Expression and role of PDGF-BB and PDGFR-β during testis morphogenesis in the mouse embryo

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Summary

The role played by PDGF in testis morphogenesis is still incompletely understood. The present study investigates the expression and potential role of platelet-derived growth factor-BB (PDGF-BB) and its receptor, PDGF receptor β (PDGFR-β), during mouse testis cord formation, and the possibility that the growth factor may be involved in the migration to the gonad of mesenchymal cells of mesonephric origin. Studies from this laboratory have previously shown that mesenchymal cells that migrate from the mesonephros into the gonad, to form peritubular myoid cells and most of the intertubular cells, can be identified by the presence on their surface of the p75 neurotrophin receptor (p75NTR), and can be isolated to near-purity by immunomagnetic selection with anti-p75NTR antibody. We show here that mesonephric p75NTR(+) cells also bear the PDGFR-β, and are able to migrate and proliferate in vitro in response to PDGF-BB. PDGF-BB is expressed at higher levels in male than female developing gonads, suggesting a role for this factor in testis development. Such a role is further supported by the observation that addition of PDGF-BB to serum-free medium is sufficient to allow organ-cultured male 11.5 days post-coitum urogenital ridges to form testis cords. Finally, we show that mesonephric cell motility and growth induced by exposure to PDGF-BB involve mitogen-activated protein kinases (MAPK) and phosphatidylinositol-3 kinase (PI3-K) pathways, as MAPK inhibitor U0126 and PI3K inhibitor Ly294002 inhibit migration and proliferation in vitro assays. The present findings support the hypothesis that the PDGF/PDGFR system plays a key role in testis morphogenesis in the mouse embryo.

Key words: Testis development, Gonadal differentiation, Mesenchymal cell migration, Platelet-derived growth factor, p75NTR, MAPK, PI3K

Introduction

Testis development is the result of a highly coordinated series of events that include cell proliferation, migration, differentiation and apoptosis, which are driven by the sequential activation of specific genes (Mackay, 2000; Parker et al., 1999; Swain and Lovell-Badge, 1999).

In mammals, although sex is chromosomally determined, gonad development begins in both sexes from a similar indifferent structure, the urogenital ridge, which is composed of a mesonephros and the attached gonadal crest. Mesonephros plays a pivotal role for testis development in the mouse embryo (McLaren, 1998), in which a mesenchymal cell population migrates from the mesonephros into the developing gonad to become peritubular myoid cells, pericytes and endothelial cells (Martineau et al., 1997; Buehr et al., 1993). Migration is a male-specific event and covers the period between approximately 11.5 and 16.5 days post-coitum (dpc) (Tilmann and Capel, 1999); in the absence of such cell influx, no organized testis cords form (Buehr et al., 1993; Merchant-Larios et al., 1993; Martineau et al., 1997). This has been clearly shown in an organ culture system: when a mesonephros from a ROSA26 'blue' mouse (a transgenic mouse that ubiquitously expresses β-gal) is cultured in vitro alongside a gonad from a 'white' one (wild-type CD1), blue cells move from the mesonephros into the gonad (Martineau et al., 1997). Migration occurs whether the mesonephros is XX or XY, but no migration is seen if the gonad is XX. Migrating cells contribute to the peritubular cell population, but not to Sertoli nor Leydig cell populations (Martineau et al., 1997).

The molecular mechanisms underlying mesonephric cell migration are unknown, but the above observations show that such migration is an essential event for testis morphogenesis, and suggest the possible presence of a chemotactic factor attracting mesonephric cells to the testis.

Platelet-derived growth factors (PDGFs) and their receptors appear to have an essential role during development. PDGF-B or PDGFR-β knockout mice die during late gestation from cardiovascular complications (Levèen et al., 1994; Soriano, 1994; Lindahl et al., 1997; Hellstrom et al., 1999). Mutant fetuses appear healthy and normal until E16-19, at which time a series of abnormalities appear, caused by the absence of vascular smooth muscle cells and/or pericytes, and of mesangial cells of the kidney glomerulus. This complex phenotype stems from the failure of mesenchymal progenitors to locate to their appropriate final destination.

Evidence has accumulated during the past few years indicating that PDGF might also be involved in testis morphogenesis. In the rat, mRNAs for PDGF-AA, PDGF-BB,

PDGFR-α and PDGFR-β are expressed in the embryonic day 18 testis, reach the highest levels by postnatal day 5 and then decline to lower levels in older animals (Gnessi et al., 1995). mRNAs for PDGF-AA and -BB, and for PDGFR-α and -β, have also been found in fetal and adult human testis (Basciani et al., 2002). In rat organ culture experiments, development of testis cords is altered by the presence of a PDGFR-specific inhibitor tyrphostin (Uzumcu et al., 2002).

Because PDGF-BB induces in vitro proliferation and migration of cells of mesenchymal origin (Heldin and Westermark, 1999), in the present study we have investigated whether this growth factor might be involved in the migration of mesenchymal cells from the mesonephros to the testis during the early stages of mouse testis development. As previous results from this laboratory had shown that such migrating cells can be identified by the presence on their surface of the p75 neurotrophin receptor (p75NTR, formerly known as the low-affinity nerve growth factor (NGF) receptor) (Russo et al., 1999; Campagnolo et al., 2001), the experiments on isolated cells reported in the present paper have been performed on mesonephric cells that had been isolated by immunomagnetic selection with anti-p75NTR antibodies.

Materials and Methods

Antibodies and reagents

Monoclonal rat anti-mouse p75NTR (MAB357), polyclonal rabbit anti-mouse p75NTR (AB1554) and Cy3 labelled goat anti-rabbit IgG were obtained from Chemicon International (Temecula, CA). Polyclonal anti-PDGFR-β antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal antibody for PDGF-BB was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to phospho-Erk1/2 (Thr202/Tyr204) and phospho-Akt (Ser473), and to nonphosphorylated corresponding molecules were purchased from New England Biolabs (Beverly, MA). Magnetic microbead-conjugated goat anti-rat IgG antibody was purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Horseradish peroxidase-conjugated anti-rabbit IgG antibody was purchased from Amersham Pharmacia Biotech (Little Chalfont, UK). Alexa 488-phalloidin was obtained from Molecular Probes (Leiden, The Netherlands). MTT, trypsin-EDTA, modified Eagle's medium (MEM), Dulbecco's MEM (DMEM), BSA, glutamine, streptomycin, collagenase type IX (1900 units/mg), gelatin and rabbit immunoglobulins were purchased from Sigma Chemicals (Milano, Italy). RPMI 1640 was obtained from Life Technologies Italia (Milano, Italy). DNAse grade II was obtained from Roche (Basel, Switzerland). Multidish plates were purchased from Nunc (Roskilde, Denmark). Falcon cell culture inserts were obtained from BD Biosciences (Erembodegem, Belgium). Millicell CM filters (PICMORG50) were obtained from Millipore (Bedford, MA). OCT embedding medium was obtained from Sakura Finetek (Torrance, CA). U0126 and Ly294002 were obtained from Promega (Milano, Italy). ^{3}[H]-TdR was from NEN (Boston, MA).

