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GLIAL CELL–DERIVED NEUROTROPHIC FACTOR (GDNF) PROMOTES INVASIVE BEHAVIOR IN TESTICULAR SEMINOMA CELLS.

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ABSTRACT

The 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 tumors in older animals, which suggests that although the underlying mechanisms are unknown, deregulation of the GDNF pathway may be implicated in germ cell carcinogenesis. To explore the involvement of the GDNF pathway in the onset and progression of testicular germ cell tumors, we firstly analyzed the expression pattern of GFRA1 and Ret, the two major co-receptors for GDNF, in seminoma samples. We report that GFRA1 was expressed more extensively in *carcinoma in situ* (CIS) cells and intratubular invasive seminoma compared with normal testis.

Functional analysis of the GDNF biological activity was performed on TCam-2 human seminoma cell line. 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. It is well known that GDNF plays a central role in spermatogonial stem cell self-renewal and proliferation (Hoffman MC et al., 2008). Therefore, in order to evaluate if GDNF could act as a mitogenic factor in TCam-2 cells, we performed proliferation assays and cell cycle analyses. Interestingly we observed that GDNF doesn't induce increase in total cell number or S-phase entry in TCam-2 cell line.

Several evidences suggested that GDNF is able to induce cell migration and invasion in several normal and tumor cell types (Okada Y et al., 1999; Veit C et al., 2004; Su CM et al., 2009; Song H et al., 2006; Paratcha G et al., 2006; Tang MJ et al., 1998; Young HM et al., 2001). Therefore we have hypothesized that GDNF can act as a chemoattractant also in seminoma cells. On this purpose we stimulated directional TCam-2 cell migration and invasion in the presence of GDNF gradients and we investigated the downstream pathways responsible for the GDNF-induced invasive behaviour. We demonstrated that GDNF is able

to induce migration, possibly through the Src and MEK pathways. It is documented that tumor cell invasion can occur through two modalities: a proteolytic mesenchymal-like or non-proteolytic amoeboid-like modality (Sahai E and Marshall CJ, 2003; Wolf K et al., 2003). GDNF is able to induce TCam-2 seminoma cell invasion in a mesenchymal-like metalloprotease-dependent manner.

In conclusion, 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

1. Testicular germ cell tumors (TGCTs)

1.1 Origin and development

Testicular germ cell tumors (TGCTs) are the most frequent solid malignant tumors among young men. Based on epidemiology, clinical presentation, phenotypic characterization, chromosomal constitution and genomic imprinting, TGCTs are classified into three groups (Oosterhuis JW et al., 2005). Type I comprises the teratomas and yolk-sac tumors of newborns and infants (Figure 1); type II comprises the seminomatous and non-seminomatous tumors of adolescents and young adults (Figure 1); and type III comprises the spermatocytic seminomas of elderly people (Figure 1). During the carcinogenic process, the primordial germ cells or gonocytes are converted into a pre-invasive lesion, known as *in situ* carcinoma (CIS) (Kristensen DM et al., 2008; Skakkebaek NE et al., 1972). CIS cells further develop to give rise to invasive TGCTs (Figure 2). However, not all TGCTs develop from a CIS, as is the case of spermatocytic seminoma of elderly men and infantile germ cell tumors (Kristensen DM et al., 2008).

The fetal origin of CIS cells is not only supported by the morphological similarities among CIS cells, primordial germ cells and gonocytes but also by immunohistochemical studies of proteins present in all these cell types such as the octamer binding transcription factor 3/4 (OCT3/4), the placental-like alkaline phosphatase (PLAP), the activating enhancer-binding protein 2 gamma (AP-2γ), and the stem cell factor receptor c-KIT (van de Geijn GJM et al., 2009). Moreover CIS lesions have been identified in prepuberal patients, who later developed TGCTs, indicating that these cells originated prior to puberty (Muller J et al., 1984). Thus CIS cells are most likely the result of a delayed or blocked differentiation of embryonic germ cells.

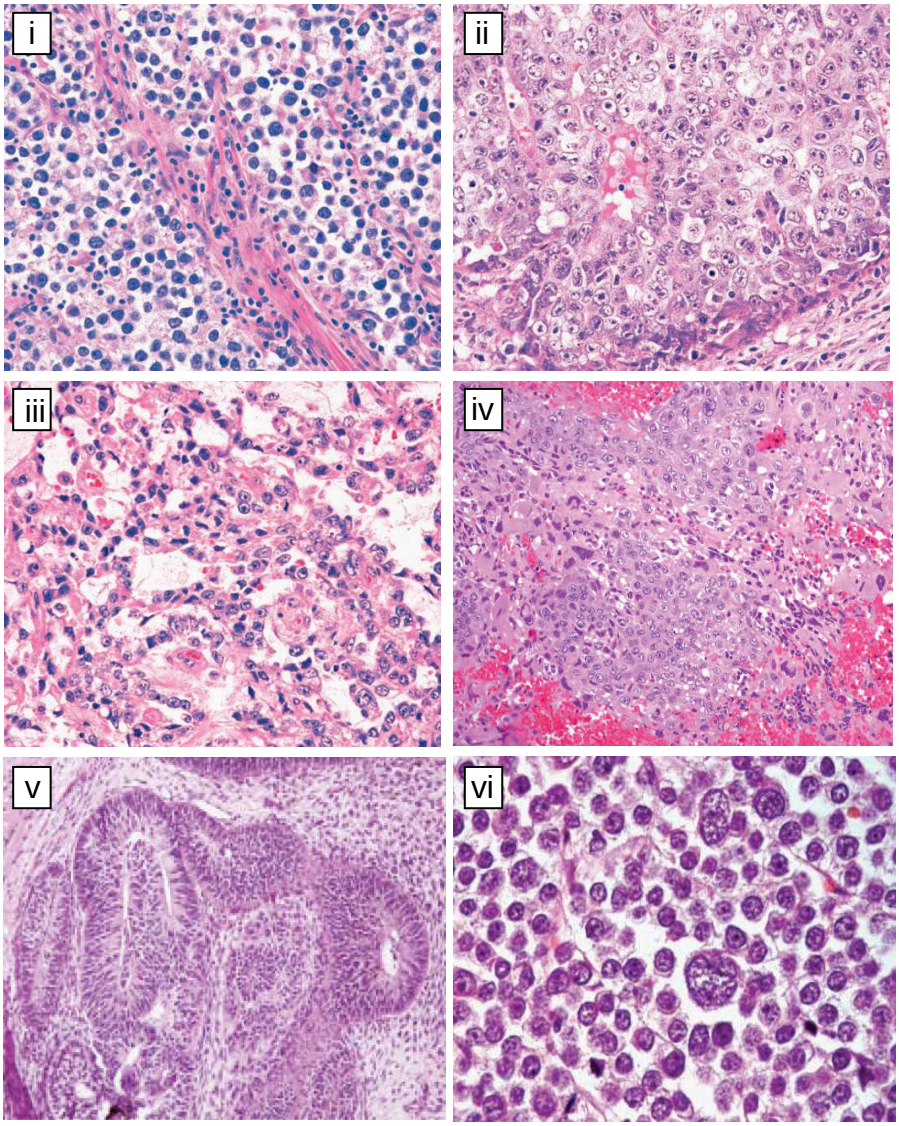

Fig. 1 – Testicular germ cell tumors (TGCTs).
Hematoxylin & Eosin stained section of: (i) seminoma, (ii) embryonic carcinoma, (iii) yolk-sac tumor, (iv) chorioncarcinoma, (v) teratoma and (vi) spermatocytic seminoma. (Modified from Wang P et al., 2010 Am J Clin Pathol 134:604-612).

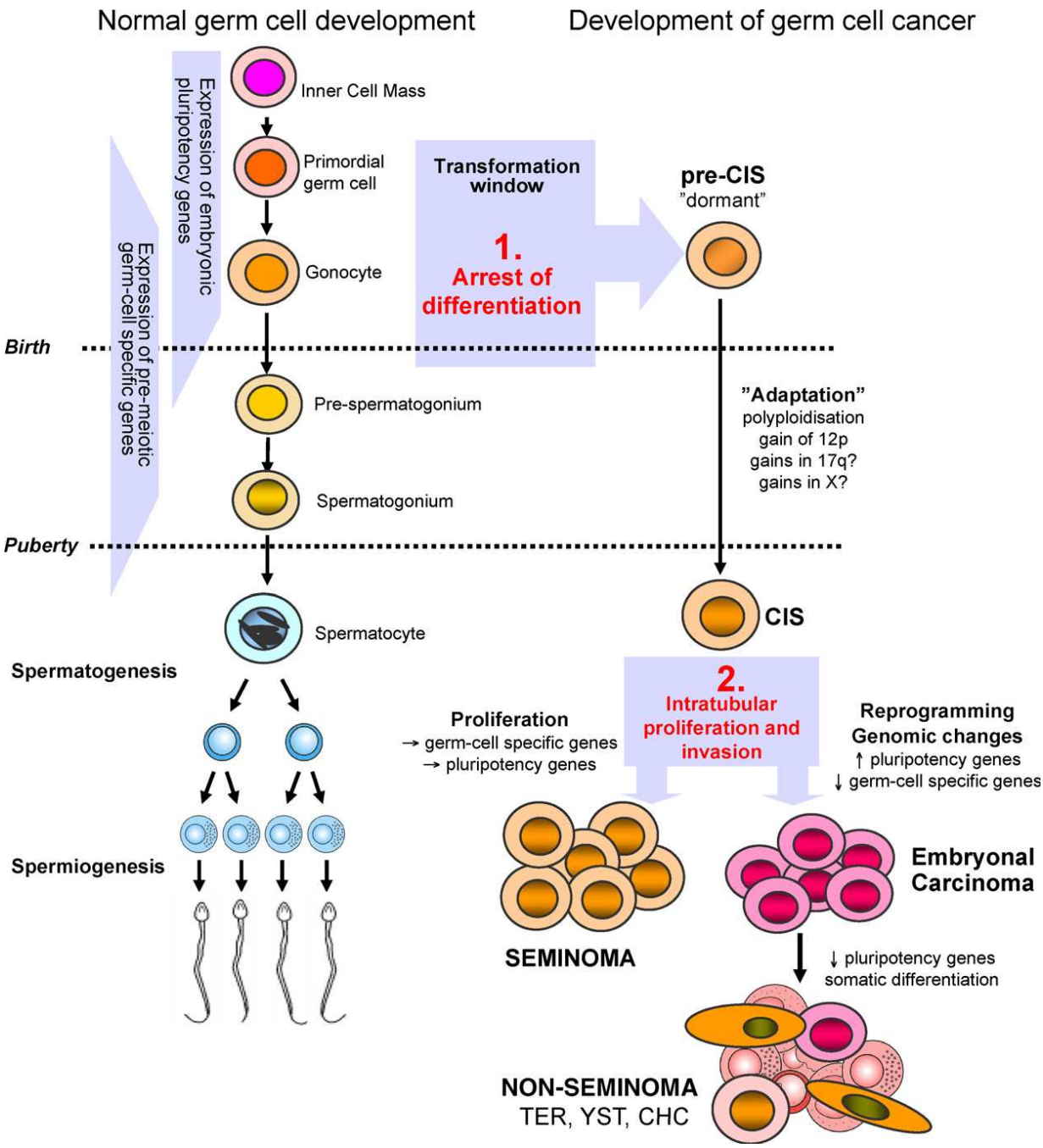

Fig. 2 - Germ cells transformation and tumor progression.
During the carcinogenic process, primordial germ cells or gonocytes undergo arrest of differentiation and are converted into a pre-invasive lesion, known as carcinoma in situ (CIS) (Skakkebaek et al., 1972, Kristensen et al., 2008). CIS cells further develop to give rise to the invasive testicular germ-cell tumors (TGCTs) of the adolescent and young adults, that are seminomatous and non-seminomatous tumors. All these lesions are characterized by an up-regulation of germ cell pluripotency genes with an expression pattern more similar to embryonic germ cells. However not all TGCTs develop with a CIS, as in the case of spermatocytic seminoma of elderly men and infantile germ cell tumors. (From Kristensen DM et al., 2008 Mol.Cell Endocrinol. 288: 111-118).

CIS lesion appears in the form of enlarged cells, usually placed in the basal portion of seminiferous tubules, with clear cytoplasm and a round, hyperchromatic nuclei (significantly larger than those of spermatogonia) and one or more prominent nucleoli. Supporting its neoplastic nature, CIS cells are aneuploid. CIS seminiferous tubules show decreased tubular diameter, thickened peritubular basement membrane and absence of normal spermatogenesis. Another observation that supports the precursor role of CIS in TGCTs development is that CIS cells are found with high frequency in the seminiferous tubules adjacent to invasive germ cell tumors. In the absence of an invasive tumor, CIS is asymptomatic and is usually detected in testicular biopsies performed for other purposes. About the 50% of the patients with CIS lesions develop an invasive TGCT within 5 years from diagnosis and the 70% within 7 years (Giwercman A et al., 1993).

