluted medium tested for scatter activity. Four day-conditioned medium was able to clearly induce scattering of MDCK cell clusters, and the same medium, at a 1:3 dilution, was still effective in inducing partial MDCK cluster dissociation (Fig. 3B). According to Stoker and Perryman (1985) we calculated that 30 U/ml of scatter activity was present in our undiluted conditioned medium and, as a control, we verified that 30 U/ml of recombinant HGF was able to induce a similar dissociation of MDCK clusters. On the contrary, conditioned medium obtained from 13.5 dpc ovary cultures failed to have any




B

Conditioned media dilution

| | 1:1 | 1:2 | 1:3 | 1:4 |
|---|---|---|---|---|
| 13.5 dpc testis | + + + | + + | + | N.S. |
| 13.5 dpc ovary | N.S. | N.S. | N.S. | N.S. |

Fig. 3. Scattering of MDCK epithelial cells induced by testicular cell conditioned medium. (A, top panel) Morphological appearance of cells cultured for 16 h in DMEM (a), in medium added with HGF (30 U/ml) (b), in ovarian cell conditioned medium (c), in testicular cell conditioned medium (d). Bar: 67 μm. (B, bottom panel) Scattering obtained with diluted gonadal conditioned medium. N.S. = no scatter.

effect on the scatter activity of MDCK cells even when supplemented with serum (Fig. 3A(c)).

### 2.2. HGF effects during testis development

#### 2.2.1. Testicular cord formation and polarized laminin deposition

HGF exerts a biological function in testis development since, as we previously demonstrated, UGRs isolated from 11.5 dpc male embryos cultured in the presence of a chemically defined medium supplemented with HGF (Ricci et al., 1999) were able to differentiate and in vitro testicular cord formation was obtained after 4 days of culture. After culture in the presence of HGF, the gonadal morphology was similar to that obtained by culturing UGR in medium supplemented with serum (Merchant-Larios et al., 1993; Buehr et al., 1993; Moreno-Mendoza et al., 1995). Laminin distribution during cord formation was studied. At 11.5 dpc laminin is present in the 'indifferent' testis distributed almost equally in the different portions of the gonadal tissue (g) (Fig. 4A). Culturing UGRs in medium without any supplementation, we obtained gonadal ridge survival and size increase without well evident morphological differentiation and only very partial laminin accumulation (arrow) in specific portions of the gonad (Fig. 4B). Usually laminin accumulation was localized outside differentiated epithelial cells, that is polarized cells, looking like cells starting to form an organized structure. The UGRs cultured in medium supplemented with HGF or serum after culture formed well organized cords and laminin was localized, as in vivo (data not shown), in the basement membrane around the newly formed seminiferous cords (Fig. 4C, D, respectively).