Animals

CD1 Swiss mice (Charles River, Calco, Italy) were housed and mated under standard laboratory conditions that complied with Italian regulations for laboratory animal care. Embryos at various developmental stages were obtained by killing the mothers by cervical dislocation at various post-coital times (between 11.5 and 13.5 dpc). At midday of the day on which the vaginal plug was found was considered to be day 0.5 of pregnancy. At 11.5 dpc, a stage at which no sexual dimorphism is apparent, the sex of the embryo was determined by examining sex chromatin in amniotic cells (Burgoyne, 1983).

Urogenital ridges were isolated by stereomicroscopical dissection and collected in ice-cold Hepes-buffered MEM additioned with 1 mg/ml BSA. When needed, mesonephroi and testes were separated using a very fine needle.

Immunomagnetic cell sorting of mesonephric cell suspension

Mesonephric cells bearing the p75 neurotrophin receptor were immunomagnetically selected as previously described (Campagnolo et al., 2001). Briefly, mesonephroi isolated from 12.5 dpc male mice embryos were incubated in trypsin-EDTA, followed by collagenase-DNAse digestion. The obtained cell suspension was pelleted, washed and filtered through a 20 μm nylon screen to obtain a single cell suspension. The cells were incubated (1 hour, 4°C) with monoclonal rat anti-p75NTR antibody (5 μg/ml), washed and incubated with magnetic microbead-conjugated goat anti-rat IgG. p75NTR(+) cells were sorted out by the use of a magnetic field device (miniMACS, Miltenyi Biotec), following the manufacturer's specifications. All experiments involving isolated mesonephric cells were performed on such immunomagnetically selected cells

Cell culture

Immunomagnetically sorted cells were made quiescent by a 6-8 hour culture in RPMI 1640 supplemented with 2 mM glutamine (at 37°C, in a humidified atmosphere of 5% CO_2 in air). When required by protocol, cells were then treated with 20 μM Ly294002 for 1 hour, or 5 μM U0126 for 15 minutes, before the addition of PDGF-BB.

Organ culture

Male urogenital ridges, dissected from 11.5 dpc embryos, were cultured for 4 days on Millicell CM filters, floating on 1 ml DMEM medium additioned with 2 mM glutamine, 100 U/ml penicillin and 50 μg/ml streptomycin; PDGF-BB (100 ng/ml) was added to the culture medium when indicated; medium and factors were changed every day.

Cell migration assay

The chemotactic response to PDGF-BB was assayed using the cell culture inserts (Repesh, 1989). The wells of a 24-well plate were loaded with 0.7 ml medium. Cell culture inserts were placed in the wells and loaded with 0.2 ml of medium containing 80,000 cells. Various PDGF-BB concentrations were added to the lower wells and the cells were incubated for 16-18 hours. In some assays, before the addition of PDGF-BB, the cells were preincubated with either 5 μM U0126 or 20 μM Ly294002, or vehicle. Cells attached to the upper surface were removed by scraping, and cells that had migrated through the 8 μm pores were fixed with 10% trichloroacetic acid (TCA) (1 hour, 4°C) and stained with 6% Giemsa (30 minutes, RT). Membranes were removed, washed and mounted on a glass slide, and migrating cells visually counted using a microscope with a 20× objective.

To test for chemotaxis vs chemokinesis, experiments were done in which cell migration was compared between wells in which PDGF-BB (10 ng/ml) had been added to the lower well only, upper well only, or both wells.

Measurement of DNA synthesis

DNA synthesis was measured as [^{3}H]-TdR incorporation into TCA-insoluble material. Immunomagnetically selected cells were plated in 96-well plates (30,000 cells/well) and cultured for 16-18 hours in the presence of various concentrations of PDGF-BB and 1 μCi/ml [^{3}H]TdR The medium was removed and the cells were washed twice with ice-cold PBS and twice with ice-cold 5% TCA to remove unincorporated [^{3}H]TdR. Cells were solubilized by adding 0.25 M NaOH and the cell lysate was transferred to scintillation fluid and

counted by a spectrometer (LS 301, Beckman). Each measurement consisted of a four-well replica.

MTT test

Possible deleterious effects of kinase inhibitors on cell viability were tested for by the MTT-cell proliferation assay. The assay is based on the ability of viable cells to reduce the yellow tetrazolium salt, MTT, to water-insoluble, dark blue formazan crystals, which can be spectrophotometrically measured. The assay was carried out by plating 10,000 cells in 96-well plates and exposing them to kinase inhibitors before the addition of various concentrations of PDGF-BB for 16-18 hours. At the end of incubation, MTT was added to each well to a final concentration of 1 mg/ml. The reaction was stopped after 4 hours at 37°C by adding 100 μl of lysis buffer (20% w/v SDS in 50% *N*,*N*-dimethylformamide, pH 4.7) and the samples were analysed at 595 nm on Biorad multiscan plate reader. Usually four replicate wells were used for each group. Control included untreated cells, whereas medium alone was used as a blank. A standard curve with increasing amounts of untreated cells was performed to normalise the results.

Immunohistochemistry and immunocytochemistry

Isolated urogenital ridges were embedded in OCT, frozen on nitrogen vapours and stored at –80°C until use. Cryostat sections, 8 μm, collected on gelatine-coated slides, were fixed in methanol (10 minutes, –20°C) and air dried. Antibody aspecific binding was blocked with 10% goat serum in PBS (30 minutes, RT), and the sections were immunostained (1 hour, RT) with polyclonal rabbit anti PDGFR-β (5 μg/ml in PBS, 0.1% BSA) followed by Cy3-labelled goat anti-rabbit IgG (1:500). Control sections were incubated with nonspecific rabbit IgG at the same concentration as primary antibody. To visualize the entire cell population, cell nuclei were routinely stained with Hoechst 33258 (0.5 μg/ml, added to the secondary antibody solution). Slides were mounted with Mowiol (Heimer and Taylor, 1974) and examined by epifluorescence with a Zeiss Axioplan 2 microscope.