A morphological analysis of parenchyma adjacent to invasive TGCTs suggests that the intermediate lesions between CIS and invasive TGCTs are either the intratubular seminoma or the intratubular non-seminoma (Oosterhuis JW et al., 2003). During development of seminomas, CIS cells become independent from the micro-environment (i.e. the Sertoli cells produced factors) and fill up the lumen of the seminiferous tubules. Cell spreading out of the seminiferous tubule constitutes the last step for the formation of the overt tumor. Conversely, even though non-seminomas are thought to develop from CIS, intratubular non-seminoma is invariably composed of pure embryonal carcinoma (EC) and when invasion starts the differentiation into its derivatives occurs (teratoma, yolk-sac tumour and chorioncarcinoma) (Oosterhuis JW et al., 2005). In this case EC has the ability to differentiate into a wide range of cell types and represents the presumed stem cell compartment. EC may undergo down-regulation of the pluripotency genes and activation of the somatic lineage (Kristensen DM et al., 2008). Hence, while seminoma is a uniform tumor resembling early fetal germ cells and CIS cells, the more malignant non-seminomas appear heterogeneous and can also contain elements of seminoma (Ulbright TM et al., 1999).

1.2 Epidemiology and risk factors

TGCTs account for up to 60% of all malignancies affecting men between 20-40 years of age. The rates of TGCTs display geographic and ethnic differences. The incidence of TGCTs in Caucasian populations is 6–11/100,000 males but in the last decades an annual increase of about 3-6% was registered (Oosterhuis JW and Looijenga LH, 2005). Black populations show a lower risk for TGCTs than Caucasians (Moul JW et al., 1994). Moreover high oestrogen levels relate with an increased risk of developing a TGCT while high testosterone levels reduce the risk. These observations could possibly explain the different incidence rates among Caucasians and Blacks (Henderson BE et al.,1988). However, even within the white populations, marked differences in the rates of TGCTs development were observed, with Denmark detaining the higher incidence. The relatively rapid rise in the incidence of TGCTs and the differences among countries and ethinc groups suggest a possible involvement of environmental or lifestyle factors in the etiology of these tumors (Rajpert-De ME and Hoei-Hansen CE, 2007). The importance of this factors is supported by epidemiological studies on populations migrating from countries with high incidence (Denmark) and with significantly lower incidence (Finland) to Sweden. The first generation immigrants showed the same risk of their country of origin, while the second generation, that was born in Sweden, had the same incidence of native Swedes (Hemminki K and Li X, 2002).

In addition, one of the best known risk factor for TGCTs is cryptorchidism, suggesting that also the microenvironment plays a crucial role in the regulation of the development of the precursor lesion.

Other risk factors for TGCTs include hypotrophic (<12 ml) or atrophic testicle, Klinefelter's syndrome, familial history of testicular tumours among first-grade relatives, the presence of contralateral tumour and infertility (Albers P et al., 2005) but also unspecific factors, such as birth order, birth weight and factors related to maternal lifestyle (Rajpert-De ME and Hoei-Hansen CE, 2007).

TGCTs can occur in a sporadic or familial manner. Twin studies showed a greater concordance for disease in monozygotic than in dizygotic twins. The segregation analysis suggests a recessive mode of inheritance. (Krausz C and Looijenga LH, 2008).

1.3 TGCT gene expression

In the last decades a significant number of markers has been identified to discriminate CIS, seminoma and EC. As previously described, CIS cells share the expression of immunohistochemical markers with primordial gem cells and early gonocytes.

Among the first proteins reported to be expressed both in gonocytes and their malignant counterpart there is the stem cell factor receptor KIT (Rajpert-De ME and Skakkebaek NE, 1994). Together with PLAP is now commonly used as a clinical marker to detect CIS in surgical biopsies.

More recently OCT3/4, a well-characterized marker for primordial germ cells, was discovered to be positive in all cases of CIS, seminoma and derived invasive cancers (Looijenga LH et al., 2003; Rajpert Rajpert-De ME et al., 2004). OCT3/4 is a transcription factor of the family of the octamer-binding proteins and is a well known key regulator of pluripotency, (de Jong J and Looijenga LH, 2006). Another important gene known for its association with pluripotency in embryonal stem (ES) cells is the homeobox proteing NANOG. Similarly to OCT3/4, NANOG is expressed not only in CIS cells but also in the overt germ cell tumor (Hoei-Hansen CE et al., 2005).

Among the novel markers for TGCT there is AP-2γ, another transcription factor associated with the undifferentiated state and suggested as a possible marker for CIS in semen specimen of patient with early stages of TGCT (Hoei-Hansen CE et al., 2007).

All these stem cell-related proteins are physiologically abundantly expressed in fetal pre-meiotic germ cells of both sexes. At the onset of meiotic prophase their expression is normally rapidly down-regulated (Rajpert-De ME and Hoei-Hansen CE, 2007).

However, among the histological types of CIS-derived invasive tumors, marked differences in the expression of embryonic genes were observed (Kristensen DM et al., 2008). While seminoma phenotype is closely similar to CIS, the EC (the undifferentiated stem cell compartment of non-seminomas) is strikingly related to that of ES cells, suggesting a re-programming or a differentiation event. For example, the SRY (sex determining region Y)-box 2 (SOX-2) is highly expressed in non-seminomas, whereas seminomas are negative for this marker (Sperger JM et al., 2003; Santagata S et al., 2007). Accordingly SOX2 belong to the SOX protein family of transcription factors that regulate the development from the early embryonal stage to specific lineages (Avilion AA et al., 2003, Tay Y et al., 2008).

1.4 Genomic aberrations

CIS, seminomas, and all variants of nonseminomas are aneuploid, as demonstrated by flow cytometry as well as cytogenetic approaches. (Oosterhuis JW et al., 1989; Murty VV et al., 1990; De Graaff WE et al., 1992; Van Echten-Arends J et al., 1995). Even if altered copy number of regions on chromosome 7, 8, 14, 17 and X have also been consistently reported (Looijenga LHJ et al., 2000; Summersgill B et al.,2001; Ottesen AM et al., 2003; Ottesen AM et al., 2004), the main recurrent structural imbalance in these tumors is the gain of the short arm of chromosome 12, mostly in the form of an isochromosome (Looijenga LHJ et al., 2003). At which stage during tumour development genomic instability is introduced is uncertain (Skotheim RI and Lothe RA, 2003). However the observation that gain of 12p material is detected in CIS cells near the overt tumours (Ottesen AM et al., 2003) suggests that this event is related to the invasive growth of the type II TGCTs (Rosenberg C et al., 2000; Summersgill B et al., 2001). Interestingly, gain of 12p region is also found in *in vitro* cultured human ES cells, suggesting a relation with an improved capability to survive in a non-physiological environment (Draper JS et al., 2003; Li SS et al., 2006).

One of the genes located on 12p region that could play a relevant role in favoring TGCTs progressions is the cyclin D2 (CNND2). This gene is indeed overexpressed in type II TGCTs and CIS (Houldsworth J et al., 1997; Kukoski R et al., 2003). However, this region contains also other genes associated with pluripotency and proliferation, including the small GTPase K-RAS and NANOG, that has been suggested to be relevant in invasive TGCTs progression, although the actual proof is lacking so far (Krausz C and Looijenga LH, 2008).

1.5 TCam-2 cell line

Currently, various non-seminomatous cell lines have been established, but only one (i.e. TCam-2) (de Jong J et al., 2008; Eckert D et al., 2008; Goddard NC et al., 2007; Mizuno Y et al., 1993) has been established from seminoma, although they constitute more than 50% of all TGCTs. This is probably due to the capability of seminoma cells to undergo to rapid apoptosis after disruption of their microenvironment (Olie RA et al., 1996). TCam-2 cell line originated from a primary testicular seminoma of a 35-year-old patient (Mizuno Y et al., 1993). The initial *in vitro* culture was subcloned after 15 passages, and after 18 passages the cells were also successfully transplanted into the back of CB-17 SCID mice. Electron microscopy demonstrated the presence of significant amounts of glycogen and absence of intercellular connection structures. Karyotyping after 10 passages *in vitro* showed 90–96 chromosomes, including the lack of the Y chromosome (Mizuno Y et al., 1993).

TCam-2 cells display the characteristic gain of chromosome 12p, a chromosomal aberration common to all the invasive germ cell tumors but not spermatocytic seminoma (De Jong J et al., 2008). Original cytogenetic analysis using Giemsa banding identified five copies of chromosome 12 (Mizuno Y et al., 1993). Successively Goddard and coworkers identified by metaphase and interphase fluorescence in situ hybridization analysis further four copies of the 12 centromere and material from the 12p11.2-12.1 region but no isochromosome of 12p (Goddard NC et al., 2007). No expression of alpha fetal protein (αFP) or beta-human

choriogonadotrophin (βhCG), respectively markers for yolk-sac tumors and chorioncarcinoma, was described, while strong staining for the seminoma marker PLAP has been reported (Mizuno Y et al., 1993). As in the case of seminoma, the pluripotency genes Oct3/4 and Nanog are highly expressed in TCam-2 cells as detected by immunohistochemistry and western blot analysis (Goddard NC et al., 2007; De Jong J et al., 2008). Instead the embryonic stem cell marker SOX2, typically highly expressed in EC but absent in seminomas, is not expressed in TCam-2 cells both on protein and mRNA level (De Jong J et al., 2008). The transcription factor AP-2γ, a known specific marker used for diagnosis of seminomas, was expressed as revealed by RT-PCR, western blot analysis and immuhistochemistry (Eckert D et al., 2008). In addition, the tumor necrosis factor receptor superfamily member 8 (CD30) usually expressed in EC and EC-derived cell lines and related to inhibition of apoptosis, is not expressed in TCam-2 cells on protein level nor on mRNA level (de Jong J et al., 2008).

Moreover, the stem cell factor (SCF) receptor, KIT, and its ligand SCF were both expressed in the TCam-2 cells as determined by immunoprecipitation for KIT (de Jong J et al., 2008, Goddard NC et al., 2007), western blot analysis and immuhistochemistry (Eckert D et al., 2008). KIT mutations have been described in bilateral TGCTs (Looijenga LH et al., 2003) but sequencing analysis of exons 9, 11, 13 and 17 did not detect any activating mutations of KIT in TCam-2 cells (Goddard NC et al., 2007). Finally, the expression of the typical germ cell markers VASA (ATP-dependent RNA helicase), DAZL (DAZ (deleted in azoospermia) ligand) and BOULE (another member of the human DAZ gene family) were observed (Eckert D et al., 2008).

Unlike other solid tumors, type II TGCTs have a low mutation rate in protooncogenes, including BRAF and KRAS2 (Sommerer F et al., 2005), as well as the tumor suppressor gene TP53 (Kersemaekers AM et al., 2002). Mutation analysis of these genes in TCam-2 cells showed a mutation in the protooncogene BRAF, due a thymine to adenine transversion at

nucleotide 1796 (T1796A), leading to a substitution of glutamic acid for valine at amino acid 600 (V600E) (de Jong J et al., 2008). It has been suggested that this mutation could be the explanation for the successful propagation of these cells *in vitro*. Because of the lack of the primary tumor, it is not possible to determine whether this mutation was present in the original tumor, or was generated due to *in vitro* propagation (de Jong J et al., 2008). On the other hand, recently Goddard and coworkers, described that the TCam-2 cell line does not show evidence for a V600E BRAF mutation (Goddard NC et al., 2010). These authors performed direct sequencing of both the forward and reverse strands of the BRAF gene in TCam-2 cells and normal female DNA revealing that the sequence is wildtype at nucleotide 1796. They hypothesized that the mutation detected by de Jong J and coworkers (de Jong J et al., 2008) could be present only in a small subpopulation of TCam-2 or alternatively, a BRAF mutation may have arisen independently or the mutant allele may have been lost during prolonged culture in different laboratories. In conclusion they affirmed that the BRAF mutation can not account for the establishing of TCam-2 cell line from a seminoma sample. In their opinion rather other factors, including mutations in other genes or the overexpression of receptors or growth factors, may account for their *in vitro* growth (de Jong J et al., 2008).

2. Tumor tissue invasion and metastasis

2.1 Mesenchymal and amoeboid cell invasion

Cell migration plays a key role in metastatic dissemination of tumour cells from the primary tumour to local and distant sites (McSherry EA et al., 2007; Farrow B et al., 2008; Condeelis J and Segall JE, 2003).

