International Journal of Andrology
international journal of andrology ISSN 0105-6263

ORIGINAL ARTICLE

Glial cell line–derived neurotrophic factor promotes invasive behaviour in testicular seminoma cells

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Keywords:
cell invasion, GDNF family receptor-a 1, glial cell line–derived neurotrophic factor, human seminoma cells, immunohistochemistry

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Received 21 September 2011; revised 16 January 2012; accepted 15 February 2012

doi:10.1111/j.1365-2605.2012.01267.x

Summary

The glial cell line–derived neurotrophic factor (GDNF) has multiple functions that promote cell survival, proliferation and migration in different cell types. The experimental over-expression of GDNF in mouse testis leads to infertility and promotes seminomatous germ cell tumours in older animals, which suggests that deregulation of the GDNF pathway may be implicated in germ cell carcinogenesis. GDNF activates downstream pathways upon binding to its specific co-receptor GDNF family receptor-a 1 (GFRA1). This complex then interacts with Ret and other co-receptors to activate several intracellular signalling cascades. To explore the involvement of the GDNF pathway in the onset and progression of testicular germ cell tumours, we analysed GFRA1 and Ret expression patterns in seminoma samples. We demonstrated, via immunohistochemistry, that GFRA1, but not Ret, is over-expressed in in situ carcinoma (CIS) and in intratubular and invasive seminoma cells compared with normal human germ cells. Functional analysis of the GDNF biological activity was performed on TCam-2 seminoma cell line. Reverse transcription-PCR (RT-PCR) and immunohistochemical analyses demonstrate that TCam-2 cells express both GFRA1 and Ret mRNA, but only GFRA1 was detected at the protein level. In TCam-2 cells, although GDNF is not mitogenic, it is able to induce migration, as demonstrated by a Boyden chamber assay, possibly through the Src and MEK pathways. Moreover, GDNF promotes invasive behaviour, an effect dependent on pericellular protease activity, possibly through the activity of matrix metalloproteinases. GFRA1 over-expression in CIS and seminoma cells, along with the functional analyses in TCam-2 cells, suggests an involvement of the GDNF pathway in the progression of testicular germ cell cancer.

Introduction

Testicular germ cell tumours (TGCTs) are among the most frequent solid malignant tumours in 15- to 39-year-old men (Ferlay *et al.*, 2010). Based on epidemiology, clinical presentation, phenotypic characterization, chromosomal constitution and genomic imprinting, TGCTs are classified into three groups (Oosterhuis & Looijenga, 2005). Type I comprises the teratomas and yolk-sac tumours of newborns and infants; type II comprises the seminomatous and non-seminomatous tumours of adolescents and young adults; and type III comprises the spermatocytic seminomas of elderly people. It is generally accepted that type II TGCTs arise from a common pre-invasive lesion, known as in situ carcinoma (CIS) (Skakkebaek, 1972; Kristensen *et al.*, 2008). Currently, various non-seminomatous cell lines have been established, but only one (i.e. TCam-2) (Mizuno *et al.*, 1993;

Eckert *et al.*, 2008; de Jong *et al.*, 2008) has been established from seminoma, although they constitute more than 50% of all TGCTs (Ulbright, 2005).

Glial cell line–derived neurotrophic factor (GDNF) was first identified through its activity on midbrain dopaminergic neurons (Lin *et al.*, 1993). Subsequently, GDNF has been shown to have pleiotropic functions, promoting neuroprotection (Beck *et al.*, 1995; Tomac *et al.*, 1995), kidney development and enteric innervations (Moore *et al.*, 1996; Pichel *et al.*, 1996; Sanchez *et al.*, 1996). GDNF exerts its effect on target cells by binding to a glycosyl phosphatidylinositol–linked GDNF family receptor-a 1 (GFRA1) (Airaksinen & Saarma, 2002). This binding recruits the receptor tyrosine kinase Ret to form a multisubunit signalling complex of GDNF/GFRA1/Ret. In addition, GDNF binding is transduced by GFRA1 in a Ret-independent fashion (Airaksinen & Saarma, 2002; Sariola & Saarma, 2003). Mice with a homozygous deletion of GDNF die shortly after birth because of severe defects in renal differentiation and the absence of an enteric nervous system (Moore *et al.*, 1996; Pichel *et al.*, 1996; Sanchez *et al.*, 1996). GDNF is important for male fertility because it plays a central role in spermatogonial stem cell self-renewal and proliferation (Hofmann, 2008). Reduced GDNF dosage in heterozygous GDNF+/− animals leads to an excessive differentiation of spermatogonia and finally to an exclusive Sertoli cell phenotype, whereas over-expression of GDNF leads to a block of stem cell differentiation (Meng *et al.*, 2000). GDNF is also an essential factor in the maintenance and expansion of in vitro spermatogonial stem cells in different mammals (Kanatsu-Shinohara *et al.*, 2003, 2008; Kubota *et al.*, 2004; Wu *et al.*, 2009). In rodent testis, GDNF is produced by the Sertoli cells, the somatic cells supporting germ cell development (Tadokoro *et al.*, 2002). However, in human testis, GDNF is produced by Sertoli cells and by cells of the peritubular wall of the seminiferous tubules (Spinnler *et al.*, 2010). Less is known concerning the role of GDNF in cancer. GDNF promotes cell migration/chemotaxis and the invasion of pancreatic (Okada *et al.*, 1999; Veit *et al.*, 2004), chondrosarcoma (Su *et al.*, 2009) and glioma cell lines (Song & Moon, 2006). Interestingly, older transgenic mice that over-express GDNF frequently develop testicular tumours with the morphology and immunohistochemical profile (placental-like alkaline phosphatase positive) of classical human seminomas (Meng *et al.*, 2001). Invasion of undifferentiated spermatogonia into the interstitium occurs after a year of age (Meng *et al.*, 2000). More recently, a link between the *doublesex* and *mab-3* related transcription factor 1 (DMRT1) tumour-suppressor protein and the deregulation of the GDNF signalling pathway has been found in TGCTs. DMRT1 is a transcription factor that has been implicated in testicular development. The loss of *Dmrt1* in foetal testis induces the reduction of Ret expression and increased teratoma formation. However, increased DMRT1 expression is associated with increased GDNF signalling and spermatocytic seminoma (Krentz *et al.*, 2009). These observations suggest that deregulation of the GDNF pathway might be implicated in germ cell carcinogenesis even if the molecular alterations involved are not known (Meng *et al.*, 2001).