The expression of PDGFR-β and p75NTR was studied on immunomagnetically isolated mesonephric mesenchymal cells. Immunostaining for each receptor was performed on separate samples owing to the unavailability of antibodies from two different animal species. Isolated cells were plated on polylysine-coated coverslips, fixed in methanol (10 minutes, –20°C) and air dried. Antibody aspecific binding was blocked with 10% goat serum in PBS (30 minutes, RT), and the cells were immunostained (1 hour, RT) with polyclonal rabbit anti-p75 (1:200 in PBS, 0.1% BSA) followed by Alexa Fluor 488 goat anti-rabbit IgG (1:400) or with policlonal rabbit anti-PDGFR-β as specified above. Cell nuclei were stained with Hoechst 33258.

The effects of PDGF-BB on microfilament organization were studied on cells plated at a concentration of 40,000 cells per well in four-well Nunc multidish plates, each containing a round glass coverslip that had been previously coated for 2 hours at 37°C with 0.1% gelatin in PBS. After 6-8 hours of incubation at 37°C to allow cell attachment, cells were exposed to 10 ng/ml PDGF-BB (15 minutes, 37°C). Cells were then fixed with 4% paraformaldehyde (10 minutes, RT), permeabilized with 0.1% Triton X-100 (5 minutes, RT), blocked with PBS, 1% BSA (30 minutes, RT) and stained (15 minutes, RT) with 0.132 mg/ml Alexa488-phalloidin. The coverslips were washed with PBS and mounted with Mowiol.

Immunoblot analysis

Mesonephroi and testes were separately homogenized in lysis buffer (50 mM Hepes pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mg/ml SDS, 15 mM $MgCl_2$, 1 mM EGTA, 2 mM PMSF, 0.5 mg/ml leupeptin, 0.7 mg/ml pepstatin, 0.2 U/ml aprotinin, 50 mM benzamidine). Lysates were clarified by centrifugation at 13,000 ***g*** (30 minutes, 4°C), and protein concentration in the supernatant was determined by the Bradford assay (Bio-Rad Laboratories) using bovine serum albumin as a standard. Fifty micrograms of the extracted proteins were separated by SDS-PAGE and blotted onto ECL nitrocellulose membrane (Amersham). The filter was blocked with 10% non-fat dry milk in PBS-0.1% Tween 20 and then incubated with polyclonal rabbit anti-human PDGFR-β (5 μg/ml, 2 hours, RT) or polyclonal rabbit anti PDGF-BB (1:200, 2 hours, RT). After several washes in PBS-0.1% Tween 20, horseradish peroxidase-conjugated secondary antibody (1:10,000) was added for 1 hour at RT. The labelled bands were detected using Amersham ECL western blotting system according to manufacturer's specifications.

For the analysis of mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3-K) pathway activation by PDGF-BB, immunomagnetically sorted cells were plated in 96-well Falcon plates (50,000 cells/well) and cultured for 6-8 hours to obtain quiescence. Following exposure to Ly294002 or U0126, various PDGF-BB concentrations were added and the cells were cultured for additional 15 minutes or 16-18 hours. Culture medium was then removed, the cells were washed with PBS and were lysed by adding to the wells the loading buffer (10% glycerol, 2% SDS, 0.062 M Tris pH 6.8, 5% β-mercaptoethanol, 0.02% bromophenol blue), and pipetting several times to obtain cell lysis. The lysate was heated to 95-100°C for 5 minutes, cooled on ice, separated by 12% SDS-PAGE gel and blotted onto nitrocellulose membrane. The filters were incubated with antibody to phospho-Erk1/2 or phospho-Akt and with antibodies to the corresponding nonphosphorylated forms. Membrane blocking and antibodies incubation were performed according to manufacturer's protocol.

All immunoblotting experiments were performed at least three times on different protein extractions.

RNA isolation and northern blot analysis

Total RNA was extracted from mesonephroi and gonads by using TriPure (Roche) according to the manufacturer's instructions. RNA samples (20 μg/lane) were separated on 1.5% formaldehyde-agarose gel and blotted on Hybond-N membrane (Amersham). Hybridization in QuikHyb solution (Stratagene, La Jolla, CA) was performed according to the manufacturer's instructions. The membrane was exposed to Kodak X-ray film. Mouse PDGF-BB cDNA (kindly provided by L. E. Samelson, NCI, Bethesda, MD) was labelled using a random primer labelling kit (Roche). As an internal control, the filters were hybridized with β-actin.

RNA in situ hybridization

CD1 mouse embryonic testes were embedded, sectioned and treated for RNA in situ hybridization analysis following the protocol described previously (Sassoon and Rosenthal, 1993). Briefly, tissues were collected, immediately fixed overnight at 4°C in freshly prepared 4% paraformaldehyde and, after dehydration, embedded in paraffin at 60°C. Specimens were sectioned at 5 μm, deparaffinized and treated with Proteinase K (20 mg/ml, 7 minutes at RT). Sections were incubated with [^{35}S]-radiolabelled probes for 16 hours at 52°C. The final concentration for both antisense and sense probes was 35 cpm/μl in hybridization buffer. Probes, generated in professor Christen Betsholtz's laboratory, were kindly provided by Michelle D. Tallquist (Fred Hutchinson Cancer Research Center, Seattle, WA). Both antisense and sense probes were prepared from a pBluescript sk vector containing 800 bp of PDGF-B cDNA The antisense probe was linearized with *Sac*I and transcribed from the T7 promoter, and the sense probe was linearized with *Hin*dIII and transcribed from the T3 promoter. After several washes of increasing stringency, slides were dipped in Kodak NBT-2, dried for 1 hour and exposed for 5 days at 4°C. Photographs were taken using a Zeiss Axioplan 2 microscope.