Even if tumour cells *in vivo* can move both randomly and directionally, invasion is more efficient when the cell is involved in directed migration toward a chemokine gradient (chemotaxis) (Roussos ET et al., 2011). Thus, chemotaxis is on the base of several major events during tumour progression: metastatic invasion and dissemination, angiogenesis and immune cell extravasation (Roussos ET et al., 2011).

Different modes of invasive migration have been described for cancer cells: amoeboid migration or mesenchymal migration for single cells and collective migration of groups of cells (Roussos ET et al., 2011). Amoeboid cell motility of single tumour cells is independent from the activity of matrix metalloproteinases (MMPs) because cells can infiltrate through gaps in the extracellular matrix (ECM) because their cytoskeleton contractile capability (Wolf K. et al., 2003; Wyckoff JB et al., 2006). Intravital multiphoton imaging had permitted to observe migrating cells *in vivo* revealing that some carcinoma cells with an amoeboid morphology can move at high speeds inside the tumours (~4 μm min–1) (Condeelis J and Segall JE, 2003). On the other end, mesenchymal migration that typically involve single cells but sometimes also collective migration, is characterized by polarized cells with an elongated cell morphology and relatively low speeds of cell migration (0.1–1 μm min–1) (Sahai E, 2005). However, even if amoeboid and mesenchymal modes of migration can be observed as separated mechanisms by *in vitro* studies, evidence suggests that *in vivo* they can convert into each other in response to changes in the microenvironment (Sahai E and Marshall C, 2003; Pankova K et al., 2009). For example, a transition from mesenchymal to amoeboid tumor cell migration can be induced by inhibition of proteolysis (Wolf K et al., 2003).

Collective migration has been defined as the coordinate movement through the ECM of clusters or sheets of tumour cells with functionally intact cell–cell adhesions (Friedl P and Gilmour D, 2009). The leader cells positioned at the front of the migrating group actively migrate toward a chemotactic gradient creating a track by matrix degradation (Rorth P, 2007; Ilina O and Friedl P, 2009; Valentin G et al., 2007; Schmidt M et al., 2007; Aman A and Piotrowski T, 2008). The other cells follow the leader cells along the remodeled matrix tracks due to the physical coupling that generate drag forces (Rorth P, 2007). The leader cell can be either a tumour cell with proteolytic activity or a stromal cell from the tumour microenvironment (Gaggioli C et al., 2007; Rorth P, 2007; Wolf K, 2007).

The occurrence and frequency of these modes of migration in cancer is dependent on tumor cell types and tumour microenvironment.

Despite the various patterns of directed migration during tumour cell dissemination, the intracellular processes involved in cell motility are probably similar and are comprised of three main steps: chemo-sensing, polarization and locomotion (Iglesias PA, Devreotes PN, 2008). First, polarized intracellular signals lead to asymmetric actin polymerization resulting in extension of the cell membrane in the direction of movement, thus creating the leading-edge protrusion. This is followed by integrin-mediated adhesion to the substrate in the direction of movement, and by subsequent detachment from the substrate on the opposite side after contraction of the trailing edge of the cell (Figure 3). Nodal point for regulation of these migratory processes are the small GTPases such as Rho, cdc42, and Rac (Gupta GP and Massague J, 2006)

2.2 Contributions of stromal cells to invasion and metastasis

Cancer cells typically produce mediators that induce chemotactic recruitment and activation of a variety of cell types into the surrounding stroma (such as fibroblasts, myofibroblasts, granulocytes, macrophages, mesenchymal stem cells, and lymphocytes) which contribute to

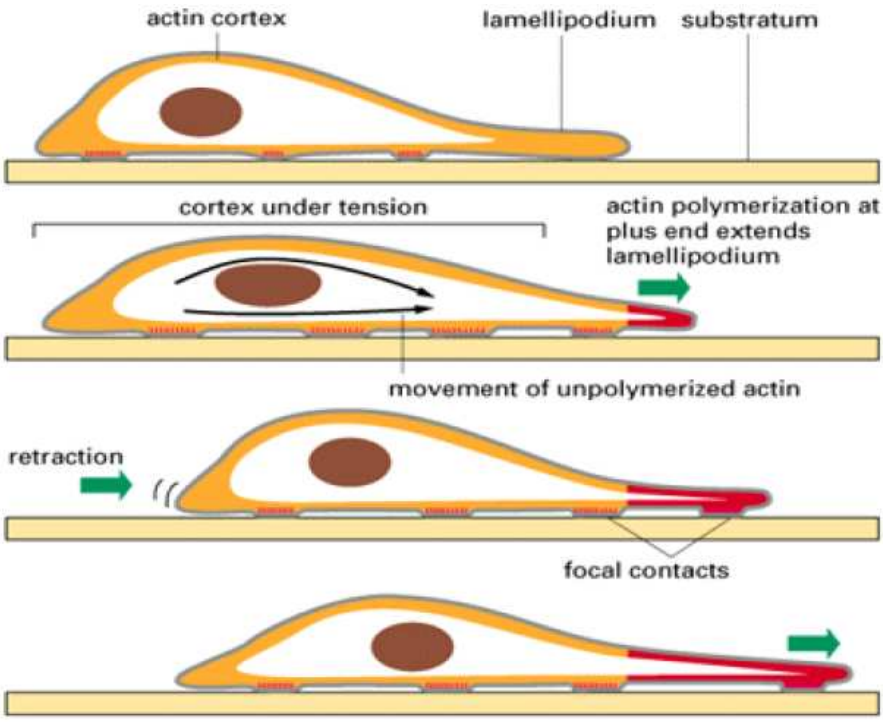

Fig. 3 - Schematic representation illustrating the different phases of cell migration.
Polarized intracellular signals lead to asymmetric actin polymerization resulting in extension of the cell membrane in the direction of movement, creating the leading-edge protrusion. This event is followed by integrin-mediated cell-substrate adhesion (focal contacts) in the direction of movement, and by subsequent detachment on the opposite side after contraction of the trailing edge of the cell. (From Molecular Biology of the Cell; Alberts).

the formation of an inflammatory microenvironment (Gupta GP and Massagué J 2006). Therefore, invasion occurs in a restricted zone of cross-talk and cooperation between this "reactive" stroma and the premalignant epithelium (Figure 4).

The proteolytic machinery of the activated stromal cells, combined with that of tumor cells, degrades extracellular matrix (ECM) proteins, uncovering cryptic sites that may display pro-migratory properties and releasing sequestered growth and survival factors (Liotta LA and Kohn EC, 2001).

In this scenario, invasive carcinoma may be viewed as "a pathology of multiple cell societies inhabiting the epithelial/mesenchymal stromal unit" (Liotta LA and Kohn EC, 2001). Thus, tumor cells could convert reactive stromal infiltrates from preservers of tissue homeostasis into accomplices in malignancy. Accordingly, leukocytic infiltration, angiogenesis, and lymphangiogenesis, all markers for stromal cells and tumor coexistence, are frequently correlated with an increased likelihood of metastatic relapse (Gupta GP and Massagué J, 2006). However the stromal reaction to invading tumor cells is variable, depending in part upon tumor cell properties and in part upon the local stromal composition (Bacac M and Stamenkovic I, 2008).

During the biological cascade of metastasis, a cancer cell from a primary tumor executes several discrete steps: i) emigration from the primary tumor and invasion of the surrounding tissue and degradation of ECM; ii) intravasation into the microvasculature of blood or lymphatic system iii) survival and translocation largely through the bloodstream and lymph to microvessels of distant tissues and finally iv) extravasation and v) metastasis formation at target tissues (Figure 5). Since cancer cells exit from the bloodstream, they have to adapt to the foreign microenvironment to survive and give rise to a macroscopic secondary tumor (colonization) (Fidler IJ et al., 2003).

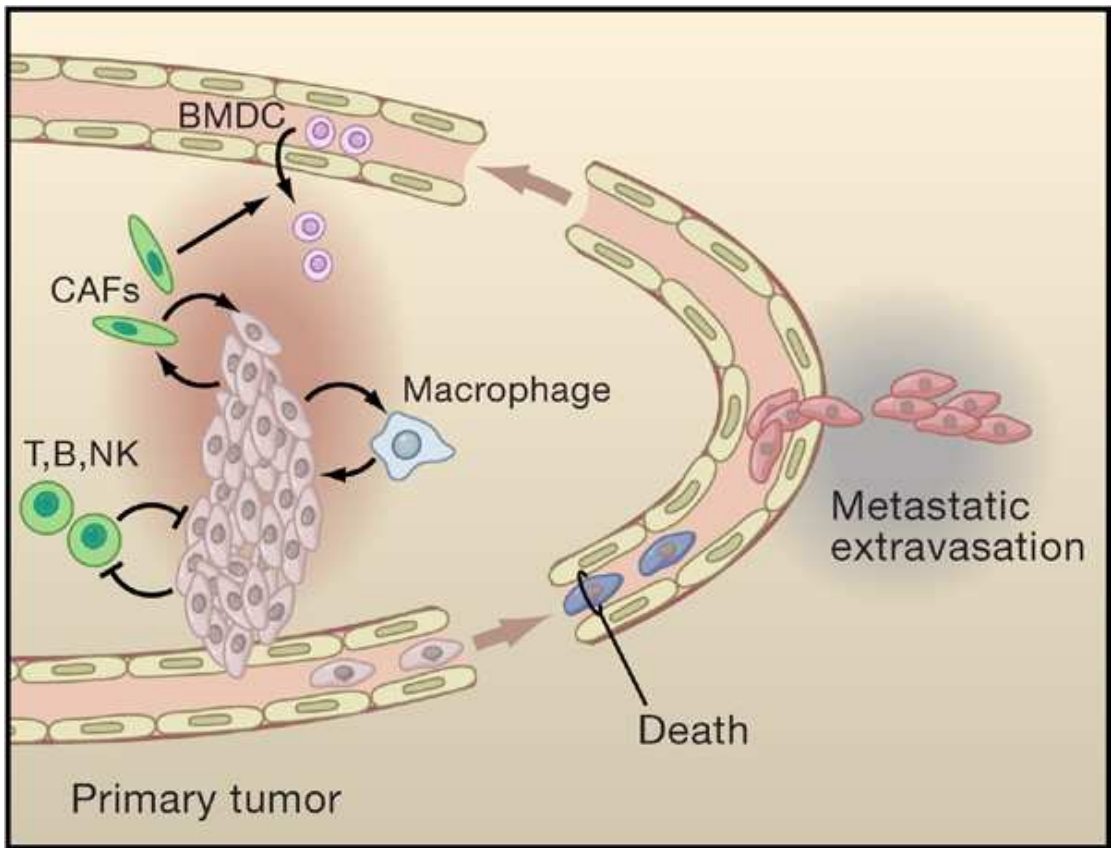

Fig. 4 – Stromal reaction.
Advanced malignancies are frequently coinhabited by different stromal cell types. The tumor-suppressive activities of lymphocytes (T, B, and NK cells) is in part antagonized through the release of immunosuppressive cytokines. Carcinoma-associated fibroblasts (CAFs) can secrete factors that promote both tumor cell growth and invasion and neoangiogenesis (through recruitment of bone marrow dendritic cells (BMDC) from circulation). Activated macrophages were recruited to tumors and can release many pro-tumorigenic growth factors. Additionally, macrophages may co-migrate with cancer cells within tumors through a paracrine growth-factor loop. Once cancer cells have invaded the blood stream, many of them will die from stresses associated with circulatory passage. The remaining survivors may attach to capillaries within a secondary organ through adhesion receptors. Subsequently, cancer cells can extravasate from capillaries and may or may not generate a viable niche at the secondary site.(From Gupta GP and Massagué J, 2006 Cell 127: 679-695).