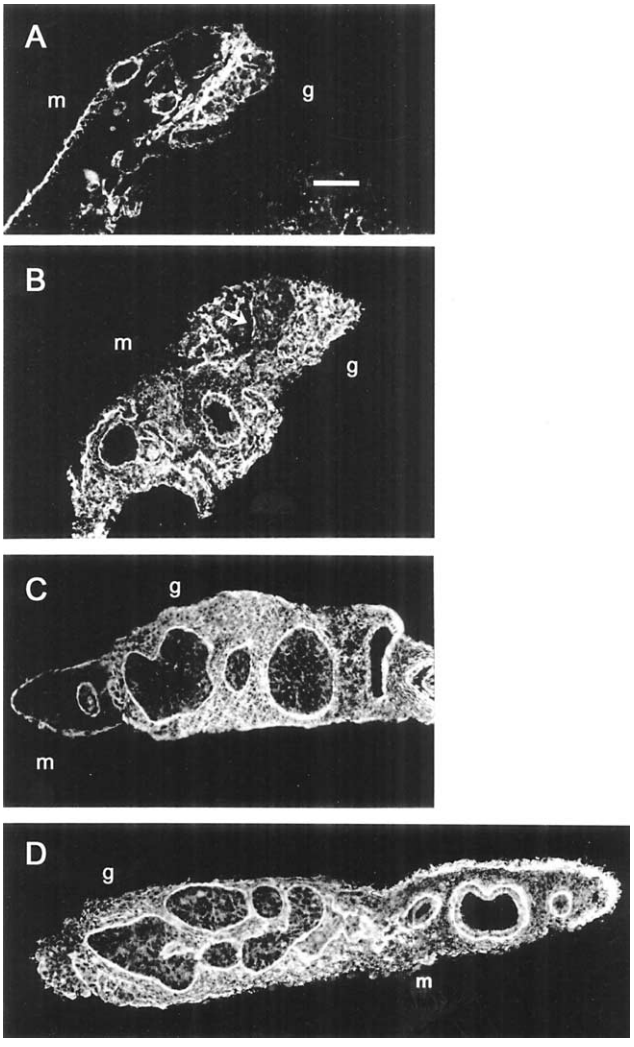



Fig. 4. Immunohistochemical detection of laminin distribution in male 11.5 dpc UGR before (A) and after 3 days of culture in medium alone (B) or supplemented with HGF (C) or serum (D). The genital ridge (g) and the mesonephros (m) are indicated. Bar: 78 μm.

#### 2.2.2. Mesonephric cell migration

Mesonephroi isolated from 13.5 dpc mouse embryos were dissociated by incubation in trypsin (Pesce et al., 1994) and $2.5 \times 10^5$ cells were seeded in the upper side of Boyden chambers. The lower chambers of the Boyden apparatus were filled with medium alone or supplemented with different amounts of HGF (100–400 U/ml). As shown in Fig. 5A, HGF strongly increases migration activity of the mesonephric cells which acquire migratory capacity as a consequence of HGF stimulation. The described effect was significant at all the doses of the factor tested. The mitogenic effect of HGF on the mesonephric cells was also studied labeling the cells for 16 h with radioactive thymidine (Fig. 5B). No significant differences were detected between control and HGF-treated cells.

#### 2.2.3. In vitro cord formation

Testes from 13.5 dpc embryos were disaggregated by incubation in trypsin (Pesce et al., 1994), plated on GFR-Matrigel and cultured in the presence or in the absence of different doses of HGF (5–100 U/ml) (Fig. 6). Either control or HGF-treated cells adhere very rapidly to the reconstituted basement membrane and then form cellular aggregates during the next hours of culture. After 16–20 h of culture, cells maintained in medium alone were organized in clusters of different sizes and shapes which maintained their morphology and remained attached to the GFR-Matrigel for at least 48 h (Fig. 6A(a)) whereas cells cultured in the presence of the factor showed higher reorganization. HGF-induced changes that are evident in the general aspect of the culture in which cord-like structures were formed (Fig. 6A(b)). In all the five experiments performed the cells treated with the highest dose of HGF (100 U/ml) reorganized in cords which formed anastomoses with neighboring cords frequently giving rise to tridimensional networks on top of the Matrigel layer (Fig. 6A(b)). Cellular reaggregation has been sometimes observed also in control cultures but consisting of very thin and short structures with respect to the dimension (width and length) of the structures formed by the HGF-treated cells. In Fig. 6B the width of the struc-