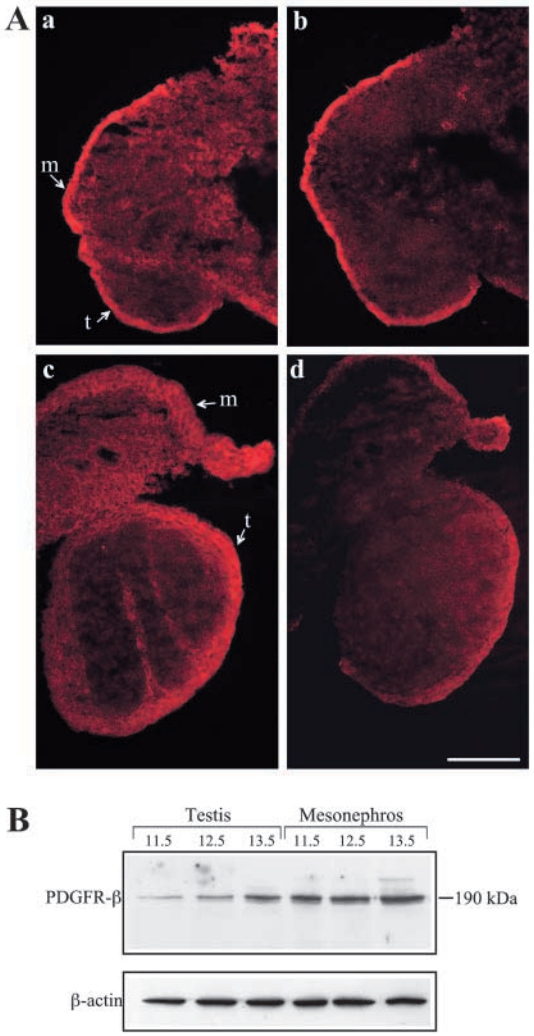

Fig. 1. PDGFR-β expression in the male urogenital ridge between 11.5 and 13.5 dcp. (A) Immunohistochemical detection of PDGFR-β localization in cryostat sections of 11.5 and 13.5 dpc male urogenital ridges. At 11.5 dpc no immunoreactivity can be detected in the gonad (a), while at 13.5 dpc cells with a strong immunoreactivity fill the interstitial compartment of the gonad (c). (b,d) control sections. m, mesonephros; t, testis. Bar, 100 μm. (B) Immunoblot analysis of PDGFR-β expression, showing the presence of a 190-kDa immunoreactive band that reacts to the same antibody as used for immunolocalization. The presence of equal amounts of proteins in each lane is confirmed by quantitatively similar β-actin immunoreactive bands.

Data analysis

All experiments were performed at least three times. Results are reported as means±s.d. The significance of the data was determined by unpaired Student *t*-test, and *P* values <0.01 were considered statistically significant.

Results

Localization of PDGFβ receptor-expressing cells in the developing male urogenital ridge

To investigate the time-course of PDGFR-β expression in vivo, sections of urogenital ridges from 11.5, 12.5 and 13.5 dpc male embryos were immunohistochemically stained with the polyclonal PDGFR-β antibody. We found that at 11.5 dpc, PDGFR-β immunoreactivity is only present in the mesonephric region, whereas the developing gonad is negative (Fig. 1A). At this early stage of development, gonads were identified on the sections by alkaline phosphatase staining of germ cells. Cells that express PDGFR-β progressively appear in the interstitial compartment of the male gonadal ridge, around developing testis cords, at 12.5 (not shown) and 13.5 dpc (Fig. 1A).

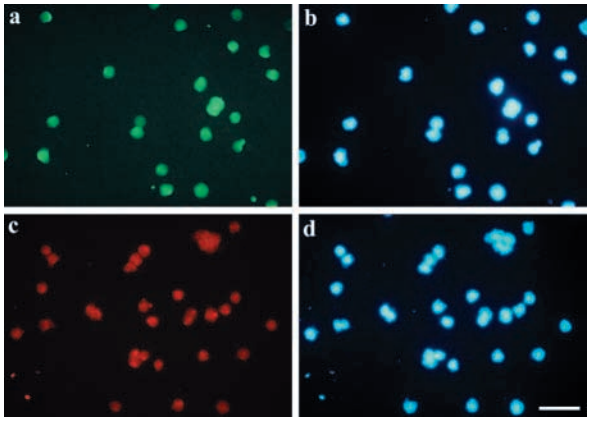

Fig. 2. Expression of p75NTR and PDGFR-β by mesonephric sorted cells. Immunoreactivity for p75NTR (a) and PDGFR-β (c) in immunomagnetically sorted mesonephric cells. Comparison of antibody (a,c) and Hoechst staining (b,d) shows that the cells are immunoreactive for both antibodies. Bar, 50 μm.

PDGFR-β expression in the developing male urogenital ridge

The presence of PDGFR-β receptor in the developing male gonad was confirmed by immunoblot analysis of proteins extracted from 11.5, 12.5 and 13.5 dpc separated gonads and mesonephroi. Fig. 1B shows the progressive increase of expression in the testis of a 190 kDa protein that is recognized by the same anti-PDGFR-β receptor antibody as used for immunohistochemistry, thus confirming the immunohistochemical results. In addition, the immunoblot shows that the amount of PDGFR-β protein in the mesonephric extract essentially does not change with developmental age.

Colocalization of PDGFR-β and p75NTR on mesonephric mesenchymal cells

We have previously shown that mesenchymal cells that migrate from the mesonephros into the testis can be identified by the presence on their surface of the p75 neurotrophin receptor, and can be isolated as an essentially pure (>96%) cell population by immunomagnetically sorting the total mesonephric cell suspension with anti-p75NTR antibody (Campagnolo et al., 2001). Because the PDGFβ receptor has been shown to play a role in the migration and proliferation of cells of mesenchymal origin, we have investigated whether this receptor molecule is expressed by p75NTR(+) cells. Because the antibodies against p75NTR and PDGFR-β were both raised in rabbit, double immunofluorescence staining could not be performed, and the

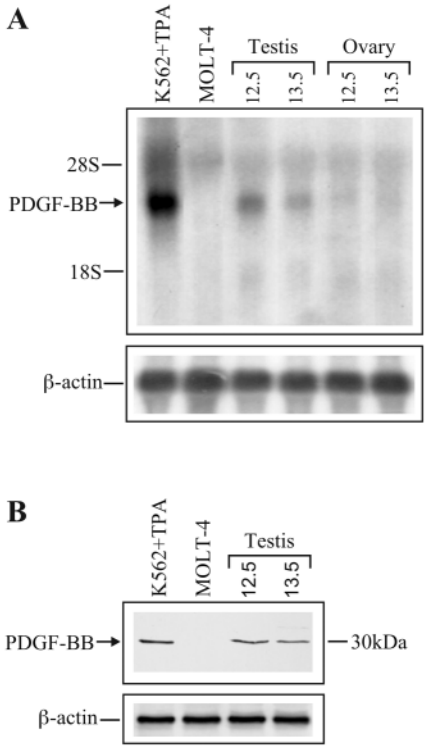

Fig. 3. PDGF-BB expression in the fetal gonad. (A) Northern-blot analysis, performed on 20 μg of total RNA extracted from 12.5 and 13.5 dpc male and female gonads. An erythroleukaemic cell line (K562) was used as a positive control, which, after treatment with TPA expresses high levels of PDGF-BB mRNA; a human lymphoma cell line (MOLT-4) was used as a negative control. Lower panel: filter reprobed with β-actin cDNA to check for the presence of equal amounts of mRNA in each lane. (B) Immunoblot analysis showing a PDGF-BB immunoreactive band of 30 kDa, detected in a nonreducing gel, which represents the PDGF-BB dimeric molecule present in fetal testis.