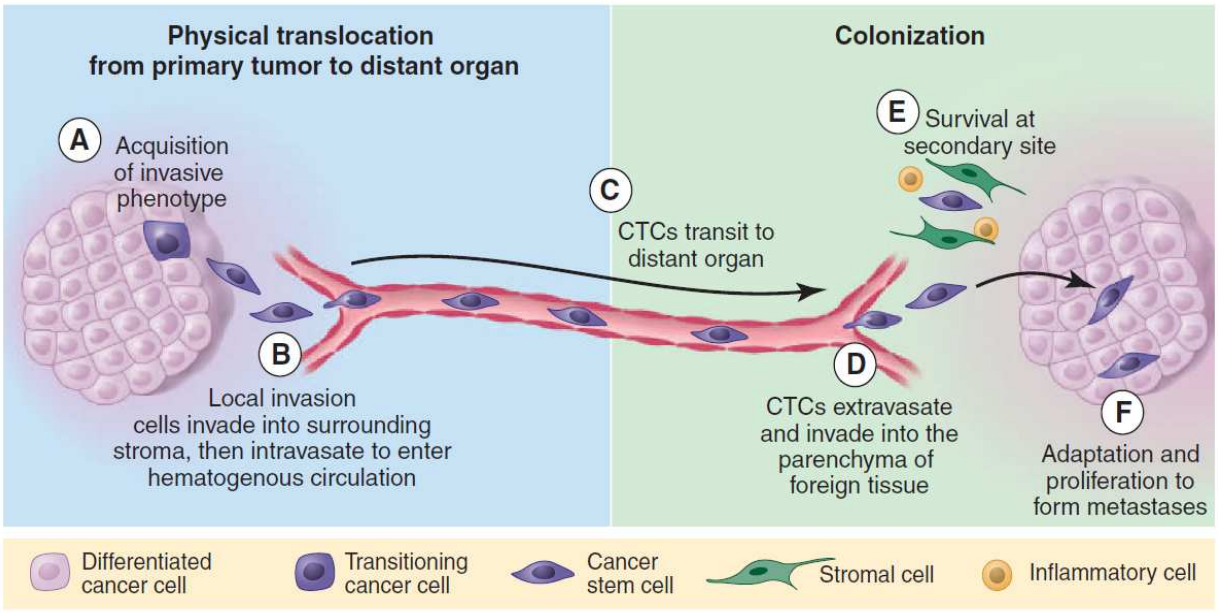

Fig. 5 - The metastatic cascade.
Metastasic process can be subdivided in two major phases: (i) physical translocation of cancer cells from the primary tumor to a distant organ and (ii) colonization of the translocated cells within that organ. (A) To begin the metastatic cascade, cancer cells within the primary tumor acquire an invasive phenotype. (B) Cancer cells can then invade into the surrounding matrix and toward blood vessels, where they intravasate to enter the circulation, which serves as their primary means of passage to distant organs. (C) Cancer cells traveling through the circulation are referred as circulating tumor cells (CTCs). (D) At the distant organ, CTCs exit the circulation and invade into the microenvironment of the foreign tissue. (E) At that foreign site, cancer cells must be able to evade the innate immune response and also survive as a single cell (or as a small cluster of cells). (F) To develop into an active macrometastatic deposit, the cancer cell must be able to adapt to the microenvironment and initiate proliferation. (From Chaffer CL and Weinberg RA, 2011 Science 331, 1559).

3. TGCTs and seminoma invasion

Although the cure rate for TGCT is high, presence of metastases reduces survival rates. Interestingly, metastasis is a prevalent feature of human TGCTs showing a very high number of metastases compared with other tumours, averaging 5.8 metastases per primary tumour (De Giorgi U et al., 2008). Approximately 30% of patients affected by TGCTs presents metastases at the time of diagnosis (Powles TB et al., 2005), and 15–20% of patients have subclinical metastases in stage I seminoma (Benne R et al., 1986), the most common presentation among TGCTs. While tumors remain confined to the testis in about 75% of patients, a retroperitoneal involvement is observed in the 20%, and the remaining 5% shows supradiaphragmatic or organ metastases (Ulbright TM et al., 2009).

Therefore metastases represent important factors directly affecting treatment modality, tumour surveillance, and survival (Zechel JL et al., 2011). Treatment for TGCTs involves surgery and chemotherapy with bleomycin, etoposide and cisplatin (BEP), obtaining a relative low mortality risk of 2.3–4.5% (Fossa SD et al., 1998; Williams SD et al., 1987). Although many TGCTs and their metastases respond readily, 10–25% of patients with metastases are resistant to chemotherapy (Piulats JM et al., 2009), further complicating treatment and worsening prognosis. Moreover, patients with a metastatic disease suffer frequently of a higher incidence of relapse (5–10%) after treatment (Lutke Holzik MF et al., 2008) and a lower survival rate (Loehrer PJ et al., 1998).

Even though the clinical and epidemiological aspects of TGCTs are well described, very scant informations are available about the molecular mechanisms for tumor spreading and metastasis. Testicular cancer metastases exhibit a conserved pattern targeting various tissues, such as lymph nodes, lung, liver, bone and spleen (De Giorgi U et al., 2008).

It has been observed that this TGCT pattern of metastases abundantly overlap with that of CXCL12 expression. In fact, metastases target organs that express high levels of the chemokine CXCL12 (SDF-1), resembling patterns observed in other CXCR4 over-expressing

cancers (breast, colorectal and prostate cancers) (Gilbert DC et al., 2009). In normal human testes, CXCL12 is expressed by Sertoli cells and its receptor CXCR4 by the germ cell population. CXCL12 is able to stimulate invasive cell migration of TCam-2 seminoma cell line in a CXCR4-dependent fashion via activation of ERK. More interestingly high-level CXCL12 expression in tumors correlates with reduced risk of TGCT occult metastases and relapse after surgical treatment (Gilbert DC et al., 2009).

Even if seminoma is generally pain-less, about 2,5% of patients presenting a metastatic involvement suffers most commonly of lumbar pain due to retroperitoneal metastasis, even if gastroinstestinal bleeding, bone pain, dyspnea and cough, a supraclavicular mass, neurological symptoms, and lower extremity edema may also be presenting symptoms due to spread to other sites. Such metastases, however, can be asymptomatic (Ulbright TM et al., 1999).

4. Glial derived neurotrophic factor (GDNF)

4.1 GDNF and co-receptors

GDNF is a distant member of the transforming growth factor beta (TGF-β) superfamily (Lin LF et al., 1993). GDNF was first identified through its activity on midbrain dopaminergic neurons but subsequently, it has been shown to have pleiotropic functions, promoting neuroprotection, cell proliferation, and migration (Sariola H and Saarma M, 2003).

Mice lacking GDNF die within the first day of birth due to severe defects in renal differentiation and the absence of an enteric nervous system (Moore MW et al., 1996; Pichel JG et al., 1996; Sanchez MP et al., 1996). The animals lacking GDNF receptors show the same phenotypes, indicating that GDNF pathway is essential for postnatal survival in the mouse (Schuchardt A et al., 1994; Enomoto H et al., 1998; Cacalano G et al., 1998).

GNDF, along with the other members of the GDNF family of neurotrophic factors (neurturin, artemin and persephin), is a basic, dimeric, secretory protein. GDNF is first synthesized as an inactive 211 amino acids long preproGDNF. Subsequently the secreted proGDNF is proteolytically cleaved to form the mature GDNF protein of 134 amino acids (Saarma M and Sariola H, 1999). The specific proteases that cleave and activate GDNF precursors have not yet been identified. The GDNF mature protein is N-glycosylated at two amino acid residues, even if the role of these carbohydrates in the biological activity of GDNF has not yet been established (Lin LF et al., 1993). Moreover GDNF contains seven cysteine residues in the same relative spacing as in the members of the TGF-b superfamily. Although the amino acid sequence homology between the GDNF and the TGF-b superfamily is less than 20%, the pattern of cysteine residues makes GDNF a distant member of this family (Lin LF et al., 1993). According to the crystal structure, the GDNF monomer forms two finger-like (Finger 1 and Finger 2) structures by pairs of antiparallel β-strands with a α-helix at the opposite end (Eigenbrot C and Geber N, 1997). The GDNF homodimer is formed by head-to-tail dimerization, which is supported by one interchain disulphide bond among the unpaired

cysteines in monomers that are not part of the cysteine knot. Only the homodimer of GDNF is biologically active (Saarma M and Sariola H, 1999).

GDNF exerts its effect on target cells by binding to a glycosyl phosphatidylinositol–linked GDNF family receptor-a 1 (GFRA1); this binding recruits the tyrosine kinase receptor Ret to form a multi-subunit signaling complex of GDNF/GFRA1/Ret.

While the other members of TGF-b superfamily signal through transmembrane serine-threonine kinase receptors, the GDNF receptor Ret is a typical tyrosine kinase receptor. Ret is a single-pass transmembrane protein that contains four cadherin-like repeats in the extracellular domain (607 amino acids), a cysteine rich domain, a hydrophobic transmembrane region and a cytoplasmic intracellular part with a kinase domain (Anders J et al., 2001; Iwamoto T et al., 1993; Kuma K et al., 1993). Moreover a Ca^{2+}-binding site is localized between cadherin-like domain 2 and 3 (Anders 2001).

The GPI-anchored receptor GFRA1 contains a N-terminal hydrophobic domain codifying for the secretory signal sequence and a C-terminal sequence with 23 hydrophobic amino acids. A group of three small amino acids proceeding the stretch of hydrophobic amino acids indicates a possible GPI-binding/cleavage site (Undenfriend S and Kodukula K, 1995).

GFRA1, as most GPI-linked proteins, can be localized to specialized detergent-resistant membrane fractions, named lipid rafts. These structures are described as floating membrane platforms in the exoplasmic leaflet of the membrane bilayer enriched in cholesterol and sphingolipids (Simons K and Ikonen E, 1997). Several protein families have high affinity for lipid rafts, such as Src-family kinases and small GTP-ases, making them signaling platforms for various transmembrane and GPI-linked proteins (Saarma M, 2001; Simons K and Toomre D, 2000).

The original model described the GDNF/GFRA1 binding and activation of Ret receptor as an *in cis* interaction between two receptors expressed on the same cell (Jing S et al., 1996). This model is supported by the overlapping expression of Ret receptor and GFRA1 in the same cell

population (Golden JP et al., 1998; Tansey MG et al., 2000). However also another mechanism has been reported where the GFRA1 receptor can be released from the cell surface by cleavage of the GPI-anchor and acts as a soluble receptor. According to this *in trans* model, the soluble GFRA1 molecule presents the ligand to Ret located in the membrane of another cell (Figure 6). It has been suggested that the *in trans* signaling can sustain and increase the response achieved through the canonical *in cis* pathway (Paratcha G et al., 2001). In this last case, GDNF binds to the GPI-linked GFRA1 receptor into lipids rafts, recruiting Ret in this compartment which binds and activates the lipid-anchored adaptor protein FRS2 (fibroblast growth factor receptor substrate 2). During the *in trans* activation, instead, GDNF binds to soluble GFRA1 and this complex binds and activates Ret on neighboring cells outside the lipid rafts. The activated receptor associates with and phosporylates SHC (Src-homologous and collagen-like protein) (Paratcha G et al., 2001; Airaksinen MS and Saarma M, 2002).

Once GDNF/GFRA1 complex binds to Ret, two transmembrane Ret molecules dimerize and transphosporylate cytoplasmic tyrosines, triggering several cell signaling cascades (Airaksinen MS and Saarma M, 2002).

4.2 GDNF transduction pathways

The GDNF-GFRA1 binding to Ret extracellular domain leads to Ret autophosphorylation at many tyrosine residues on its intracellular domain (Airaksinen MS and Saarma M, 2002). The phosphorylated tyrosine residues of activated Ret act as docking sites for various adaptor proteins that in turn may activate different pathways in target cells (Airaksinen MS and Saarma M, 2002).