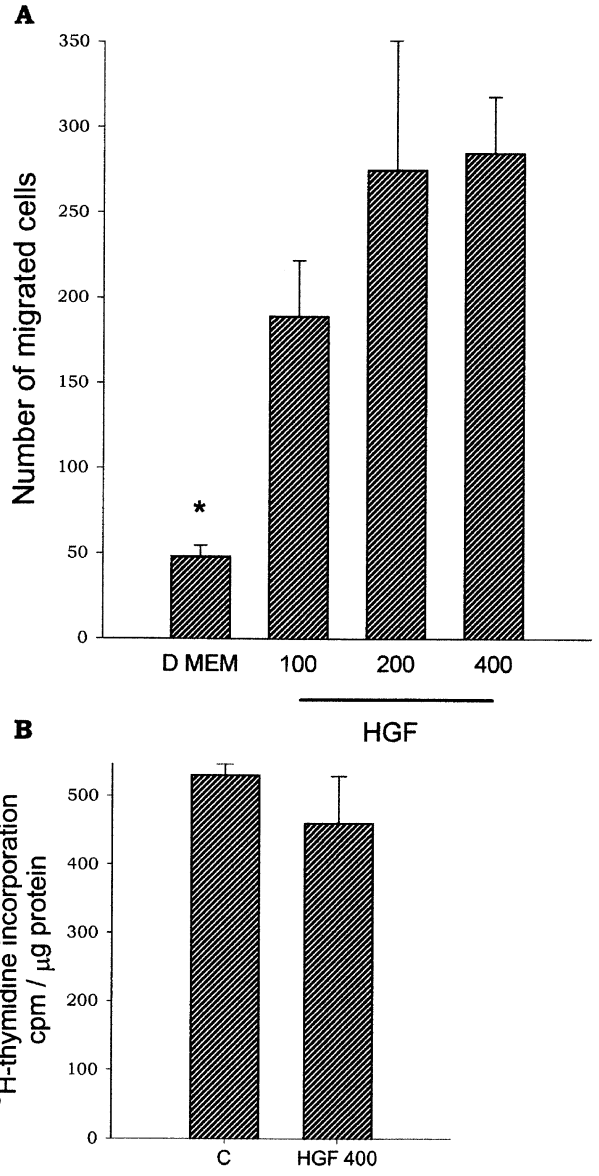



Fig. 5. (A) Migration activity of mesonephric cells isolated from 13.5 dpc male embryos. The number ±SE of control (DMEM) and HGF (100–400 U/ml) treated cells with migration capacity is reported. *, $P < 0.001$ vs. HGF treated samples. (B) $^3$H-thymidine incorporation of mesonephric cells isolated from 13.5 dpc male embryos cultured in DMEM or treated with HGF (400 U/ml). $P$ = n.s. (not significant).

tures formed by control (30 ± 2.5 μm) and treated cells (74 ± 7 μm) is reported.

### 2.2.4. Mitogenic effects

Testes from 13.5 dpc embryos were disaggregated as indicated previously and plated on GFR-Matrigel. The cell suspension was cultured in the presence of medium alone or supplemented with HGF (100 U/ml) for 16 h in the absence of serum (Fig. 7). At the end of the incubation time, Matrigel was digested by collagenase-dispase (3 mg/ml, 37°C for 30 min), the cells present in the culture dishes were counted (Fig. 7A) and the amount of DNA evaluated (Fig. 7B). The

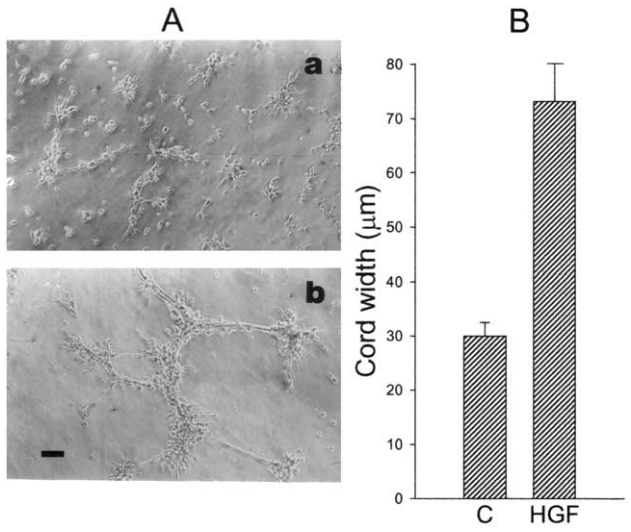



Fig. 6. Phase-contrast microscopy (A) of embryonic testicular cells cultured for 20 h on GFR-Matrigel in the absence (a) or in the presence (b) of HGF (100 U/ml). In (B) the width of the cord-like structures formed (means ± SE, $n = 5$) is reported; $P < 0.001$. Bar: 87 μm.

results obtained clearly show that HGF induces a parallel increase of DNA synthesis and cellular duplication. Similar results were obtained by labeling the cells with radioactive thymidine (data not shown).