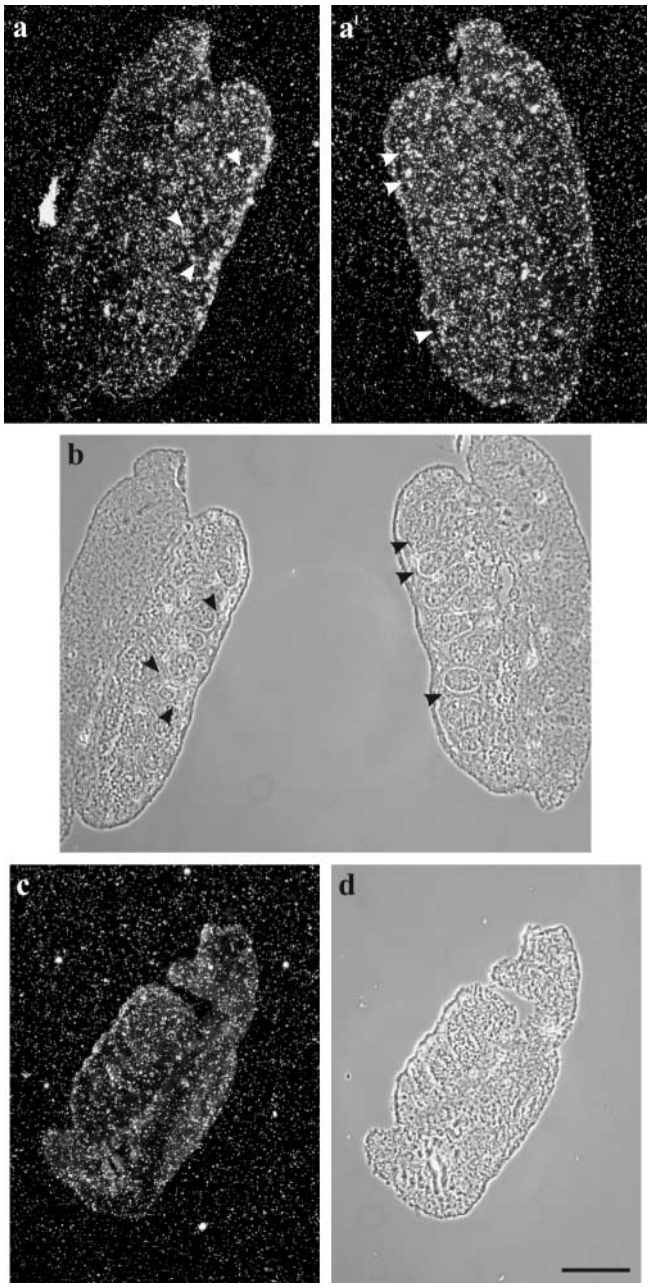

Fig. 4. Expression of *Pdgf-B* in 12.5 dpc male urogenital ridge. The in situ hybridization (a,a′) shows that the growth factor is mainly expressed by cells in the interstitial compartment of the developing testis, as indicated by arrowheads; b, phase contrast. c, hybridization with sense probe and, d, its phase contrast. Bar, 100 μm.

immunolocalization experiment was carried out on two separate samples from the same immunoselected cell population. We found that essentially all of such selected cells also express readily detectable levels of PDGFβ receptor (Fig. 2). Only a small fraction (less than 2%) of the flow-through cells, i.e. cells that are not retained in the immunomagnetic selection column, are labelled by the PDGFR-β antibody. The experiments reported in the present paper were therefore carried out on cells sorted by the presence of p75NTR, rather than PDGFR-β, to avoid any possible antibody-induced perturbation of the receptor under study.

Expression of PDGF-BB in fetal urogenital ridges

Because the PDGF-β receptor is expressed by mesonephric mesenchymal cells, and its ligand PDGF-BB is a potent mitogen and chemoattractant for cells of mesenchymal origin, we next asked whether PDGF-BB was expressed by the developing gonads, to possibly direct the migration of mesonephric precursors. Northern blot analysis of total RNA extracted from 12.5 and 13.5 dpc male and female gonads shows that PDGF-BB mRNA is present in embryonic gonads, and that the mRNA content is higher in male than in female gonads (Fig. 3A). PDGF-BB mRNA is translated into protein in the developing testis, as shown by western blot analysis of 12.5 and 13.5 dpc testis lysates (Fig. 3B), which revealed the presence, in a nonreducing gel, of an immunoreactive 30 kDa protein band, representing the dimeric form of PDGF-BB. The same band was detected in extracts from phorbol ester TPA-stimulated K562 cells, which were used as a positive control. In situ hybridization experiments on *Pdgf-b* expression in 12.5 dpc mouse urogenital ridges (Fig. 4) showed a higher expression of PDGF-BB mRNA in the testis than in the

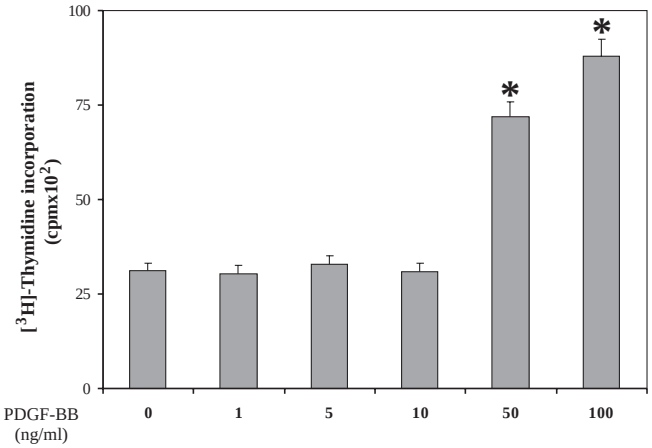

Fig. 5. Effect of PDGF-BB on mesenchymal mesonephric cell proliferation. Immunoselected quiescent mesenchymal cells were treated for 16-18 hours with PDGF-BB (various concentrations) and [^{3}H]TdR (1 μCi/well). Results are mean±s.d. of four independent experiments, each done in quadruplicate. *P<0.01 vs untreated control.

mesonephros, and that the growth factor is mainly expressed by cells in the interstitium of the developing testis.

Effect of PDGF-BB on DNA synthesis and on migration by mesonephric mesenchymal cells

Having found that PDGF-BB is expressed in the developing testis, we next investigated whether this growth factor stimulates proliferation and migration in mesonephric cells. The proliferation experiment was performed by measuring [^{3}H]TdR incorporation in immunomagnetically selected cells, cultured for 16-18 hours in the presence of various PDGF-BB concentrations. We found that, at concentrations of 50 and 100 ng/ml, PDGF-BB causes a 2.5-fold increase in [^{3}H]TdR incorporation above basal level, indicating that it acts as a mitogenic factor for mesonephric cells (Fig. 5).