We undertook this study to determine the potential role of the GDNF pathway in the onset and progression of human seminomas. For this purpose, we first analysed the expression pattern of GFRA1 and Ret, the two major co-receptors for GDNF, in CIS and seminoma samples. Apart from a single study (Viglietto *et al.*, 2000), there is scant information on the expression of GDNF and its co-receptors in human TGCTs. We report that GFRA1 was expressed more extensively in CIS cells and invasive seminoma compared with normal testis, via immunohistochemical analysis. Thus, the potential function of the activation of the GDNF pathway in seminoma cells was investigated using the TCam-2 human seminoma cell line as a model system.

Materials and methods

Tumour samples

Surgical samples were obtained from 10 patients with testicular seminoma (mean age: 35.5 years, range: 23–50 years). The specimens were fixed in 10% formalin-buffered solution and paraffin embedded. Morphological analysis of haematoxylin-eosin stained sections indicated that tumour samples contained variable amounts of seminiferous tubules with qualitatively normal spermatogenesis (8/10), atrophic seminiferous tubules (8/10), seminiferous tubules with pre-invasive CIS cells (8/10) and invasive seminoma cells (10/10). Informed written consent was obtained from all subjects; the study was approved by the Sapienza University of Rome Ethics Committee.

Immunohistochemistry (IHC)

Immunohistochemistry was performed as described (Muciaccia *et al.*, 2010). Briefly, 3 μm thick paraffin tissue sections were serially collected and mounted into poly-lysine-coated slides. Deparaffinized and rehydrated sections were subjected to an antigen retrieval procedure, that is heating in a microwave oven (800 W) in 10 mM sodium citrate buffer (pH 6.0) for 10 min and incubated with primary antibodies diluted in phosphate buffered saline (PBS) (1 : 20 anti-human monoclonal RET, 1 : 100 anti-human CD34, anti-human inhibin and anti-PLAP

from Novocastra, Milan, Italy; 1 : 200 anti-human GFRA1 from R&D Systems, Milan, Italy; 1 : 400 anti-human CD117 from Dako, Milan, Italy). The sections were then processed using the avidin-biotin peroxidase complex (ABC) procedure, according to the manufacturer's instructions (UltraTek HRP Anti-Polyvalent Kit; ScyTek Laboratories, Rome, Italy). Negative controls were performed by omitting the primary antibodies (data not shown). Peroxidase activity was revealed using 3,3-diaminobenzidine tetrahydrochloride (Roche, Milan, Italy), and the nuclei were counterstained using haematoxylin solution. For IHC of the TCam-2 cells, the cells were harvested, collected by centrifugation, fixed in 4% paraformaldehyde at 4 °C for 10 min and paraffin-embedded. Paraffin sections, 5 μm thick, were processed as described above for tissue sections. Negative controls were performed by omitting the primary antibodies.

TCam-2 cell cultures

The TCam-2 human cell line was derived in 1993 from a primary testicular tumour sample of pure classical seminoma (Mizuno *et al.*, 1993). TCam-2 cell line is the only validated seminoma cell line (Eckert *et al.*, 2008; de Jong *et al.*, 2008). TCam-2 cells were cultured as described in RPMI 1640 (Lonza, Treviglio, Italy) supplemented with 10% foetal bovine serum (Lonza) and penicillin/streptomycin (Invitrogen, Monza, Italy) at 37 °C in a humidified atmosphere with 5% carbon dioxide (Goddard *et al.*, 2007). To test the effect of GDNF on cell proliferation and survival, TCam-2 cells were maintained for 16 h under serum-free conditions and then treated in the presence or absence of 100 ng/mL GDNF (R&D Systems) or 10% (v/v) foetal bovine serum. After 24, 48 and 72 h, the cells were trypsinized, harvested and counted. Dead cells were evaluated using trypan blue exclusion staining. To test the effect of GDNF on cell cycle entry, the cells were maintained for 16 h under serum-free conditions and then cultured in the absence or in the presence of 100 ng/mL GDNF or 10% foetal bovine serum as positive controls for 12, 24 and 48 h. The cells were recovered, fixed overnight in 50% EtOH at 4 °C and stained with a propidium iodide/RNase solution (50 μg/mL and 100 U/mL) for at least 3 h. The cell suspensions were analysed with a Beckman Coulter Epics XL Flow Cytometer. To test for gelatinolytic activity, TCam-2 cell conditioned media were collected after 48 h, and the cells were lysed in 0.5% Triton X-100 in 0.1 M Tris–HCl, pH 8.1 (Catizone *et al.*, 2010).

Reverse transcription-PCR analysis

The cells were harvested and total RNA was extracted with a phenol-chloroform extraction (TRIreagent; Sigma-Aldrich, Milan, Italy). One microgram of total RNA per sample was used for cDNA synthesis with random hexamers and Transcriptor reverse transcriptase (Roche). In control samples, reverse transcriptase was omitted to monitor genomic DNA contaminations. PCR was performed in a volume of 25 μL containing 2 μL cDNA, 50 μM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM MgCl, 50 pmol of each amplification primer, 200 mM dNTPs and 0.5 U Taq DNA polymerase (Roche). The primers and PCR details are described in Table S1. The PCR products were fractionated by 1.8% agarose gel electrophoresis and visualized by ethidium bromide staining. Experiments were performed three times, using different RNA preparations.