## 3. Discussion

In this study we report the evidence that HGF is expressed in the embryonic testis and plays multiple roles during mammalian testicular differentiation. We demonstrate that this growth factor is expressed in the mouse UGR at the

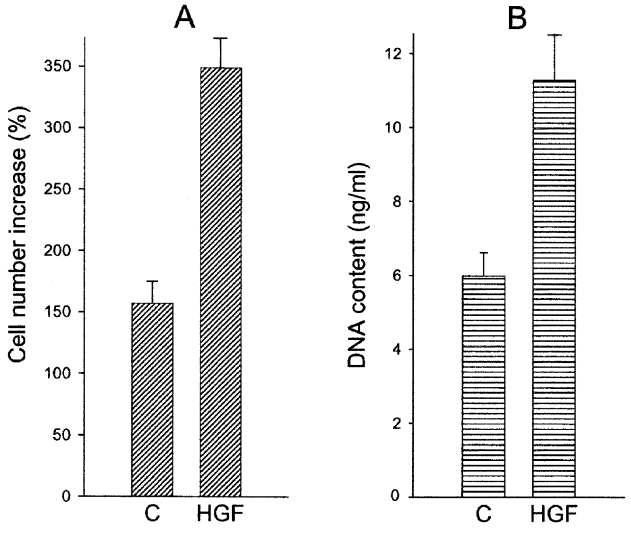



Fig. 7. Mitogenic effect of HGF on testicular embryonic cells cultured on GFR-Matrigel for 20 h. The cell number increase (A) and the DNA content (B) evaluated on control (C) and HGF-treated (100 U/ml) cells are reported. $P < 0.001$ in (A) and $P < 0.01$ in (B).

beginning of the morphological differentiation of the gonad (11.5 dpc). In the subsequent days of development (13.5–15.5 dpc) a HGF specific message is detectable in the differentiated testes. In the developing testis the factor is exclusively localized outside the cords, clearly detectable in the tunica albuginea cells, in the interstitial compartment and in the peritubular cells. In the developing gonads HGF expression appears to be male specific not being detectable in the ovaries isolated from embryos of the same age. Although in the adult female HGF exerts a role in the follicle development (Parrott and Skinner, 1998a,b; Parrott et al., 2000; Nilsson and Skinner, 2001), it seems not to play a role in the early prenatal development of the ovary. Moreover, we report that the factor is secreted in a biologically active form since the culture medium of the testicular cells is able to induce the scattering of the MDCK cells. The here-reported demonstration that HGF is locally produced in the testis, allows to suppose that the in loco production is, at least in part, responsible for the in vivo testicular differentiation. We previously reported that HGF is able to support in vitro male gonad differentiation (Ricci et al., 1999) and we now report that, as a consequence of the HGF-induced cord formation in the indifferent testis, the secretion of laminin starts to be polarized being detectable only outside the cords, and heavily localized all around the newly formed cords and in the interstitium. The described effect on the secretory activity of the testicular cells is presumably only one of the different effects exerted by the factor on the functional activities of the testicular cells. In fact it is well known that HGF is a pleiotropic cytokine able to induce multiple biological activities in a variety of cellular types (Matsumoto and Nakamura, 1993, 1996; Zarnegar and Michalopoulos, 1995; Trusolino et al., 1998). It is reported that during embryonic development HGF acts as a factor able to induce proliferation, scattering and morphogenesis (Birchmeier and Gherardi, 1998). In the testis cell proliferation is strongly required: size increase is male specific and rapid after cord formation (Mittwoch et al., 1969). The increase in the number of Sertoli cells, which occurs during the embryonic and early postnatal life, is also necessary for a correct sperm production, which is correlated to the number of Sertoli cells present in the testis (Orth, 1982, 1984, 1986). It is known that several growth factors such as transforming growth factors and neurotrophins regulate rat testis growth (Olaso et al., 1998; Cupp et al., 1999a, 2000; Levine et al., 2000). In this paper we demonstrate that HGF induces cell proliferation of the embryonic testicular cells being in this way responsible for the size increase of the mouse testis, necessary for its correct subsequent differentiation. At the moment, FGF9 (Colvin et al., 2001) and HGF are the only known growth factors able to induce proliferation of the mouse embryonic testicular cells.