The ability of PDGF-BB to influence the migration of mesonephric cells was tested by the cell culture inserts method. We found that at concentrations between 1 and 50 ng/ml the growth factor stimulates migration of the cells in a dose-dependent fashion; a sharp decline was seen when the concentration was raised to 100 ng/ml (Fig. 6A).

To determine whether PDGF-BB-induced cell motility is the consequence of a directional chemotactic response or of random movements induced by the growth factor (chemokinesis), migration assays were performed in which PDGF-BB (10 ng/ml) was added to the lower well only, the upper well only, both wells, or neither. As shown in Fig. 6B, cell migration only occurred when PDGF-BB was present in the lower well, showing that PDGF-BB primarily stimulates a chemotactic response.

An essential requirement for a chemotactic response is the reorganization of the actin microfilament network, leading to the formation of lamellipodia (flattened veil-like menbranes) and membrane ruffling, as the cell establishes a migratory leading edge. In agreement with the results on cell migration, we found that exposure of mesonephric cells to PDGF-BB (10 ng/ml, 15

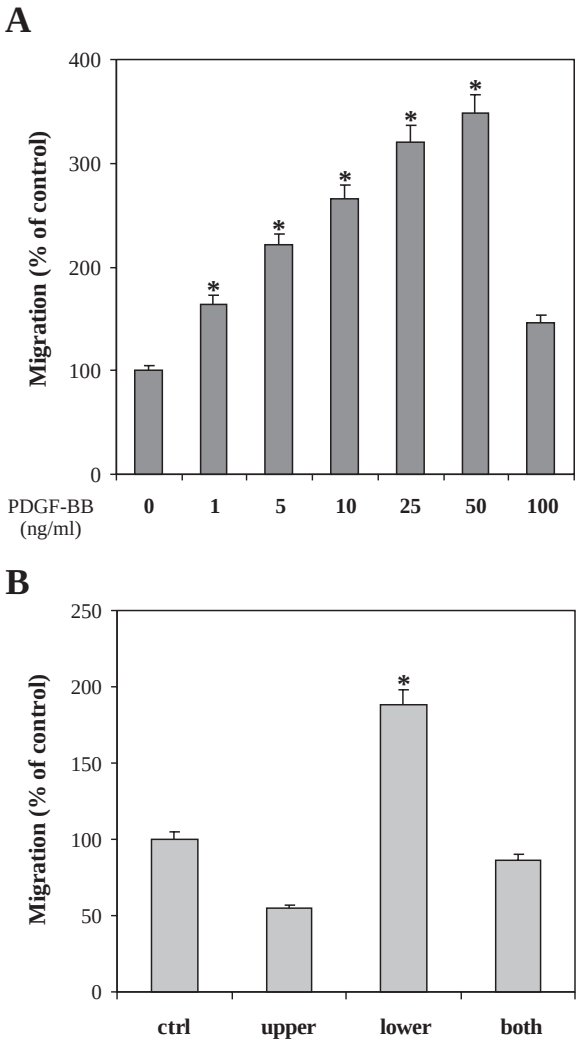

Fig. 6. Effect of PDGF-BB on mesenchymal mesonephric cell migration. Assays were performed by incubating cells for 16-18 hours in cell culture inserts with 8 μm porosity membrane. Cells that had migrated to the lower surface of the filters were counted. (A) Dose-response curve. Various concentrations of PDGF-BB were added to the lower wells only. (B) Chemotaxis vs chemokinesis analysis. PDGF-BB (10 ng/ml) was added to the upper well, to the lower well, or to both set of wells. Results are mean±s.d. of three independent experiments, each performed in duplicate. *P<0.01 vs untreated control.

minutes) causes rapid shape changes, with reorganization of the actin cytoskeleton and lamellipodia formation, as detected by labelled phalloidin staining of F-actin (Fig. 7).

Activation of kinase pathways by PDGF-BB

Because PDGF-BB elicits both proliferation and migration in mesonephric cells, we investigated the signal transduction pathways involved in these processes. Two of the pathways that are most commonly activated by PDGF-BB are the Ras•MAP kinase kinase•Erk1/2 signalling cascade, triggered by the association of activated PDGFR-β with Grb2/Sos, and the PI3-K•Akt pathway. Western blot analysis using antibodies directed against unphosphorylated and phosphorylated forms

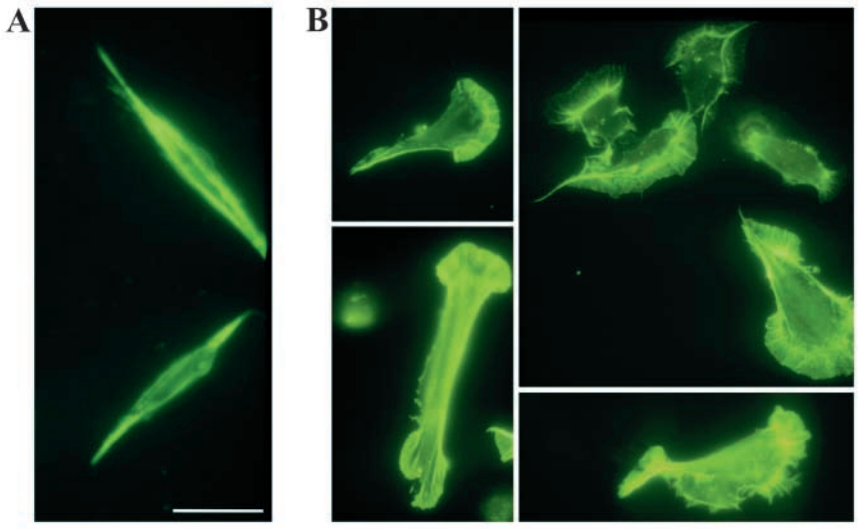

Fig. 7. Effect of PDGF-BB on microfilament reorganization, as revealed by phalloidin staining. Quiescent mesonephric mesenchymal cells (A) were stimulated for 15 minutes (B) with PDGF-BB (10 ng/ml). Note that treated cells exhibit a rapid change in microfilament reorganization and in cellular morphology (phalloidin staining at the leading edge of the cell and extensions of lamellipodia). Bar, 20 μm.