For instance, the Src signaling pathway, activated by Ret Tyrosine 981 phosphorilation, elicits neurite outgrowth, neuronal survival and ureteric branching (Airaksinen MS and Saarma M, 2002) as well as spermatogonial stem cells (SSCs) self-renewal and proliferation in mouse

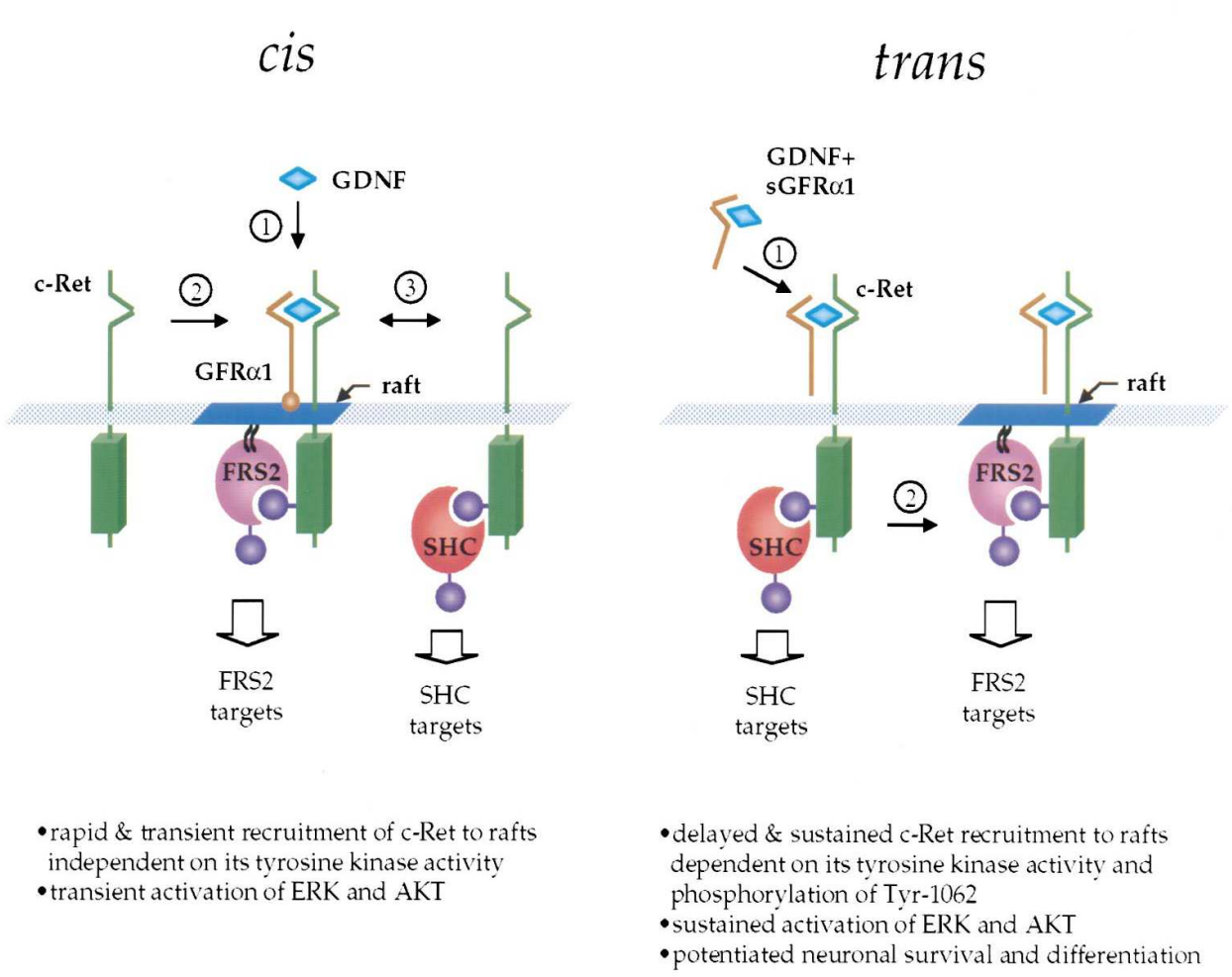

Fig. 6 - GDNF receptor complex signaling.
In *cis* pathway (left): GDNF binds to GPI-anchored GFRa1 receptors in lipid rafts (1), resulting in the recruitment and activation of c-Ret in this compartment (2). c-Ret associates with and activates FRS2 inside and SHC outside lipid rafts. Activated c-Ret is in equilibrium between raft and non-raft compartments (3). During activation in *trans* (right), a complex of GDNF and soluble GFRa1 (sGFRa1) released from neighbouring cells binds to and activates c-Ret outside rafts (1), where the activated receptor associates with and phosphorylates SHC. c-Ret is then recruited to rafts by a mechanism dependent on its tyrosine kinase activity and phosphorylation of Tyr-1062 (2). Inside lipid rafts, c-Ret associates with and activates FRS2. Both c-Ret and GFRa1 are believed to function as homodimers; however, for simplicity, only monomers are represented in this cartoon. (From Paratcha G et al., 2001 Neuron 29: 171–184).

testes (Hoffman MC, 2008). Four Src family kinases have been so far implicated in the Ret mediated SSCs proliferation: Src, Yes, Lyn and Fyn. Further Src activates the PI3K/Akt signaling pathway leading to N-myc expression and promoting SSCs self-renewal (Hoffman MC, 2008).

Another important transduction pathway activated by GDNF/GFRA1/Ret complex is the Ras signaling pathway. In the developing enteric nervous system, the developing kidney, and in neuroblastoma it is triggered by Ret Tyr 1062 phosphorylation (Worby CA et al., 1996; Jijiwa M et al., 2004; Hayashi H et al., 2000).

Concerning SSCs, instead, the rapid and transient activation of the Ras/ERK1/2 pathway was induced by GDNF binding and activation of the protein adaptors Shc and Grb2. At the end of this signaling cascade, some transcription factors such as Creb-1, Atf-1, and Crem-1 were phosphorilated and therefore activated. Finally, the Gdnf/Ret/Ras axis up-regulates the transcription levels of the immediate-early gene c-fos, the cell cycle activator cyclin A, as well as Cdk2 (Hoffman MC, 2008). Cyclin A is a key regulatory protein of the cell cycle S-phase entry and associates with Cdk2 in mammalian cells (Cardoso MC et al., 1993). Therefore, like in other cell types, Creb and c-Fos enhance the expression of cyclin A and favor the G1/S cell cycle transition in GFRA-positive spermatogonia (Desdouets C et al., 1995; Sunters A et al., 2004).

4.2.1 Ret-indipendent transduction pathways

Recently, some observations suggest that the GDNF/GFRA1 complex can transduce also in a Ret-independent fashion (Sariola H and Saarma M, 2003). In support of this hypothesis, the GFRA1 expression is widespread in many areas of the nervous system, and especially in the forebrain, cortex and inner ear where no expression of Ret is detected (Trupp M et al., 1997; Kokaia Z et al., 1999).

In RET deficient cell lines and primary neurons, GDNF is able to trigger Src family kinase activation and phosphorylation of the ERK/MAP kinase, the phospholipase C-gamma (PLC-γ) and the nuclear transcription factor cAMP Responsive Element Binding Protein (CREB), and induction of the protoncogene Fos (Poteryaev D et al., 1999; Trupp M et al., 1999). In RET-deficient mice exhibiting severe renal hypodysplasia, GDNF partially restores ureteric branching morphogenesis (Popsueva A et al., 2003). In MDCK cells expressing GFRA1 but not RET, GDNF stimulates branching but not chemotactic migration (Sariola H and Saarma M, 2003). In several RET-deficient but GFRA1-positive cells GDNF induces Met phosphorylation suggesting a contribute of Met to RET-independent GDNF signaling (Sariola H and Saarma M, 2003). However, since GDNF does not immunoprecipitate Met, a direct interaction between GDNF and Met seems improbable. Instead the GDNF-triggered RET-independent Src and Met activation might be mediated by neural cell adhesion molecule (NCAM). Ibañéz C. and co-workers investigated the Ret-indipendent GDNF signaling mechanisms in the immortalized neuronal precursors RN33B and in primary Schwann cells, which are reported to express relatively high levels of GFRA1, but no Ret receptor. They observed that, in the absence of GFRA1, GDNF interacts with NCAM with low affinity (Paratcha G et al., 2003). When GFRA1 is associated with NCAM, instead, GDNF binds with high affinity to p140NCAM and activates Fyn and focal adhesion kinase (FAK) in the cytoplasm (Paratcha G et al., 2003). According to these observations, GDNF effects on midbrain dopaminergic neurons *in vitro* and *in vivo* are inhibited by an NCAM blocking antibody, giving support to the physiological relevance of GDNF signaling through NCAM (Chao CC et al., 2003).

4.3 GDNF and spermatogenesis

In rodent testis, GDNF is produced by the Sertoli cells, the somatic cells supporting germ cell development (Tadokoro Y 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 K et al., 2010). GDNF is important for male fertility because it plays a central role in spermatogonial stem cell self-renewal and proliferation (Hofmann MC et al., 2008). Reduced GDNF dosage in heterozygous GDNF+/- animals leads to an excessive differentiation of spermatogonia and finally to an exclusive Sertoli-cell-only phenotype, whereas over-expression of GDNF leads to a block of stem cell differentiation (Meng X et al., 2000) (Figure 7).

Although mice lacking GDNF or its receptors GFRA1 and Ret die within the first day of birth, due to severe defects in renal differentiation and the absence of an enteric nervous system (Moore MW et al., 1996; Pichel JG et al., 1996; Sanchez MP et al., 1996), the testicular morphology of these mice is normal before birth.

Transplantation of GDNF, GFRA-1 and Ret deficient neonatal testes under the back skin of immunodeficient mice to monitor the development of the grafted testes revealed that any disruption of GDNF-mediated Ret signaling results in a lack of SSCs self-renewal and induces the progressive loss of spermatogenesis by germ cell depletion (Naughton CK et al., 2006). On the contrary, normal spermatogenesis was observed in the grafted wild-type testes.

In the last decade, GDNF has been described as an essential factor in the maintenance and expansion of *in vitro* SSCs in different mammals (Kanatsu-Shinohara M et al., 2003; Kanatsu-Shinohara M et al., 2008; Kubota H et al., 2004; Wu Z et al., 2009). Microarray analysis on *in vitro* cultured SSCs showed that GDNF administration induces the upregulation of some transcription factor-encoding genes, including *Bcl6b*, *Etv5* and *Lhx1* and interestingly of *Gfra1* (Oatley JM et al., 2006). In order to determine whether these GDNF-induced transcription factors are relevant to SSCs functions, their expression was transiently reduced by RNA interference in cultured mouse SSCs. After cell transplantation in recipient mouse testes, it has been observed that SSCs expansion *in vitro* was impaired, suggesting that BCL6B, ETV5 and LHX1 are important factors for SSCs self-renewal (Oatley et JM al., 2006; Oatley JM et al., 2007).

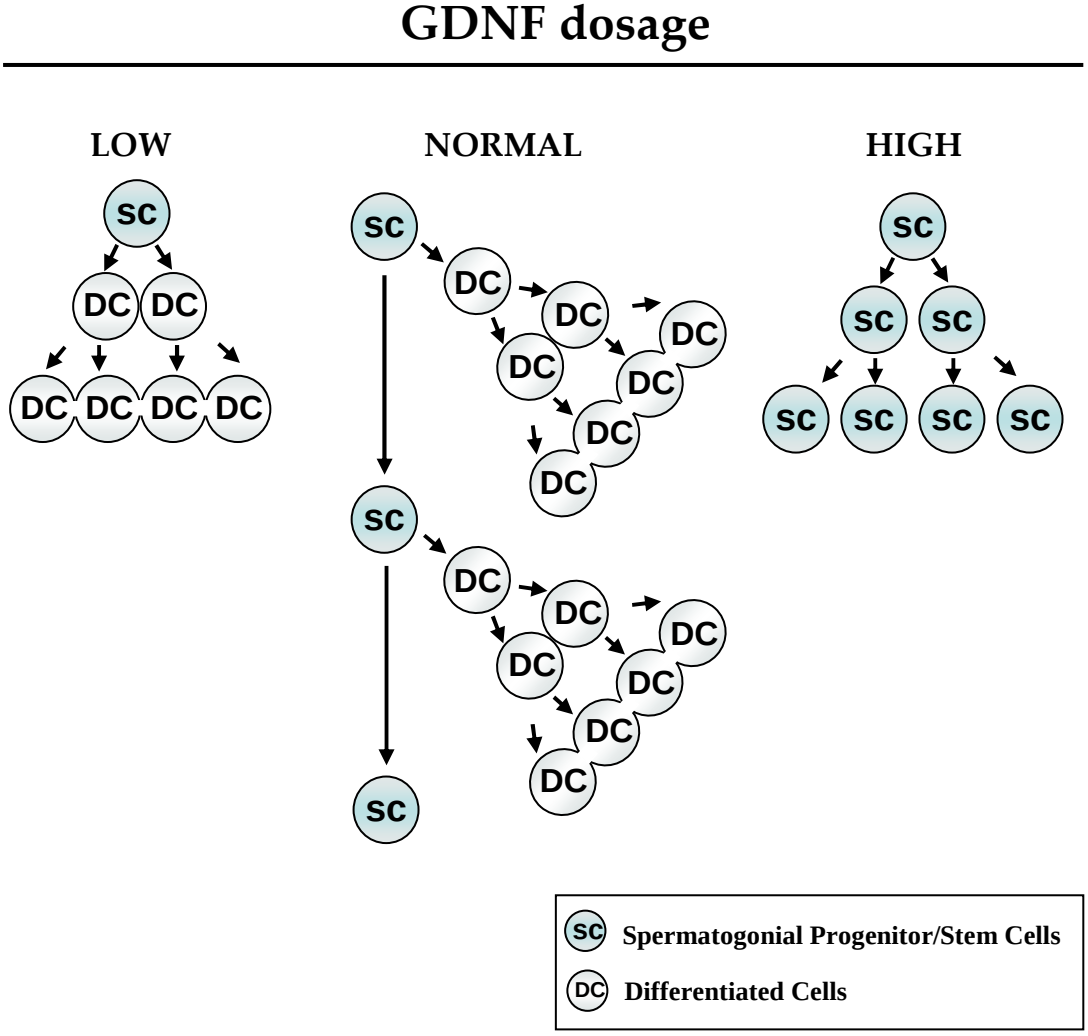

Fig. 7- GDNF dosage rule the correct balance between stem cell self-renewal and differentiation.
When GDNF level is low, the sprematogonial stem cells undergo differentiation while high GDNF dosage induces stem cell self-renewal. Transgenic mice with loss or gain of function of Gdnf show disturbed spermatogenesis. (Modified from Sariola and Saarma, 2003 J. Cell. Sci. 116: 38-59).