We also demonstrate that HGF is able to induce in vitro the migration of the mesonephric cells and thus we hypothize that HGF acts as a migratory factor during embryonic morphogenesis. It is well known that cellular migration from mesonephroi into the genital ridge is essential for the correct development of the testis and plays a critical role in the formation of testis cords (Martineau et al., 1997; Tilmann and Capel, 1999). This migration is male specific and, accordingly with this specificity, we found that HGF is not expressed in the female gonad. However, HGF is not the only factor able to induce cell migration. PDGF, for example, is a well-known chemotactic factor for vascular smooth muscle cells (Facchiano et al., 2000) and a strong chemoattractant for smooth muscle cells (Koyama et al., 1992; Bornfeldt et al., 1994). In the postnatal testis PDGF exerts chemotactic activity on peritubular myoid cells, the smooth muscle cells which surround the seminiferous tubules (Gnessi et al., 1995). In the rat embryo PDGF (Burton et al., 1993) and the PDGFβ receptor is expressed early in the mouse embryo when cord formation occurs, which is localized in the mesenchyme of the gonads (Shinbrot et al., 1994). Recently, Colvin et al. (2001) have suggested that FGF9 could directly or indirectly induce mesonephric cell migration into developing gonads. Therefore several factors are probably involved in the migration of the mesonephric cells into the developing testis.

In addition, we present data suggesting that HGF acts as a morphogen during testicular development. Branching morphogenesis induced by HGF has been described during metanephros and lung differentiation (Woolf et al., 1995; Ohmichi et al., 1998) and we have demonstrated that HGF is a morphogenetic factor during embryonic (Ricci et al., 1999) and postnatal (Catizone et al., 2001) testis development being able to reorganize postnatal Sertoli cells into cord-like structures. We now demonstrate that in the mouse embryo also HGF induces reaggregation of dissociated testicular cells and the formation of a tridimensional network of cord-like structures. This finding, together with the demonstration that in organ culture HGF alone is able to sustain cord formation (Ricci et al., 1999), allows to conclude that HGF is a morphogenetic factor able to induce testicular morphogenesis during embryonic life. It has been reported that neurotrophins are involved in rat testis cord formation, however, cord formation was only partially inhibited by treating the organ culture with the antisense NT3 oligonucleotides (Cupp et al., 2000) and it has to be clarified if the factor is essential for testicular morphogenesis. Retinoids, during lung morphogenesis (Malpel et al., 2000), negatively regulate testis growth and seminiferous cord formation (Cupp et al., 1999b; Ricci et al., 1999; Livera et al., 2000) essentially acting via RARα (Livera et al., 2001).

Moreover, we analyzed the testes of three c-met null 13.5 dpc mouse embryos (kindly provided by Dr Ponzetto) and we did not find, at least at morphological level, clear evidence of alteration in the testicular cord formation (data not shown). However, it is possible that macrophage stimulating protein (MSP), the other known scatter factor present in the adult testis (Oshiro et al., 1996), could induce in the embryonic testis the same effects induced by HGF in regu-

lating testicular cord formation. Our observations, together with the literature data mentioned earlier, suggest the existence of redundancy and interaction between the growth factors regulating embryonic development of the testis.

All together the data presented in this paper demonstrate that HGF is expressed during the embryonic development of the testis and clarify the multiple roles exerted by this factor during the morphogenesis of the male gonad. Our in vitro studies suggest that in the embryonic testis HGF acts not only as a mitogenic factor but also as a morphogenetic factor and a scatter factor for the mesonephridic cells. In addition our results give new insights on the molecular mechanisms regulating the process of cord formation which is fundamental for a correct development of the embryonic testis.

## 4. Materials and methods

### *4.1. Animals*

CD-1 mouse embryos were used for the experiments. For determination of the age of the embryos, the morning after vaginal plug formation was considered as day 0.5 of embryonic development. On day 11.5 of pregnancy, male and female gonads are morphologically indistinguishable and the embryos were sexed scoring the presence of the Barr bodies according to Palmer and Burgoyne (1991).