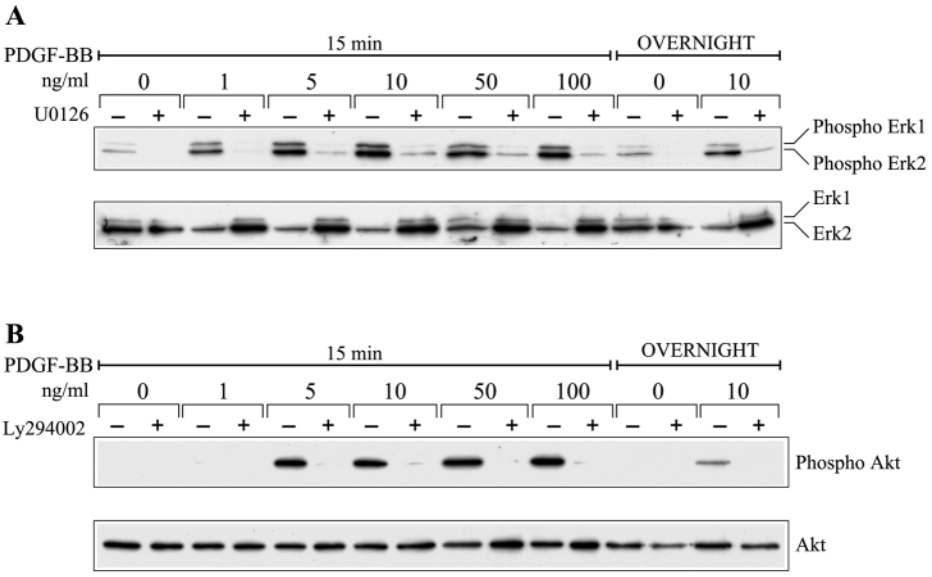

Fig. 8. Erk1/2 and Akt phosphorylation in PDGF-BB-stimulated mesonephric cells: inhibition by U0126 and Ly294002. Quiescent cells were incubated with U0126 or Ly294002 and then stimulated with different concentrations of PDGF-BB for 15 minutes or overnight, as described in Materials and Methods. Lysed cells were separated by SDS-PAGE and blotted filters were incubated with (A) antibody for phospho-Erk1/2 (upper panel) and for nonphosphorylated forms as control (lower panel); or (B) antibody for phospho-Akt (upper panel) and for nonphosphorylated form as control (lower panel).

of Erk1/2 or Akt showed that PDGF-BB triggers activation of both pathways in mesonephric cells (Fig. 8A,B). The phosphorylation of both kinases is sustained, as protein phosphorylation is still over basal levels 16-18 hours after the addition of the growth factor (Fig. 8).

PDGF-BB-induced activation of Erk1/2 in mesonephric cells is MEK-dependent, as pre-incubation with U0126, a specific inhibitor of MEK activity, is able to block its phosphorylation. Likewise, the inhibition of Akt phosphorylation by Ly294002 shows that PI3K is involved in Akt activation. At the concentrations used, U0126 and Ly294002 had no deleterious effects on the cells, as assessed by the MTT test (data not shown). The data also show that PDGF-BB-induced protein phosphorylation continues to be inhibited after overnight culture in the presence of inhibitors.

Effects of MEK and PI3K inhibitors on cell proliferation and migration

We next investigated the involvement of the above signalling pathways on PDGF-BB-induced cell proliferation and migration. The experiments were carried out on cells cultured overnight in the presence of PDGF-BB, with or without MEK or PI3K inhibitors which, as shown in Fig. 8, maintain their inhibitory effect at these culture times. The results obtained show that exposure to either Ly294002 or U0126 prevents both proliferation and migration of mesenchymal cells induced by PDGF-BB in vitro (Fig. 9A,B). These results indicate that activation of both PI3-K and MEK is required for PDGF-BB-induced stimulation of proliferation and migration of mesonephric cells.

Effect of PDGF-BB on testis cord formation in urogenital ridge organ culture

To assess the biological function of PDGF-BB in testis development, male urogenital ridges from 11.5 dpc embryos were organ-cultured in the presence of DMEM alone or DMEM supplemented with 100 ng/ml PDGF-BB. Three different experiments were performed, in which each experimental point (control or PDGF-BB culture) contained five or six urogenital

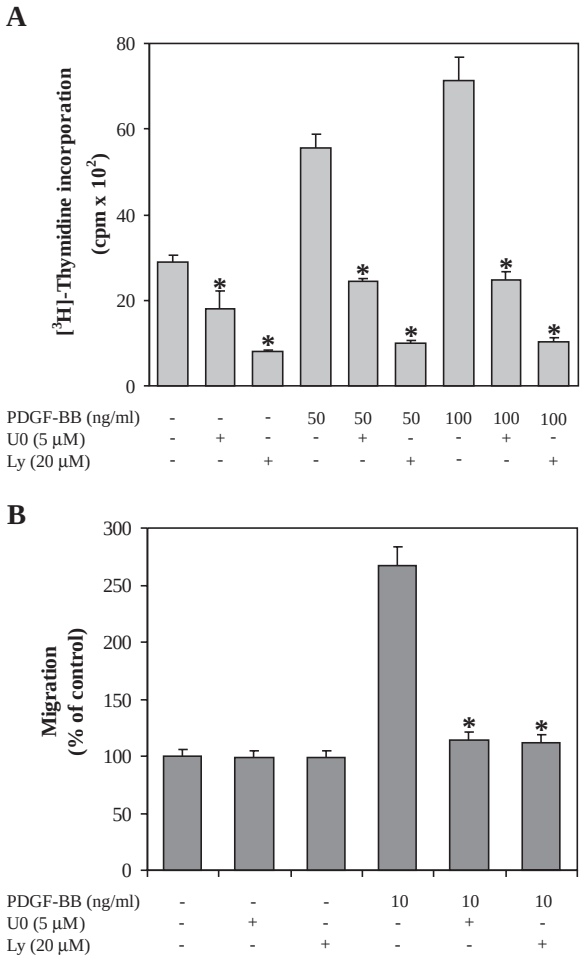

Fig. 9. Effect of U0126 and Ly294002 on proliferation and migration of mesonephric mesenchymal cells. (A) Quiescent cells were pretreated with U0126 (U0) or Ly294002 (Ly) and then stimulated with PDGF-BB. [^{3}H]TdR incorporation was measured as an index of cell proliferation. Results are means±s.d of four independent experiments, each done in quadruplicate. *P<0.01 vs its respective control (no inhibitor). (B) Quiescent cells were pretreated with U0126 or Ly294002 and then tested for directed migration towards 10 ng/ml PDGF-BB. Results are means±s.d. of four independent experiments, each done in duplicate. *P<0.01 vs PDGF-BB 10 ng/ml.