Boyden chamber assays

The cells were assayed for their ability to migrate through a polycarbonate filter (pore size, 8 μm; Whatman International, Maidstone, UK) using Boyden chambers (NeuroProbe, Gaithersburg, MD, USA). The cells (5×10^4/well) were added to the upper chamber, and GDNF, 15 μg/mL anti-GDNF antibody (R&D Systems), CXCL12 (SDF-1 from Peproteck, London, UK), 15 μg/mL isotype control antibody (R&D Systems) or FBS (control wells) were added alone or in combination in the lower chamber. To analyse the GDNF-triggered pathways, 10 μM U0126 (MEK inhibitor), 10 μM or 15 μM LY294002 (PI3K inhibitor), 10 μM PP-2 (Src-inhibitor) were added to both the upper and bottom chambers. All inhibitors were dissolved in dimethyl sulphoxide (DMSO) and subsequently diluted in the medium. The various concentration of inhibitors used did not affect cell viability as assessed by FACS analysis of propidium iodide stained cells (data not shown). The chambers were incubated for 5 h at 37 °C. The cells were assayed for their ability to invade through filters (8 μm) coated with Matrigel Basement Membrane Matrix, Growth Factor Reduced (BD Biosciences, Buccinasco, Italy) using Boyden chambers. The cells (5×10^4/well) were added to the upper chamber, and GDNF or CXCL12 was added to the lower chamber. GDNF and CXCL12 were dissolved in PBS containing 0.1% BSA, and subsequently diluted in the medium. To obtain pericellular protease inhibition, a cocktail of 250 μM E64, 2 μM leupeptin, 100 μM pepstatin A, 2.2 μM aprotinin and 50 μM GM6001, termed the protease inhibitor mix (prot. inh. mix) was prepared as previously described and added to both the upper and bottom chambers (Carragher *et al.*, 2006). E64, pepstatin A and GM6001 were dissolved in DMSO, leupeptin and aprotinin were dissolved in water. All inhibitors were subsequently diluted in the medium. Invasion was allowed to proceed for 24 or 48 h at 37 °C in a humidified atmosphere with 5% carbon dioxide, at

which time filters were fixed and stained. The cells from the upper side of the filter were carefully removed using a cotton swab. The cells that had migrated or invaded to the lower side of the filters were quantified by bright-field microscopy using a 40× objective, and the average number of cells per field was calculated. Data are expressed as a migration index and calculated as the fold increase over the control. The control cells were either in serum-free RPMI 1640 containing 0.1% BSA or in serum-free RPMI 1640 containing 0.1% BSA and DMSO. The results are reported as the means ± standard error.

Gelatin zymography for matrix metalloproteinases (MMPs) detection

Gelatinolytic activity of the TCam-2 conditioned media and cell extracts were assayed as previously described (Catizone *et al.*, 2010). Briefly, 20 μL aliquots of conditioned media and cell extracts were fractionated by 10% SDS-polyacrylamide gel electrophoresis in the presence of 0.1% gelatin under non-reducing conditions. Following electrophoresis, the gels were washed twice in 2.5% Triton X-100 for 30 min at room temperature to remove SDS. The gels were incubated at 37 °C overnight in substrate buffer, stained with 0.5% Coomassie Brilliant Blue R250 and destained in 30% methanol and 10% glacial acetic acid (vol/vol).

Statistical analysis

All quantitative data are presented as the mean ± standard error of the mean (SEM). Data were analysed for significant differences between different subsets of migrated or invading cells using a one-way analysis of variance (ANOVA) followed by a post hoc Dunnet test. The significance level was fixed at $\alpha = 0.05$.

Results

GFRA1 is over-expressed in CIS and invasive seminoma cells

Investigation of the expression patterns of GFRA1 and Ret was performed by IHC on ten different testicular seminoma samples (Fig. 1). Adjacent serial sections were stained with antibodies against c-Kit (CD117) and PLAP, two diagnostic markers routinely used to identify CIS and seminoma cells, and with anti-CD34 to detect endothelial cells (Figure S1). In seminiferous tubules displaying qualitative normal spermatogenesis near a CIS lesion, GFRA1 expression was detected, as previously described, in subsets of dark and pale type A spermatogonia (Grisanti *et al.*, 2009) and in interstitial Leydig cells (Davidoff *et al.*, 2001) (Fig. 1A). Compared with preserved testicular parenchyma, GFRA1 expression was up-regulated in both CIS and intratubular seminoma cells, whereas expression levels varied from moderate to strong in invasive seminoma cells (Fig. 1A). In preserved testicular parenchyma, Ret expression was not detected in seminiferous tubules, whereas it was clearly detected in interstitial tissue, possibly in the Leydig cell lineage (Fig. 1B). In some instances, a strong Ret expression was detected in Sertoli cells of atrophic seminiferous tubules, with a thickened peritubular wall and lack of germ cells, as well as in Sertoli cell cytoplasm of seminiferous tubules containing pre-invasive CIS cells. Finally, in invasive seminoma lesions, no Ret expression was detected in seminoma cells, whereas Ret-positive staining was detected in both endothelial cells (identified morphologically and by CD34 staining) and in cell clusters, possibly endothelial/pericyte precursors, located in perivascular areas (Fig. 1B).

The TCam-2 seminoma cell line expresses the co-receptors for GDNF

Because CIS and seminoma cells highly express the GDNF co-receptor GFRA1, we tested the hypothesis that GDNF is involved in tumoural germ cell proliferation. To this end, we took advantage of TCam-2 cells, a seminoma cell line. First, expression of GDNF co-receptors was analysed by RT-PCR analysis (Fig. 2A) and by IHC (Fig. 2B). *GFRA1* is alternatively spliced into two highly homologous isoforms, GFRA1a and GFRA1b, which differ at five amino acids (Shefelbine *et al.*, 1998). Both GFRA1 isoforms are expressed in the TCam-2 cells (Fig. 2A). *Ret* undergoes alternative splicing, which generates the Ret9 and Ret51 isoforms, differing at the carboxyl-termini (Arighi *et al.*, 2005). As observed by RT-PCR analysis, both Ret9 and Ret51 transcripts are present in the TCam-2 cells (Fig. 2A). GFRA1, but not RET, was detected by IHC (Fig. 2B). We next addressed whether GDNF modulates TCam-2 proliferation or survival. Serum-starved cells were treated in the absence and in the presence of 100 ng/mL GDNF with or without 10% FBS for 24, 48 and 72 h. The total number of cells, as well as the number of dead cells, did not vary in GDNF-treated cells compared with the control at each time point analysed (Figure S2). FACS analysis was performed to determine the percentage of TCam-2 cells in S phase (Figure S3). Although FBS significantly increased the percentage of cells in S phase compared with the control, GDNF did not induce S-phase entry of TCam-2 cells at any time points analysed (Figure S3).