### *4.2. RNA isolation and Northern blot analysis*

Various tissues from CD-1 mouse embryos were dissected and RNA was extracted according to Chomczynski and Sacchi (1987). 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 (Amersham-Italia, Milan, Italy). Pre-hybridization, hybridization and washings were performed according to the conditions suggested by the supplier. The membrane was exposed to X-ray film. Mouse HGF cDNA (kindly provided by Dr C. Ponzetto, Torino University) was labeled using a random primer labeling kit (Gibco BRL, Life Technologies, USA). Relative differences in hybridization were determined by scanning densitometry of autoradiograms. HGF expression in total RNA was normalized to the signal for the constitutively expressed glyceraldeyde-3-phosphate dehydrogenase (GAPDH).

### *4.3. Immunolocalization of HGF*

Male UGRs isolated from 11.5 days post coitum (dpc) embryos and testes isolated from 13.5 and 15.5 dpc embryos were fixed overnight in paraformaldehyde 4% (PFA 4%), then washed twice in phosphate buffered saline (PBS), dehydrated, embedded in paraffin and sectioned at a thickness of 5 μm. Sections were dewaxed, hydrated and rinsed with PBS. Endogenous peroxidases were blocked by incubation with 3% hydrogen peroxide in distilled water for 20 min at room temperature. The primary antibody, generated against human-HGF (DV-14 monoclonal antibody kindly provided by Dr M. Prat), was used undiluted. Sections were incubated with the primary antibody at 4°C overnight. The following steps of the immunolocalization were performed according to the manufacturer's instructions (Histostain-Plus kit; Zymed laboratories). The avidin–biotin immunoperoxidase system with 3-amino-9-ethylcarbazole as chromogen was used to visualize bound antibodies. The preparations were counterstained with hemalum, mounted with glycerol and analyzed using a Zeiss Axioscope. Negative controls were processed in the absence of the primary antibody (data not shown).

### *4.4. Scatter activity of gonadal cell culture medium*

The scatter activity of gonadal cell culture medium was measured on colonies of canine kidney epithelial cells (MDCK cells) as previously described (Bhargava et al., 1991). A suspension of MDCK cells in Dulbecco's modified Eagle's medium (DMEM) alone or supplemented with 10% fetal calf serum (FCS) was prepared and 9000 cells were plated in 24-well plates containing 0.5 ml of culture medium. Plates were incubated at 37°C. Undiluted or diluted culture medium, obtained after 4 days of gonadal cell culture, was added to MDCK cells either at plating or after 24 h of culture. To obtain conditioned medium, $2 \times 10^5$ gonadal cells isolated from testes or ovaries of 13.5 dpc embryos were usually seeded in a 24-well plate containing 0.5 ml of the medium. As control of scatter activity, HGF (30–100 U/ml) was added to the cells.

### *4.5. Organ culture and laminin detection*

The UGRs were isolated from 11.5 dpc male embryos and cultured for 4 days on steel grids previously coated with 2% agar. Grids were then placed in organ culture dishes (Falcon) with 0.8 ml of medium necessary to wet the grid. The chemically defined medium utilized was DMEM (Gibco) supplemented with glutamine (2 mM), Hepes (15 mM), non-essential aminoacids, penicillin (100 IU/ml) and streptomycin (100 μg/ml). FCS (10%, Gibco), or HGF (Sigma, 200–400 U/ml) were added to the culture medium when indicated. UGRs were cultured at 37°C in a humidified atmosphere of 5% $CO_2$ in air. After culture, tissues were fixed overnight in 4% PFA in PBS, pH 7.4, at 4°C and washed twice in PBS for 1 h. For sectioning, the samples were maintained in PBS, 7% sucrose for 5–10 min at 4°C and then in PBS, 15% sucrose for 5–10 min at 4°C; successively the samples were maintained in PBS, 15% sucrose, 7% gelatin at 37°C to achieve the complete embedding of the samples. The samples were then included in the latter solution which is solid at room temperature, frozen in liquid nitrogen and sectioned at a thickness of

7 μm. The sections were utilized for indirect immunofluorescence.