The GDNF receptor GFRA1 has been found to be expressed by SSCs isolated from immature mouse testes (Buageaw A et al., 2005) but not by SSCs from cryptorchid adult testes (Ebata KT et al., 2005; Orwig KE et al., 2008). According to dependence of SSCs self-renewal in *in vitro* culture upon GDNF administration, GFRA1 expression is maintained by cultured SSCs. However both rodent and human SSCs do not uniformly express GFRA1 (Grisanti L et al., 2009). In particular, in humans, where SSCs are among Adark (Ad) and Apale (Ap) spermatogonia (De Rooij DG and Russell LD, 2000), only a fraction of the Ad and Ap spermatogonia expresses GFRA1 (Grisanti L et al., 2009). In the same year, the Schlatt's group published a related paper about GFRA1 expression in human SSCs. Four distinct subpopulations of GFRa1-positive cells were observed which could be distinguished according to differences in the cell sizes and additional morphological criteria (Gassei K et al., 2010).

In the testis, the regulation of GDNF expression is still poorly understood. However the GDNF production by Sertoli cells is known to be dependent on the follicle-stimulating hormone (FSH) (Tadokoro Y et al., 2002). In order to study the GDNF/FSH pathway, male Sl/Sl^d mutant mice, whose testes produce only undifferentiated type A spermatogonia, were used. FSH stimulation mediated by Sertoli cells was abolished by injecting a gonadotropin releasing hormone antagonist (Nal-Glu) resulting in a decrease of GDNF production and undifferentiated spermatogonia proliferation. Moreover primary cultures of Sertoli cells respond to FSH increasing the GDNF production. This observation was also confirmed by Simon and colleagues, who demonstrated that FSH increases the GDNF level expression in the Sertoli cell line TM4 (Simon L et al., 2007). In addition, they observed that the GDNF production by primary Sertoli cells *in vitro* is dependent on fibroblast growth factor 2 (FGF2), tumor necrosis factor alpha (TNF-α) and interleukin-1beta (Il-1β) (Simon L et al., 2007).

4.4 GDNF and cancer

Even though physiological functions of GDNF in normal cells and tissues are well documented, less is known concerning the role of GDNF in cancer. GDNF promotes cell migration/chemotaxis and invasion of human pancreatic (Okada Y et al., 1999; Veit C et al., 2004), chondrosarcoma (Su CM et al., 2009) and glioma cell lines (Song H et al., 2006). Interestingly, older transgenic mice that over-express GDNF frequently develop testicular tumors with the morphology and immunohistochemical profile (placental-like alkaline phosphatase positive) of classical human seminomas (Meng X et al., 2001). The GDNF transgene has been expressed in testes under the translation elongation factor 1α promoter. All male GDNF overexpressing mice are infertile and after two weeks of age start to accumulate undifferentiated spermatogonia which form large cell clusters occluding seminiferous tubules. Around ten weeks of age, these clusters undergo apoptosis with subsequent testis atrophy. However a rim of spermatogonia still remains in the periphery of the tubules. Invasion of undifferentiating spermatogonia into the interstitium occurs after a year of age (Meng X et al., 2000) and 89 percent of mice develops testicular tumors. No distant metastases were found by autopsy or histological analysis (Meng X et al., 2001).

Even if the murine seminomatous tumors resemble human seminomas for many aspects, some differences are also been observed. Firstly, in most transgenic mice tumors developed bilaterally (56%), while in humans they were mainly unilateral. Secondly, whereas distorted spermatogenesis was detected in mouse, a normal spermatogenetic process was maintained in man. Moreover, tumors appeared at old age in mouse and at young age in man. Finally, the large lymphocyte infiltrate present in most of human seminomas is absent in mouse tumors. However, in spite of these differences, at the present time this is the only available animal model for seminoma (Meng X et al., 2001).

More recently, a link between the DMRT1 (*doublesex* and *mab-3* related transcription factor 1) tumor-suppressor protein and the deregulation of the GDNF signaling pathway has been

found in TGCTs. DMRT1 is a transcription factor implicated in testicular development. The loss of *Dmrt1* in fetal testis induces the reduction of Ret expression and increased teratoma formation. However, increased DMRT1 expression is associated with increased GDNF signaling and spermatocytic seminoma (Krentz AD et al., 2009). Thus, it is possible to speculate that a reduced GDNF signaling, together with high expression of pluripotency genes, leads to somatic differentiation programs and teratoma insurgence. On the contrary, the postnatal overactivation of GDNF pathway may block differentiation of germ cell and spermatocytic tumor formation (Krentz et al., 2009).

These observations suggest that a deregulation of the GDNF pathway might be implicated in germ cell carcinogenesis even if the molecular alterations involved are not known (Meng X et al., 2001).

Apart from a single study (Viglietto G et al., 2000), there are scant informations on the expression of GDNF and its co-receptors in human TGCTs and their possible role in the pathogenesis remains to be completely established.

AIM OF THE PRESENT STUDY

The neurotrophic factor GDNF has multiple functions involved in cell survival, proliferation and migration of different cell types. The experimental over-expression of GDNF in mouse testis leads to infertility and promotes seminomatous germ cell tumors in older animals. This observation suggests that although the underlying mechanisms are unknown, deregulation of the GDNF pathway may be implicated in germ cell carcinogenesis. However, apart from a single study (Viglietto G et al., 2000), little is known about the expression of GDNF and its co-receptors in human TGCTs.

The aim of the present study is to determine the potential role of the GDNF pathway in the onset and progression of human seminoma germ cell tumors. For this purpose, we first analyzed the expression pattern of GFRA1 and Ret, the two major co-receptors for GDNF, in CIS and seminoma samples. We found that GFRA1 was expressed more extensively in CIS cells and invasive seminoma compared with normal testis.

Therefore, to gain insights into the potential function of the activation of the GDNF pathway in seminoma cells we used the TCam-2 human seminoma cell line as an experimental model system.

MATERIAL AND METHODS

5.1 Tumor samples

Surgical samples were obtained from ten 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 hematoxylin-eosin stained sections indicated that tumor 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).

5.2 Immunohistochemistry (IHC)

Immunohistochemistry was performed as described (Muciaccia B et al., 2010). Briefly, 3 μm thick paraffin tissue sections were serially collected, treated for antigen retrieval and incubated with primary antibodies (1:20 anti-human monoclonal RET; 1:100 anti-human CD34, anti-human inhibin and anti-PLAP from Novocastra; 1:200 anti-human GFRα1 from R&D Systems; 1:400 anti-human CD117 from Dako). 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). Negative controls were performed by omitting the primary antibodies (data not shown). Peroxidase activity was revealed using 3,3-diaminobenzidine tetrahydrochloride (Roche), and the nuclei were counterstained using hematoxylin solution. For immunohistochemistry 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.

5.3 TCam-2 cell cultures

The TCam-2 human cell line was derived in 1993 from a primary testicular tumor sample of pure classical seminoma (Mizuno Y et al., 1993). TCam-2 cell line is the only validated seminoma cell line (de Jong, J et al., 2008; Eckert D et al., 2007; Goddard NC et al., 2007; Mizuno Y et al., 1993).TCam-2 cells were cultured as described in RPMI 1640 (Lonza) supplemented with 10% fetal bovine serum (Lonza) and penicillin/streptomycin (Invitrogen) at 37°C in a humidified atmosphere with 5% carbon dioxide(Goddard NC et al., 2007). To test the effect of GDNF on cell proliferation, TCam-2 cells were maintained for 16 hours under serum-free conditions and then treated in the presence or absence of 100 ng/ml GDNF (R&D Systems) or 10% (v/v) fetal bovine serum. After 24, 48 and 72 hours, 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 hours under serum-free conditions and then cultured in the absence or in the presence of 100 ng/ml GDNF or 10% fetal bovine serum as positive controls for 12, 24 and 48 hours. The cells were recovered and stained with a propidium iodide/RNase solution. The cell suspensions were analyzed with a Beckman Coulter Epics XL Flow Cytometer. To test for gelatinolytic activity, TCam-2 cell conditioned media were collected after 48 hours, and the cells were lysed in 0.5% Triton X-100 in 0.1 M Tris-HCl, pH 8.1 (Catizone A et al., 2010).

5.4 Reverse transcription-PCR (RT-PCR) Analysis

The cells were harvested and total RNA was extracted with a phenol-chloroform extraction (TRIreagent, Sigma-Aldrich). 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 1. 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.

5.5 Boyden chamber assays

The cells were assayed for their ability to migrate through a polycarbonate filter (pore size, 8 μm; Whatman International) using Boyden chambers (NeuroProbe). The cells (5 X 10^4/well) were added to the upper chamber, and GDNF, anti-GDNF antibody (R&D Systems), CXCL12 (SDF-1 from Peproteck), isotype control antibody or FBS (control wells) were added alone or in combination in the lower chamber. To analyze 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. 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 hours 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) using Boyden chambers. The cells (5 X 10^4/well) were added to the upper chamber, and GDNF or CXCL12 were added to the lower chamber. 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 NO et al., 2006). Invasion was allowed to proceed for 24 or 48 hours 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 40x

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 (SE).

5.6 Gelatin zymography for metalloproteinases (MMPs) detection

Gelatinolytic activity of the TCam-2 conditioned media and cell extracts were assayed as previously described (Catizone A 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).

5.7 Statistical Analysis

All quantitative data are presented as the mean ± standard error of the mean (SEM). Data were analyzed 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

6.1 GFRA1 is extensively 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 (Figure 8, 9). 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 10). 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 L et al., 2009) and in interstitial Leydig cells (Davidoff MS et al., 2001) (Figure 8). Compared with preserved testicular parenchyma, GFRA1 expression was expressed more extensively in both CIS and intratubular seminoma cells, while expression levels varied from moderate to strong in invasive seminoma cells (Figure 8). In preserved testicular parenchyma, Ret expression was not detected in seminiferous tubules, while it was clearly detected in interstitial tissue, possibly in the Leydig cell lineage (Figure 9). 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, while 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 (Figure 9).

6.2 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 tumor germ cell proliferation. To this end, we took