GDNF induces TCam-2 cell migration

In other cellular systems, GDNF has been implicated in the migration and invasion of normal and tumour cells

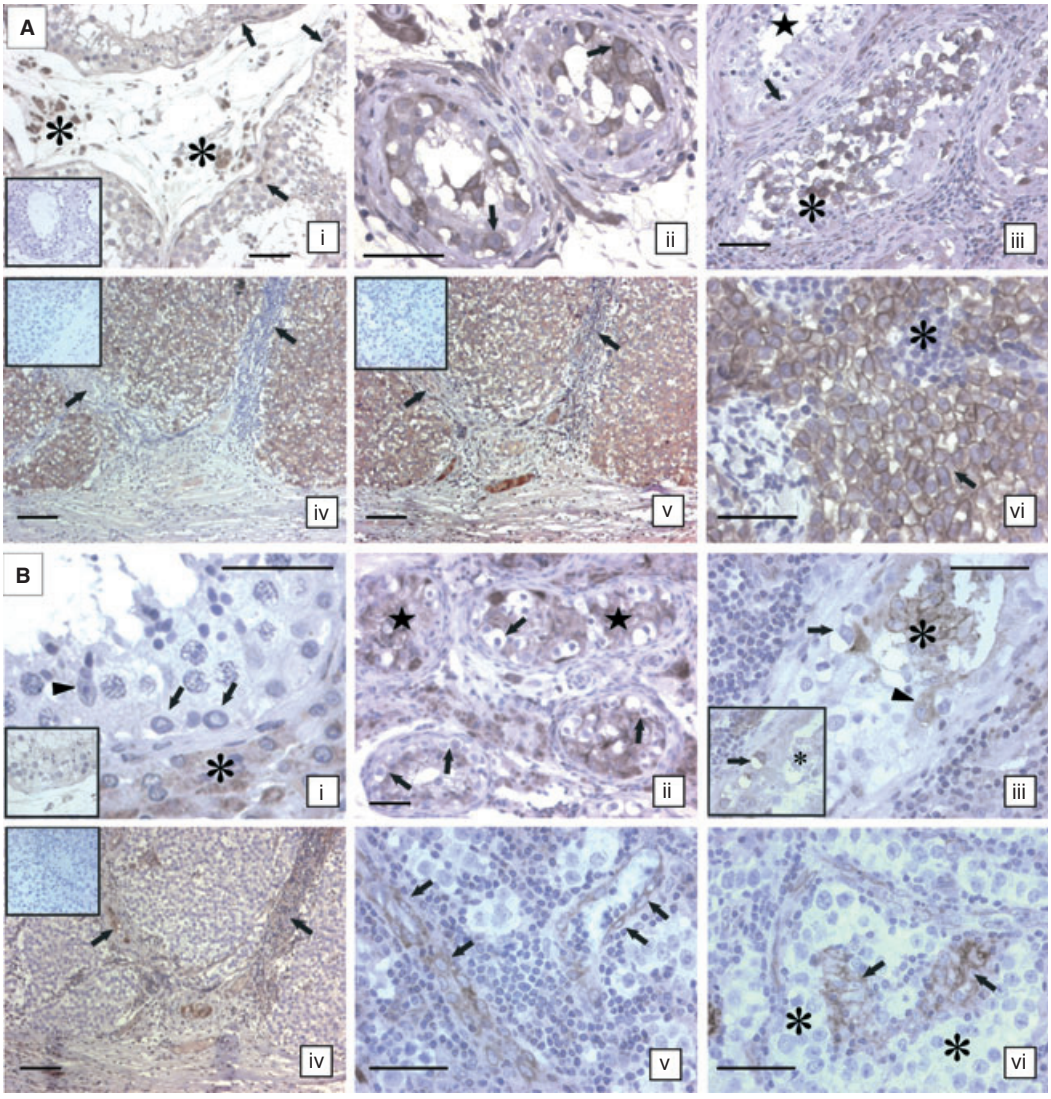

Figure 1 Immunohistochemical detection of GDNF co-receptors in seminoma samples. Images are from six representative seminoma tissue samples. (A) GFRA1 cellular localization: (i) seminiferous tubules with preserved spermatogenesis adjacent to CIS lesion. GFRA1 expression was detected in Leydig cells (asterisks) and in the basal compartment of seminiferous tubules where subsets of dark and pale type A spermatogonia were positive (arrows); (ii) seminiferous tubules with CIS cells showing high GFRA1 staining (arrows). CIS cells were recognized morphologically or with anti-c-Kit immunodetection on serial sections (not shown); (iii) GFRA1-positive intratubular seminoma cells (asterisk). GFRA1 expression was up-regulated in seminoma cells compared with spermatogonia (arrows) in adjacent seminiferous tubules with preserved spermatogenesis (star); (iv) c-Kit (CD117) and (v) GFRA1 immunostaining in adjacent sections of the same seminoma tissue sample. Note the fibrous septa rich in infiltrating inflammatory cells (arrows); (vi) high magnification of GFRA1-positive invasive seminoma cells (arrows), infiltrating lymphocytes are negative (asterisks). (B) Ret cellular localization: (i) lack of Ret staining in preserved testicular parenchyma Ret of seminiferous tubules both in germ and somatic cells, while Ret-positive cells (possibly Leydig cells) are detected in the interstitial compartment (asterisk). Dark type A spermatogonia are indicated by arrows, and a Sertoli cell nucleus is indicated by an arrowhead; (ii) high Ret cytoplasmic staining was detected in Sertoli cells (stars) present in seminiferous tubules containing Ret-negative CIS cells (arrows); (iii) in some seminiferous tubules, clusters of Ret-positive Sertoli cells were found sloughing into the lumen (asterisk). Arrowhead points to a Ret-positive Sertoli cell properly arranged in the epithelium. Arrows indicate the same CIS cell identified on adjacent sections, which is Ret-negative and GFRA1-positive (inset). An asterisk indicates the same cluster of Sertoli cells shown in (iii); (iv) in invasive seminoma cells, no Ret expression was detected. Arrows point to fibrous septa containing Ret-positive cells; (v) Ret expression was detected in endothelial cells (arrows) lining the lumen of vessels. Endothelial cells show a morphological activated phenotype with large euchromatic nuclei; and (vi) Ret staining was detected in clusters of possibly endothelial/pericyte precursors (arrows). Seminoma cells are Ret-negative (asterisks). No staining is present by omitting the primary antibodies as shown in insets presented in A (i, iv, v) and in B (i, iv). Fig. A (i) and B (i) are from sample no. 10; Fig. A (iv-vi) and B (iv) are from sample no. 07; Fig. A (ii) is from sample no. 05. Fig. A (iii) is from sample no. 06. Fig. B (iii), (v) and (vi) are from sample no. 08. Fig. B (ii) is from sample no. 02. Bars, 50 μm.