Before immunostaining with the antibody the sections were treated with 0.15% glycine for 15 min and then with 10% goat serum for 1 h at room temperature to minimize the non-specific binding. Sections were then exposed to a polyclonal antibody against laminin (1:40 dilution, Sigma–Aldrich Co., St. Louis, MO, USA) for 16 h at 4°C. At the end of the incubation the sections were washed extensively with PBS supplemented with 0.2% Triton X-100 and 1% bovine serum albumin (BSA) (PBS-BSA-T) and incubated for 45 min at room temperature with a fluorescein isothiocyanate-conjugated goat anti-rabbit antiserum (Sigma–Aldrich Co.). The sections were extensively rinsed again with PBS-BSA-T and mounted in buffered glycerol (pH 9). As negative control, the primary antibody was omitted. Samples were analyzed and photographed using a light fluorescence microscope (Zeiss Axioplan).

### 4.6. In vitro migration assay

Cells were obtained by dissociating according to Pesce et al. (1994); 13.5 dpc male mesonephroi ($2.5 \times 10^5$ cells) were seeded on the upper side of a Boyden's migration chamber (Blind well, Neuro Probe Inc., Bethesda, MD, USA) on a porous polyvinylpirrolidine free polycarbonate membrane (5.0 μm pore size, Nucleopore Co., Pleasanton, CA, USA). The lower chamber of the Boyden apparatus was filled with 200 μl of DMEM containing different amounts of HGF (100–400 U/ml). Medium alone served as negative control. The assay was incubated at 37°C in a humidified atmosphere of 5% $CO_2$ in air and after 16 h of incubation cells attached to the upper side of the filter were mechanically removed whereas the cells migrated to the lower side of the filter were fixed and stained in Diff-Quick (Dade AG, Dudingen, Switzerland) and counted. All experiments were performed in triplicate.

### 4.7. Reaggregation experiments

Testes were isolated from 13.5 dpc embryos and dissociated by incubation in trypsin-EDTA (Pesce et al., 1994). The resulting cell suspension was seeded in 24-well tissue culture dishes coated with 200 μl per well of reconstituted basement membrane containing a reduced amount of growth factors (GFR-Matrigel, Collaborative Biomedical Products, Pittsburgh, PA, USA) mixed in a 1:1 ratio with culture medium (MEM). Usually $1.8–2 \times 10^5$ cells were plated in each well and cultured in DMEM alone or supplemented with HGF (5–100 U/ml). After 16–20 h of culture, cells were extensively washed with PBS, fixed and photographed to evaluate cellular reaggregation.

### 4.8. DNA assay

Testicular cells prepared and cultured as described in 'reaggregation experiments', were used to evaluate the amount of DNA in control and HGF (100 U/ml) treated cells. The DNA content was evaluated by a fluorimetric assay using Hoechst 33258 (Fluka) as a fluorescent dye (0.1 μg/ml Hoechst in 0.1 M NaCl, 10 mM Tris–HCl, 1 mM EDTA, pH 7.4). Cells were sonicated in water (500 μl/well) and dye solution was added (1:1000 ratio). Fluorescence was measured by a Perkin–Elmer fluorimeter at 365/460 nm (excitation/emission) wavelengths using salmon sperm DNA as a standard. Alternatively, $^3$H-thymidine (1 μCi/ml; New England Nuclear Corp., Boston, MA, USA; sp act 20 mCi/mmol) was added to the culture (16 h incubation). At the end of the incubation time, cells were extensively washed with PBS and the amount of labeled thymidine incorporated into the cells was evaluated by TCA precipitation.

$^3$H-thymidine incorporation was also used to test the proliferating effect of HGF on male mesonephric cells. Briefly, cells obtained as described in Section 4.6 were seeded in 24-well culture dishes (300,000 cells/well) in DMEM alone or supplemented with HGF (400 U/ml). The incubation time and the assay used were the same as described for testicular cells.

### 4.9. Statistical analysis

The significance of the results was determined by using the Student's *t*-test.

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