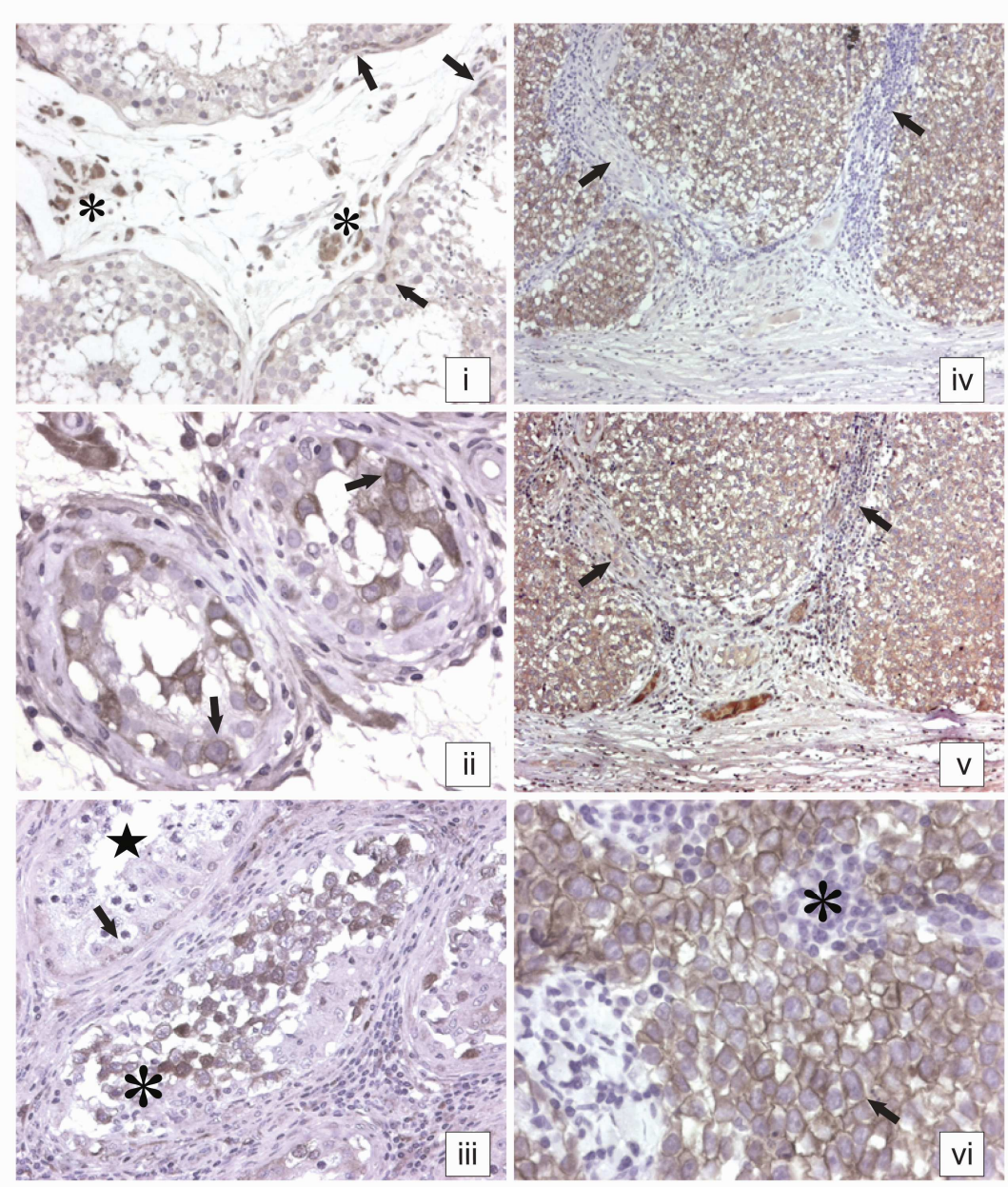

Fig. 8 - Immunohistochemical detection of GFRA1 receptor in TGCTs.
Images are from six representative seminoma tissue samples. GFRA1 cellular localization: (i) seminiferous tubules with preserved spermatogenesis adjacent to CIS lesion. GFRA1 expression was detected in Leydig cells (asterisks) and in 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).

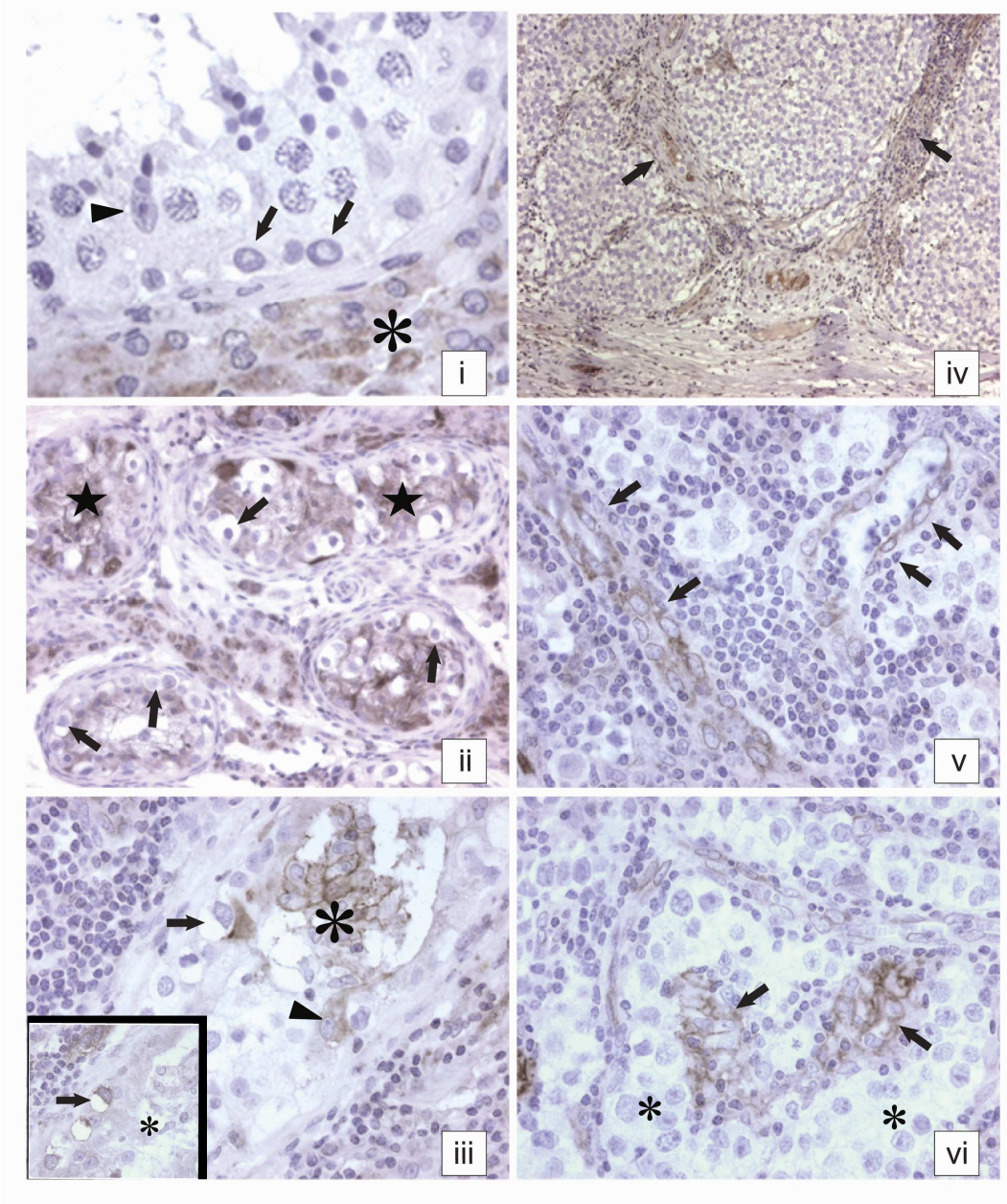

Fig. 9 - Immunohistochemical detection of Ret receptor in TGCTs.
Images are from six representative seminoma tissue samples. Ret cellular localization: (i) lack of Ret staining in preserved testicular parenchyma of seminiferous tubules both in germ and somatic cells, while Ret-positive cells (possibly 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 heterochromatic nuclei; and (vi) Ret staining was detected in clusters of possibly endothelial/pericyte precursors (arrows). Seminoma cells are Ret-negative (asterisks).

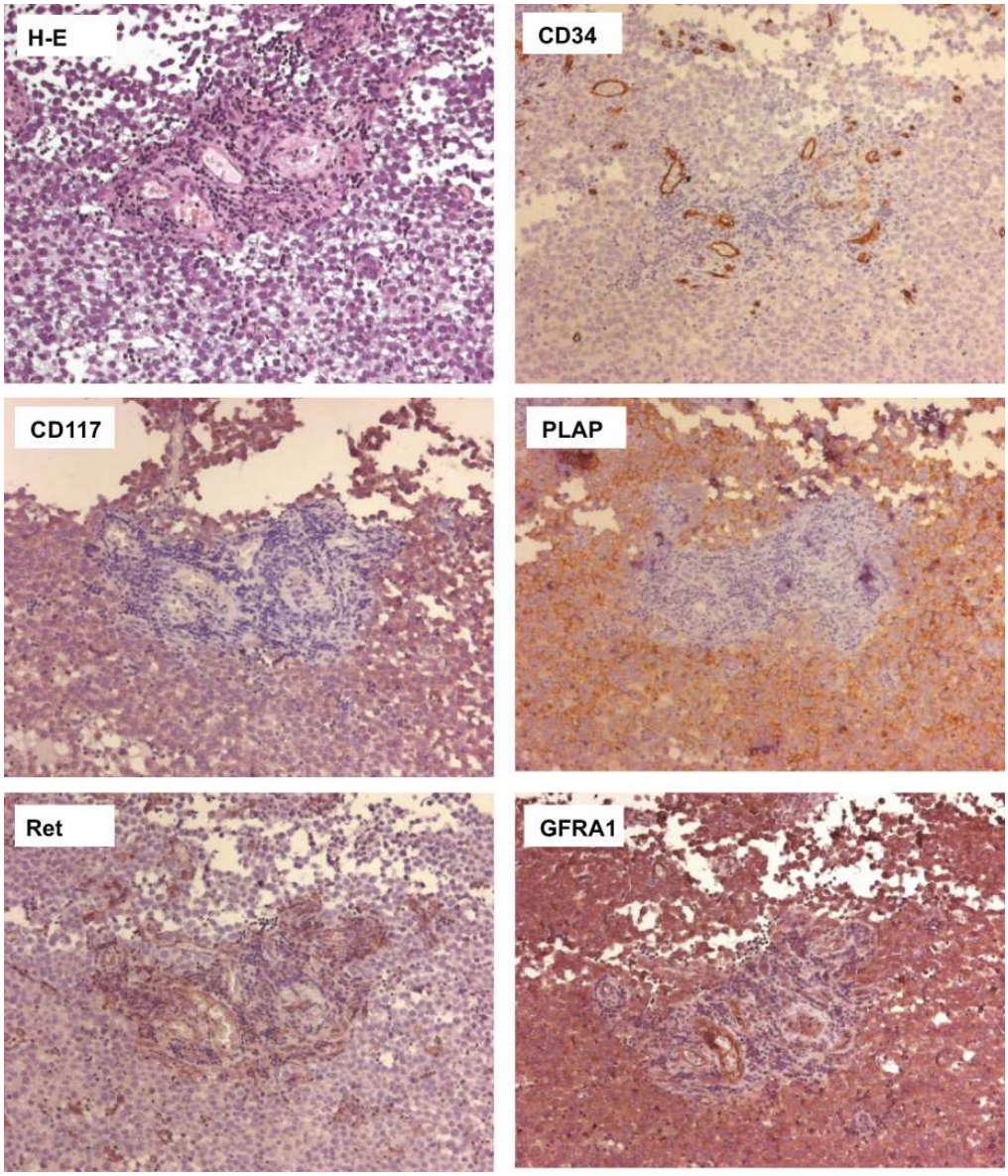

Fig. 10 - Immunohistochemical analysis performed on adjacent paraffin sections obtained from sample # 7. GFRA1 antibodies identify CIS and seminoma cells similar to two routinely used diagnostic markers, such as c-Kit antibodies (CD117) and PLAP. Anti-CD34 antibodies detect endothelial cells.

advantage of TCam-2 cells, currently, the only available validated seminoma cell line. First, expression of GDNF co-receptors was analyzed by RT-PCR analysis (Figure 11A) and by IHC (Figure 11B). *GFRA1* is alternatively spliced into two highly homologous isoforms, GFRA1a and GFRA1b, which differ at five amino acids (Shefelbine SE et al., 1998). Recently GFRA1b, but not GFRA1a, has been demonstrated to be responsible of cell migration and invasion in C6 glioma cells (Wan G et al., 2010). Both GFRA1 isoforms are expressed in the TCam-2 cells (Figure 11A). *Ret* undergoes alternative splicing, which generates the Ret9 and Ret51 isoforms, differing at the carboxyl-termini (Arighi E et al., 2005). As observed by RT-PCR analysis, both Ret9 and Ret51 transcripts are present in the TCam-2 cells (Figure 11A). In order to verify whether also GFRA1 and Ret receptor proteins were expressed, we performed IHC analysis on TCam-2 cells. GFRA1, but not RET, was detected by IHC (Figure 11B). We next addressed whether GDNF modulates TCam-2 proliferation or survival. In fact, GDNF is a known key factor regulating spermatogonial stem cell self-renewal and proliferation (Hofmann MC et al., 2008). 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 hours. 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 analyzed (Figure 12). In order to determine the percentage of TCam-2 cells in S phase FACS analysis, using the same treatment conditions for 12, 24 and 48 hours, was performed (Figure 13). While 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 point analyzed (Figure 13).