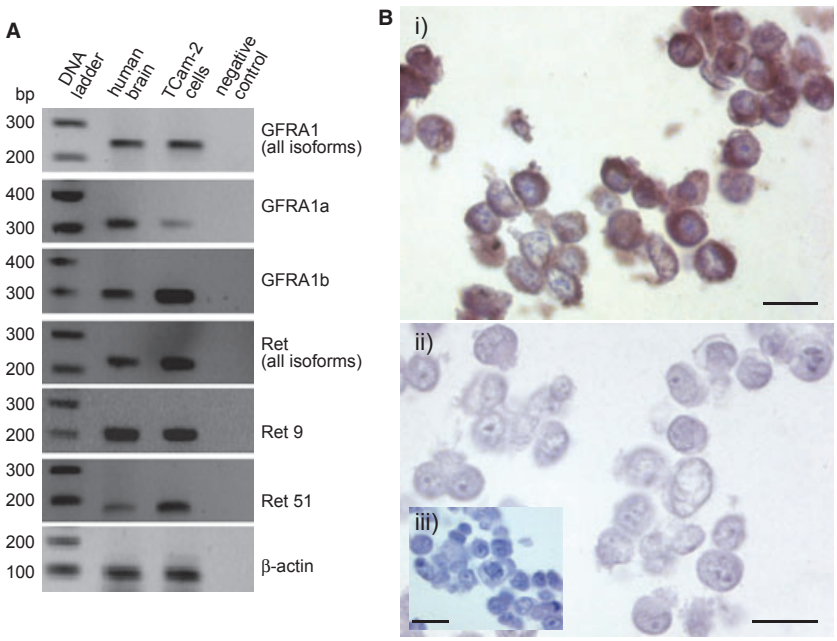

Figure 2 Identification of GDNF co-receptor variants expressed in TCam-2 cells. (A) RT-PCR analysis. Total RNA was prepared from TCam-2 cells and used as a template for RT-PCR analysis. A sample containing RNA from human brain and a sample containing no reverse transcriptase were used, respectively, as positive and negative controls. A β-actin primer set was used as a loading control. (B) Immunohistochemical detection of GDNF co-receptors in TCam-2 cells: (i) GFRA1 and (ii) Ret. No staining is present by omitting the primary antibodies (inset). Bars, 20 μm.

(Tang *et al.*, 1998; Okada *et al.*, 1999; Young *et al.*, 2001; Veit *et al.*, 2004; Paratcha *et al.*, 2006; Song & Moon, 2006; Su *et al.*, 2009). More interestingly, we recently found that in vitro GDNF is a chemoattractant for murine undifferentiated spermatogonia (Dovere. L, unpublished data). Based on these observations, we tested the hypothesis that the GDNF pathway could be involved in TCam-2 seminoma cell migration using Boyden chamber assays with increasing concentrations of GDNF (20–150 ng/mL). A significant effect on cell migration was achieved with 100 and 150 ng/mL GDNF (Fig. 3A). This effect was blocked by an anti-hGDNF neutralizing antibody but not by the antibody isotype control (Fig. 3B). When GDNF was added to both the upper and lower chambers, and therefore no chemokine gradient was present in the Boyden chamber, cell migration was decreased to the control level (Fig. 3B). Chemoattractant activity of 1% FBS was comparable with 100 ng/mL GDNF, and no synergic effect was found when the TCam-2 cells were stimulated with both 1% FBS and 100 ng/mL GDNF (Fig. 3B). Invasive migration of the TCam-2 seminoma cell line is stimulated by the chemokine CXCL12, which activates the ERK pathway (Gilbert *et al.*, 2009). By comparing the GDNF chemoattractant activity with that of CXCL12, we found that GDNF is more effective (approximately 50%) than CXCL12 (Fig. 3C). In addition to the observation that GDNF is an in vitro chemoattractant for undifferentiated mouse spermatogonia (Dovere. L, unpublished data), these data suggest that the chemoattractant activity of GDNF is conserved in both normal and transformed germ cells. We next investigated the GDNF-triggered pathway involved in TCam-2 migration. In different tumour cell types, GDNF activates the MEK and PI3K pathways (Veit *et al.*, 2004; Song & Moon, 2006; Su *et al.*, 2009). Three different inhibitors were tested as follows: PP-2 (a potent Src-inhibitor), LY294002 (a PI3K-inhibitor) and U0126 (a MEK-inhibitor). PP-2 (Fig. 4A) and UO126 (Fig. 4B), but not LY294002 (Fig. 4C), completely abolished GDNF-induced cell migration. These data suggest that GDNF-induced migration is mediated by the Src and MEK pathways but not by the PI3K pathway.