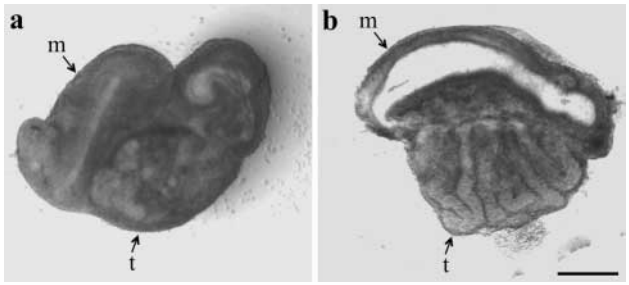

Fig. 10. Male urogenital ridge organ culture. Photomicrographs illustrating morphological changes in male urogenital ridges cultured for 4 days in the presence of serum-free medium alone (a) or also with 100 ng/ml PDGF-BB (b). Data are representative of three separate experiment, which showed similar results. Bar, 0.5 mm. m, mesonephros; t, testis.

ridges. When ridges were cultured in plain medium, no signs of morphological differentiation were seen (Fig. 10a). Interestingly, in each experiment, supplementation of the medium with PDGF-BB (100 ng/ml) was sufficient to allow testis cord formation in at least four urogenital ridges (Fig. 10b).

Discussion

In the present study we investigated some aspects of a possible involvement of the PDGF-BB/PDGFR-β system in the early stages of fetal testis morphogenesis in the mouse.

Immunohistochemical analysis of fetal urogenital ridges revealed that, between 11.5 and 13.5 dpc, cells bearing the PDGFβ-receptor progressively fill in the interstitial compartment of the developing male gonad. The histological data have been confirmed by immunoblot analysis, which showed that in the 11.5 dpc testis β-receptor protein is present as a faint band, which becomes an intense signal at later developmental stages.

As our previous studies (Campagnolo et al., 2001) had shown that mesenchymal cells that migrate from the mesonephros into the developing testis can be identified by the presence on their surface of the p75 neurotrophin receptor, we explored the possibility that these cells might be bearing the PDGFR-β as well. Cells immunomagnetically sorted for the presence of the p75NTR were assayed for the presence of the PDGFR-β, and the results showed that essentially all of the p75NTR positive cells were also positive for PDGFR-β.

Thus, we decided to perform our studies on the effect of PDGF-BB on isolated mesonephric mesenchymal cells by sorting such cells from dissociated mesonephroi with the use of an immunomagnetically labelled antibody to p75NTR rather than to PDGFR-β. This choice was based on the need to avoid possible antibody-induced perturbations of the receptor under study.

As it is known that various cells of mesenchymal origin are induced to proliferate and migrate by β-receptor ligand PDGF-BB (Heldin and Westermark, 1999), we investigated whrther PDGF signalling might be involved in the events of mesonephric cell migration and proliferation that are essential for testis morphogenesis.

Our data show that PDGF-BB plays a mitogenic effect on mesonephric cells, with a 2.5-fold increase in DNA synthesis, a finding that suggests that it may be at least in part responsible for the size increase of the developing mouse testis. Moreover, we show that PDGF-BB attracts mesonephric cells in vitro, with a dose-dependent response. Exposure to PDGF-BB is accompanied by marked shape changes, with rearrangements of actin filaments leading to the formation of edge ruffles, which are a common aspect of chemotaxis. Of particular interest are the results showing that migration already occurs at low PDGF-BB concentrations (1-10 ng/ml), whereas the mitogenic effect only occurs at higher concentrations (50-100 ng/ml). This differential response raises the possibility that a gonadally released PDGF-BB gradient might first induce chemotactic migration of mesenchymal cells; once cells enter the gonad, they might be exposed to locally secreted higher PDGF-BB concentrations, with ensuing cell proliferation.

Northern-blot, immunoblot and in situ hybridization analysis indeed show the presence, in the male gonadal ridge, of PDGF-BB mRNA transcript and protein. The mRNA is expressed at higher levels in male than female gonads, suggesting a major role for this factor in male urogenital ridge development.

Studies on PDGF-elicited signal transduction events usually correlate activation of MAPK pathways with proliferation, and of PI3-K pathways with migration (Heldin and Westermark, 1999). Here we show that both signal transduction pathways are essential for proliferation and migration of mesonephric mesenchymal cells, and neither of the two pathways are of unique importance for PDGF-stimulated cell growth or migration. This suggests a cross-talk between different signalling pathways, as previously shown to occur between Ras and PI3-K, which interact physically and can activate each other following PDGF cell stimulation (Rodriguez-Viciana et al., 1994; Hu et al., 1995; Klinghoffer et al., 1996). As both pathways appear to be involved in both proliferation and migration of mesonephric mesenchymal cells induced by PDGF-BB, it can be hypothesized that it is probably the intensity of the stimulus that induces the cells to migrate (at low PDGF-BB concentration) or proliferate (at higher concentration).

The data on the ability of PDGF-BB to induce mesonephric cell proliferation and migration are consistent with the growth factor ability to support differentiation of the mouse male gonad, as assayed by in vitro organ culture. It is known that male urogenital ridges isolated from 11.5 dpc mouse embryo and cultured in presence of serum develop testis cords (Merchant-Larios et al., 1993; Buehr et al., 1993; Moreno-Mendoza et al., 1995). Serum addition is essential for testis differentiation in culture as, as we confirmed, cord formation does not occur in its absence. The results reported in the present paper show that PDGF-BB acts as a testis morphogenetic factor, as its addition to serum-free medium is sufficient to allow testis cord formation in cultured urogenital ridges.

Altogether, our data indicate that the PDGF/PDGFR system is involved in the early stages of testis morphogenesis, during testis cord formation. Our findings are compatible with recent observations showing that treatment of the developing rat testis with a PDGFR inhibitor (tyrphostin) induces a decrease in the number of cords per testis and a fusion of testis cords (Uzumcu et al., 2002). Moreover, the same group had previously shown a role for neurotrophins in testis cord formation in the rat embryo (Levine et al., 2000). Our observations, together with the literature data mentioned above, suggest the existence of interaction and possible redundancy between the growth factors that regulate testis development, both PDGF and neurotrophins being apparently strictly involved in testis morphogenesis. Indeed, results reported in the present paper have been obtained on mesenchymal cells that bear both the neurotrophin receptor p75 and the PDGF β receptor. In addition, recently published data show that NT3 plays a chemotactic role in vitro on mesonephric mesenchymal cell migration (Cupp et al., 2003). Investigations on the cooperation between PDGFs and neurotrophins might yield important insights in the analysis of regualtory mechanism of testis development.

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