GDNF promotes TCam-2 cell invasion in a protease-dependent fashion

We next addressed whether invasion could be triggered by GDNF in TCam-2 cells. Invasion was analysed at 24 and 48 h using a modified Boyden chamber assay with Matrigel-coated filters. At both time points, GDNF administration significantly enhanced the invasion of cells compared with the control, as well as with CXCL12 (Fig. 5A). Tumour cell invasion can occur through two modalities: a proteolytic mesenchymal-like or non-proteolytic amoeboid-like modality (Sahai & Marshall, 2003;

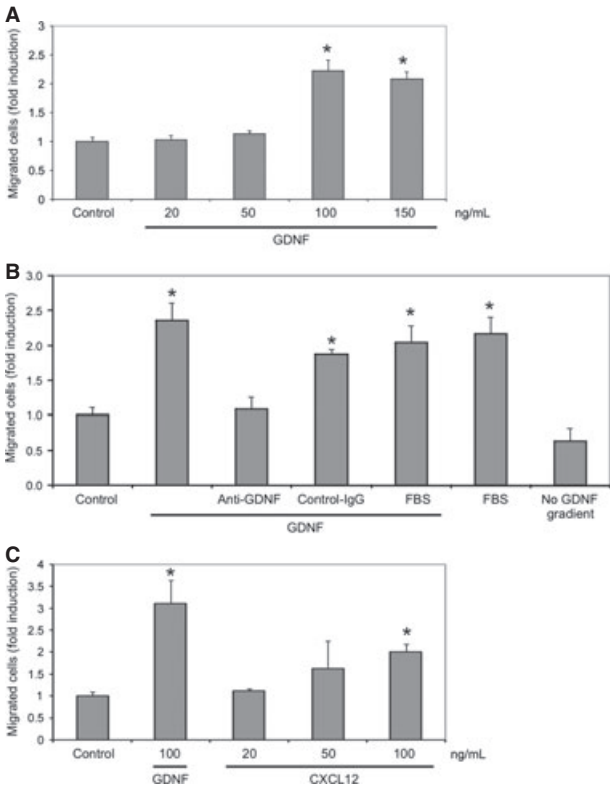

Figure 3 GDNF-dependent migration of TCam-2 cells. Cell migration was evaluated using the Boyden chamber assay as detailed in the Materials and Methods section. (A) TCam-2 cells were treated with increasing concentrations of GDNF. (B) TCam-2 cells were treated as indicated at final concentrations of 100 ng/mL GDNF, 15 μg/mL anti-GDNF antibody, 15 μg/mL control-IgG or foetal bovine serum (FBS) (1% v/v). To abrogate the GDNF gradient in the Boyden chamber, 100 ng/mL GDNF was added both in the upper and lower chamber. (C) TCam-2 cells were treated with 100 ng/mL GDNF or with increasing concentrations of CXCL12 (SDF-1). Data are expressed as the mean ± SEM ($n = 3$, measured in triplicate). *$p < 0.001$ vs. control (one-way ANOVA, Dunnet post hoc).

Wolf *et al.*, 2003). The mesenchymal-like strategy of invasion requires extracellular matrix degradation by proteases, including MMPs, and thus, it is sensitive to protease inhibitors. In contrast, the amoeboid-type modality is protease-independent, with cells that adopt a rounded morphology. To identify which of the two modalities is employed by the TCam-2 cells in GDNF-induced cell invasion, we tested a cocktail of protease inhibitors (named prot. inh. mix), which included GM6001 (a broad MMPs inhibitor), E64 (targets cysteine proteases such as cathepsins B, L, H and K), pepstatin A (inhibits aspartic proteases including cathepsin D), leupeptin (a broad inhibitor of cysteine proteases and cathepsin D) and aprotinin (an inhibitor of serine proteases, such as urokinase and tissue plasminogen activator) (Carragher *et al.*, 2006). Prot. inh. mix administration completely abolished the GDNF-induced TCam-2 cell invasion (Fig. 5B), suggesting a protease-dependent invasion strategy. To evaluate the contribution of MMPs to TCam-2 cell invasion, the MMPs-specific inhibitor GM6001 was tested alone, and its activity was compared with prot. inh. mix without GM6001. GM6001 alone, but not the other inhibitors, reduced the GDNF-induced TCam-2 cells invasion, suggesting the involvement of MMPs (Fig. 5B). MMP-2 and MMP-9 activity (Fig. 5C) was detected in TCam-2 cell extracts and conditioned medium by gelatin zymography.

Discussion

In recent years, evidence has emerged showing the importance of GDNF for proper spermatogenesis. In mice, GDNF is secreted by Sertoli cells and promotes spermatogonial stem cell self-renewal (Hofmann, 2008). Overexpression of GDNF in adult transgenic mice induces proliferation of clusters of undifferentiated spermatogonia within seminiferous tubules (Meng *et al.*, 2000). In addition, older animals develop testicular tumours that mimic human seminoma, suggesting that over-activation of this pathway may lead to the development of germ cell tumours (Meng *et al.*, 2001). Supporting this hypothesis, Ret, a co-receptor for GDNF, is a well-known oncogene that upon mutation and/or rearrangement undergoes constitutive GDNF- and GFRA1-independent activation. Activating mutations of *Ret* were previously reported in several types of cancer, including thyroid, pituitary, adrenal and melanoma cancer. However, human seminoma does not appear to be linked to mutations or relevant polymorphisms of *Ret* (Chevalier *et al.*, 2010). An alternative mechanism for driving germ cell tumour progression could be the up-regulation of wild-type GFRA1 and/or Ret proteins. Over-expression of GFRA1 and/or Ret has been found in different cancers (Takaya *et al.*, 1996; Dawson *et al.*, 1998; Nakashima *et al.*, 2007), and in some instances, Ret over-expression correlates with poor survival (Ito *et al.*, 2005) as well as the development of a subgroup of breast tumours (Esseghir *et al.*, 2007). We demonstrate, for the first time, that GFRA1 is up-regulated in CIS, and its expression is maintained in invasive seminoma. However, Ret was not detected by IHC in normal germ cells, in CIS cells, or in the invasive seminoma. In TCam-2 cells, *Ret* was detected at mRNA but not protein levels, suggesting that in CIS and seminoma cells, Ret protein may be expressed at very low levels. Alternatively, GFRA1 may transduce GDNF signal in a Ret-independent fashion (Airaksinen & Saarma, 2002; Sariola & Saarma, 2003). To date, a number of CIS and seminoma cells markers are known to be expressed in foetal gono-

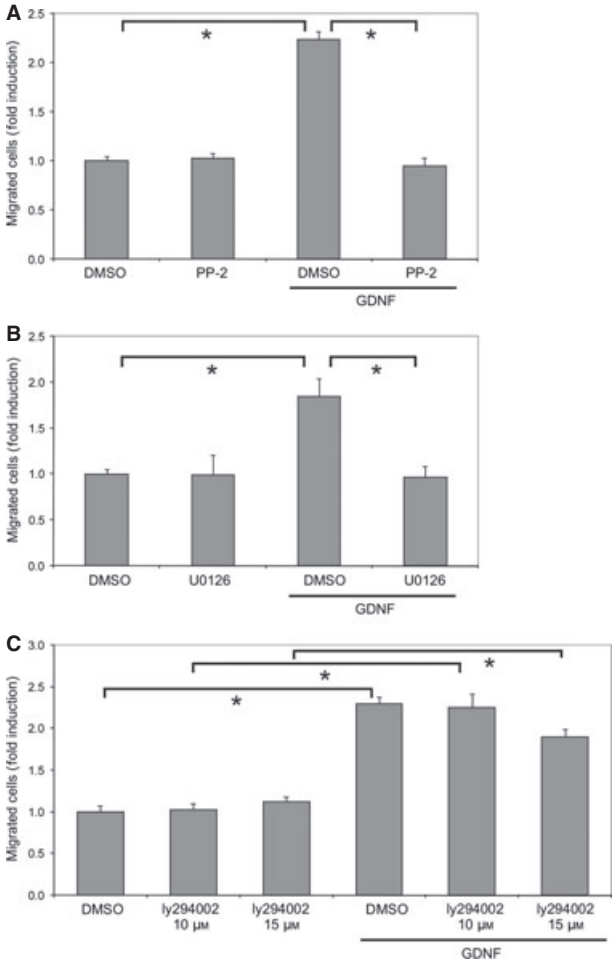

Figure 4 The effect of different inhibitors on GDNF-induced TCam-2 cell migration. Cell migration was evaluated using the Boyden chamber assay as detailed in the Materials and Methods section. TCam-2 cells were pre-treated for 30 min with 10 μm PP-2 (A), 10 μm U0126 (B) or an indicated concentration of LY294002 (C) followed by stimulation with 100 ng/mL GDNF. In the control samples, DMSO was added to the highest concentration present in the treated samples. The results are expressed as the mean ± SEM, ($n = 3$, measured in triplicate). *$p < 0.001$ vs. control (one-way ANOVA, Dunnet post hoc).

cytes but not in adult spermatogonia. However, CIS cells also express genes found in the germ cells of normal adult testis (i.e. VASA, TSPY, DAZ and DAZL) (Rajpert-De Meyts, 2006). Because GFRA1 is detected in dark and pale type A spermatogonia, GFRA1 lengthens the list of tumour germ cell markers that are expressed in normal germ cells.

To elucidate the biological activity of GDNF in seminoma, we employed the TCam-2 cell line as an experimental model system. Even though TCam-2 cells express the GDNF co-receptors, GDNF did not affect cell survival and proliferation. Because GDNF acts as a chemoattractant in several normal and tumour cell types (Tang *et al.*, 1998; Okada *et al.*, 1999; Young *et al.*, 2001; Veit *et al.*, 2004; Paratcha *et al.*, 2006; Song & Moon, 2006; Su *et al.*, 2009), we tested the hypothesis that GDNF is a chemoattractant for the TCam-2 seminoma cell line. GDNF induced TCam-2 cell migration and was mediated by the Src and MEK pathways. In contrast, PI3K does not seem to be involved in GDNF-induced migration. Our findings are consistent with the observation that in some pancreatic carcinoma cells, GDNF is not mitogenic but acts as a chemoattractant (Veit *et al.*, 2004). We found that GDNF induces TCam-2 cell invasion through GFR-Matrigel, a matrix consisting of laminin, collagen, entactin and growth factors. Invasive tumour dissemination involves proteolytic degradation and remodelling of the extracellular matrix (ECM) barriers, a process dependent upon extracellular proteases, including MMPs (Kessenbrock *et al.*, 2010). However, protease inhibition does not completely abolish tumour cell migration and dissemination, suggesting alternative compensating protease-independent mechanisms (Zucker *et al.*, 2000; Coussens *et al.*, 2002). Of note, when proteolysis is blocked, some tumour cell types switch from a protease-dependent mesenchymal type of motility to an amoeboid-like rounded mode of motility that requires Rho/ROCK signalling (Sahai & Marshall, 2003; Wolf *et al.*, 2003). By using inhibitors of a wide range of pericellular proteases, we found that the invasive strategy used by TCam-2 cells is protease-dependent, which suggests a mesenchymal-like invasion. Among candidate proteases implicated in GDNF-induced TCam-2 cell invasion are the MMPs, the most prominent family of proteases involved in tumourigenesis (Kessenbrock *et al.*, 2010). The lack of invasion upon inhibition of pericellular proteases suggest that TCam-2 cells are not able to undergo the mesenchymal-ameboid switch, thus suggesting a lack of plasticity compared with more aggressive tumour cell types (Carragher *et al.*, 2006).

We demonstrated that GFRA1 expression is up-regulated in CIS and seminoma cells. This raises the question as to the source of GDNF in seminoma. In normal human testis, GDNF is produced by the Sertoli and peritubular cells, which form the wall of the seminiferous tubules (Spinnler *et al.*, 2010). The expression level of GDNF was found to be similar in peritubular cells isolated from patients suffering from azoospermia and fibrotic remodelling of the peritubular wall (Spinnler *et al.*, 2010). At present, data are not available regarding the expression level and regulation of GDNF in normal human testis or those affected by pathological conditions, including TGCTs. However, our preliminary results indicate that GDNF mRNA levels are heterogeneous in normal testis and seminoma samples. In infertile mouse

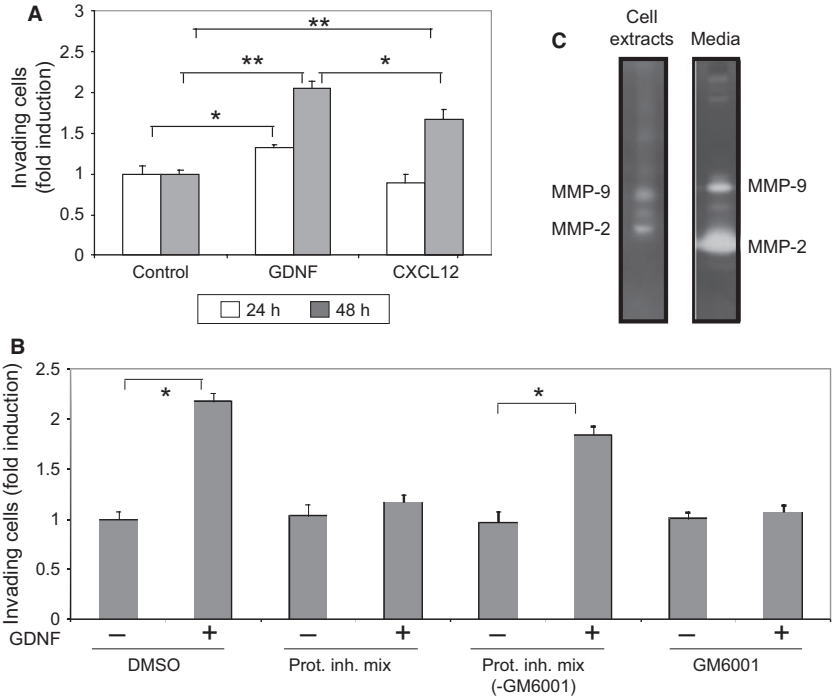

Figure 5 Glial cell line–derived neurotrophic factor (GDNF) promotes invasion in TCam-2 cells. Matrigel invasion assays were performed and evaluated as detailed in the Materials and Methods section. (A) TCam-2 cells were treated for 24 and 48 h with 100 ng/mL GDNF and 100 ng/mL CXCL12; (B) TCam-2 cells were pre-treated for 30 min with indicated protease inhibitors or DMSO alone followed by stimulation with GDNF (100 ng/mL). The results are expressed as the mean ± SEM ($n = 2$, measured in triplicate). *$p < 0.05$, **$p < 0.001$ (one-way ANOVA, Dunnet post hoc). (c) MMP-2 and MMP-9 gelatinase activity in TCam-2 conditioned media and cell extracts. Images are representative of three independent experiments.

models, GDNF levels are inversely correlated with the germ cell content of the testis, possibly through the regulation of the hypothalamus–pituitary axis (Tadokoro *et al.*, 2002). Moreover, GDNF expression in Sertoli cells is induced by inflammatory cytokines (Simon *et al.*, 2007). Therefore, it could be speculated that, as described in some breast cancers (Esseghir *et al.*, 2007), local levels of GDNF in the tumour microenvironment may be increased as a consequence of leucocyte infiltration and/or spermatogenic arrest, which are commonly found in TGCTs. Although currently it is not possible to directly address whether CIS and intratubular seminoma cells respond to local GDNF by increasing their ability to migrate and invade the interstitial compartment, our combined in vivo and in vitro observations suggests that GDNF may induce tumour cell migration and dissemination within the testis. Recently, it has been suggested that the expression level of the chemokine CXCL12 (SDF-1) in TGCT samples is a predictive marker of relapse for patients affected by a subset of type II TGCTs, that is, stage I non-seminoma. Patients with moderate to strong expression of CXCL12 have a reduced risk of relapse, suggesting that a high level of chemokines in the tumour microenvironment may impact tumour germ cell spreading and metastatic potential (Gilbert *et al.*, 2009). Following this line of reasoning, it would be interesting to assess whether or not the expression level of GDNF signalling pathway molecules have a similar prognostic value in patients affected by seminoma tumours.

Acknowledgements

We are grateful to Dirk de Rooij for critical discussion of the data and Fabrizio Padula for technical assistance.

Funding

This work was supported by grants from the Agenzia Spaziale Italiana [# I/065/08/0]; and Ministero dell'Istruzione, dell'Università e della Ricerca [PRIN #20083CBLPY_002 and 2009FW 5SP3_003].

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Immunohistochemical analysis performed on adjacent paraffin sections obtained from sample no. 7. GFRA1 antibodies identify CIS and seminoma cells similar to two routinely used diagnostic markers, such as c-Kit antibodies (CD117) and PLAP. Anti-CD34 antibodies detect endothelial cells. Inset represent a negative control. Bars, 100 μm.

Figure S2. GDNF is not a mitogenic factor for TCam-2 cells. (A) TCam-2 cells were serum-starved for 16 h and then cultured in the presence or absence of 100 ng/mL GDNF with or without 10% FBS for 24, 48 and 72 h. Percentage of dead cells at each time point is shown in (B). The results are from a representative of three experiments performed. Data are shown as the mean ± SEM of triplicate samples.

Figure S3. GDNF does not induce S-phase entry in TCam-2 cells. (A) Flow cytometry analysis reveals that TCam-2 cells have a tetraploid DNA content. LYMPHO: human lymphocytes. (B) Cell cycle phase distribution of TCam-2 cells treated in the absence or presence of 100 ng/mL GDNF or with 10% FBS (as a positive control) for 12, 24 and 48 h of culture. Percentage of cells in G1, G2 and S phase for each experimental condition is presented in (C). The results are from a representative of three experiments performed. $*p < 0.05$ (one-way ANOVA, Dunnet post hoc).

Table S1. List of primers used in RT-PCR analysis.

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