6.3 GDNF induces TCam-2 cell migration

Intriguingly, in other cellular systems, GDNF has been implicated in the directed migration and invasion of normal and tumor cells (Okada Y et al., 1999; Paratcha G et al., 2006; Song H and Moon A, 2006; Su CM et al., 2009; Tang MJ et al., 1998; Veit C et al., 2004; Young HM

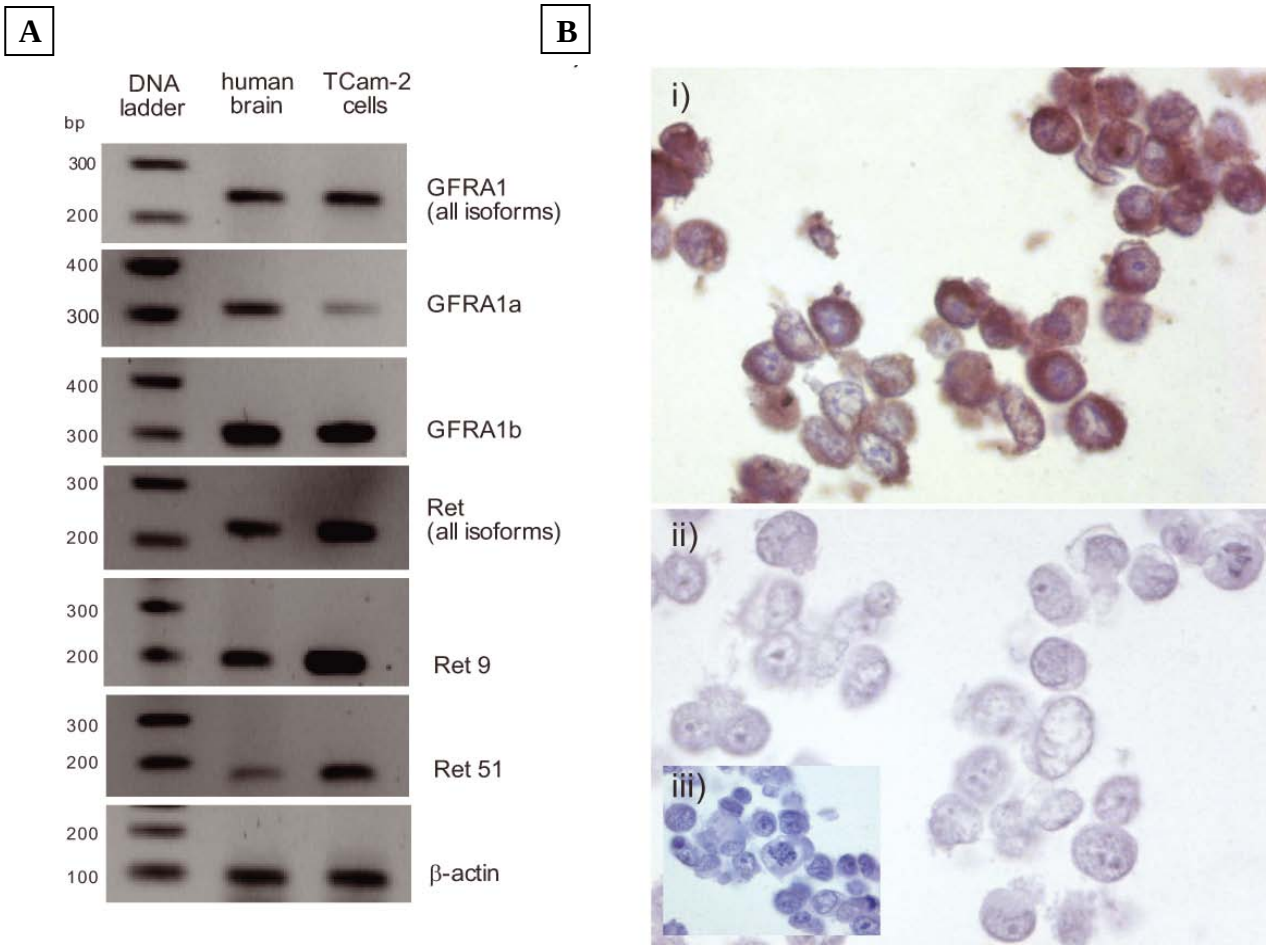

Fig. 11 - Identification of GDNF co-receptor variants expressed in TCam-2 cells.
(A) RT-PCR analysis. Total RNA was prepared from TCam-2 cells and used as a template for RT-PCR analysis. A sample containing RNA from human brain and a sample containing no RNA (not shown) were used as controls. A β-actin 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).

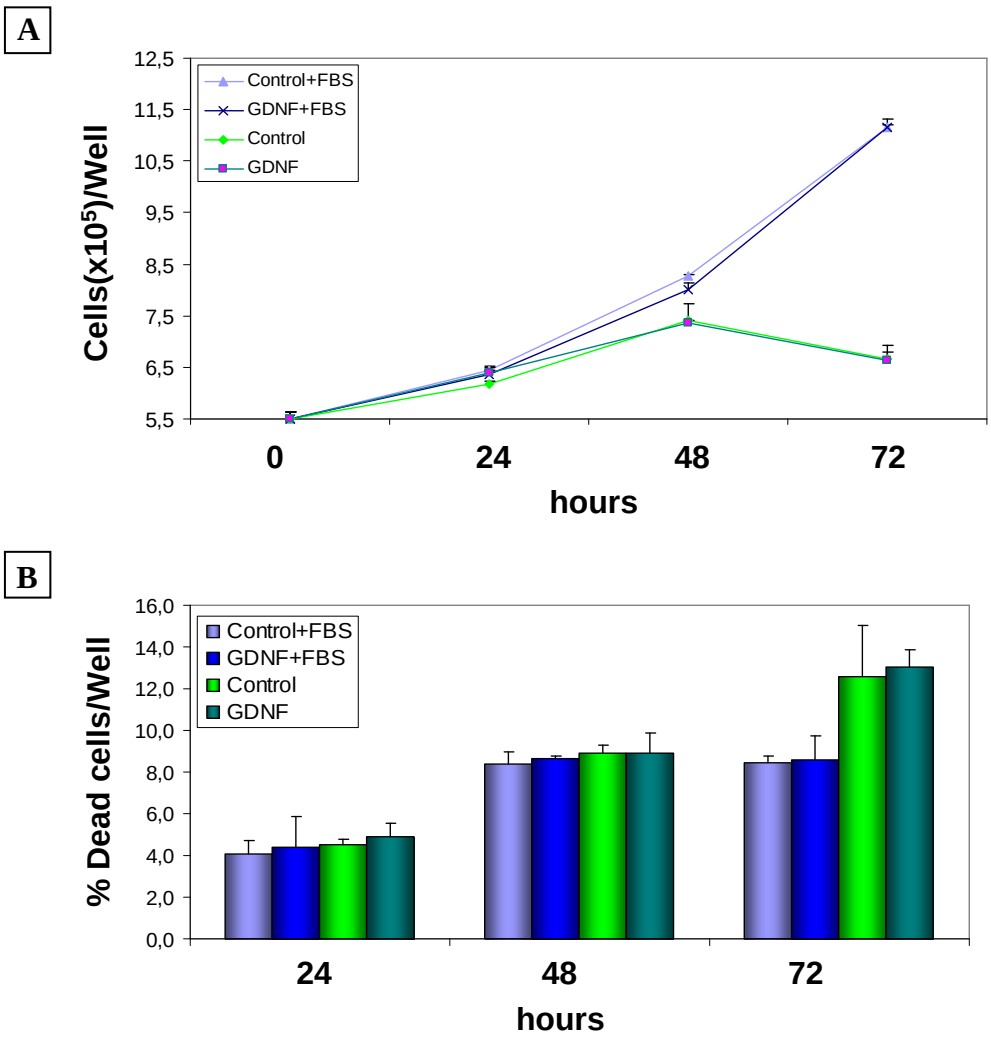

Fig. 12 - GDNF is not a mitogenic factor for TCam-2 cells.
(A) TCam-2 cells were serum-starved for 16 hours and then cultured in the presence or absence of 100 ng/ml GDNF with or without 10% FBS for 24, 48 and 72 hours. Percentage of dead cells at each time point is shown in (B). The results are from a representative of three experiments performed. Data are shown as the mean±SEM of triplicate samples.

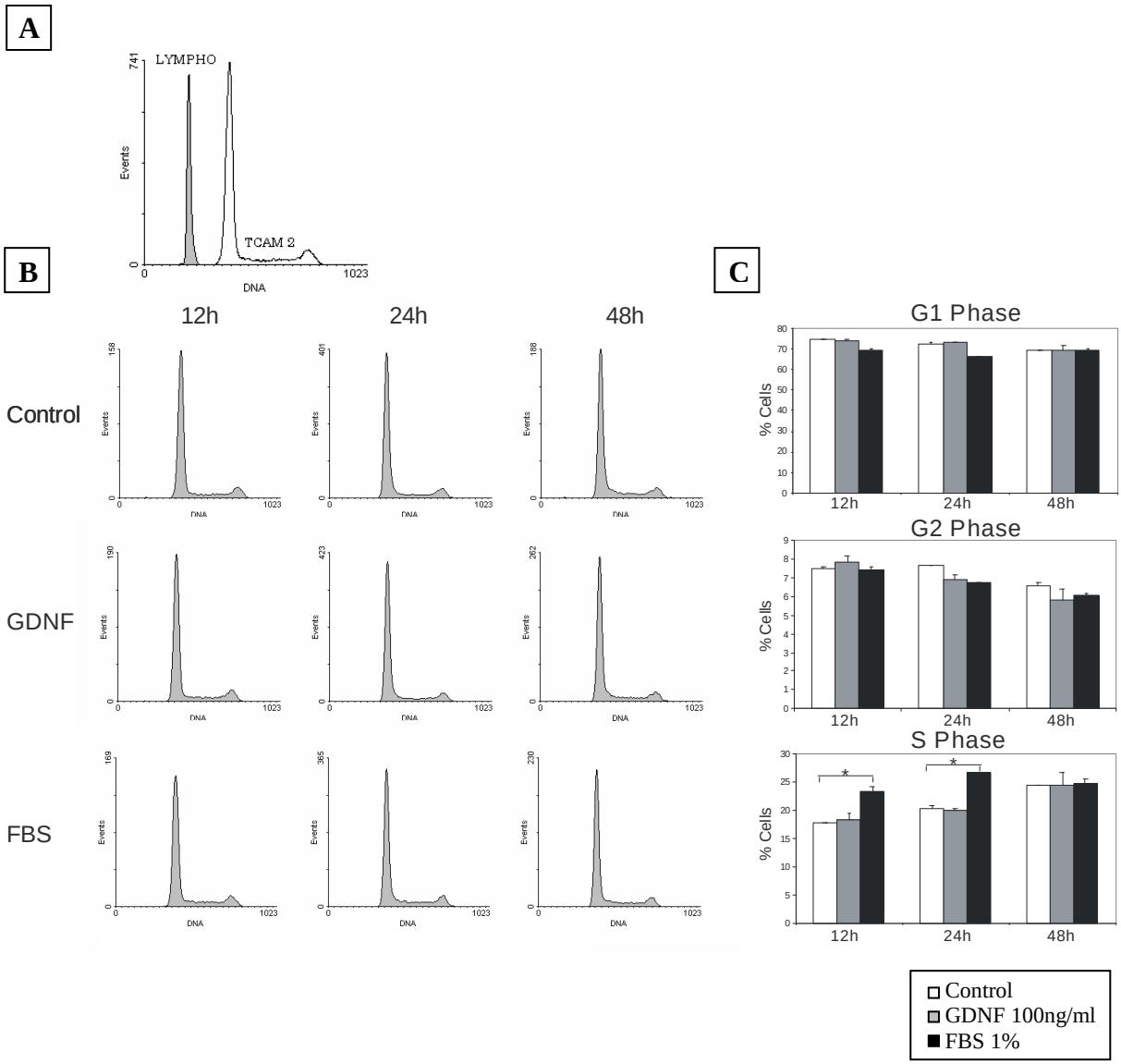

Fig. 13 - GDNF does not induce S-phase entry in TCam-2 cells.
(A) Flow cytometry analysis reveals that TCam-2 cells have a tetraploid DNA content. LYMPHO: human lymphocytes. (B) Cell cycle phase distribution of TCam-2 cells treated in the absence or presence of 100 ng/ml GDNF or with 10 % FBS (as a positive control) for 12, 24 and 48 hours 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)

et al., 2001). More interestingly, we recently found that *in vitro* GDNF is a chemoattractant for murine undifferentiated spermatogonia (Dovere L, manuscript in preparation). Based on these observations, we hypothesized that TCam-2 cells may migrate in response to a GDNF gradient. Thus, in order to test whether the GDNF pathway could be involved in TCam-2 seminoma cell migration we performed Boyden chamber migration assays with increasing concentrations of GDNF (20 to 150 ng/ml). Therefore to create a positive gradient for the putative chemoattractant, the upper compartment was filled with DMEM+0,1% BSA alone, while the lower chamber contained DMEM+0,1% BSA plus GDNF. The results showed that, consistent with our hypothesis, after 5h of incubation, a significant effect on cell migration was achieved with 100 ng/ml and 150 ng/ml GDNF (Figure 14A). As a control, we tested if the anti-GDNF antibody was able to block this effect. Addition of the blocking antibody, along with GDNF, completely neutralized the GDNF activity on TCam-2 cells, reducing cell migration at a level comparable to the control (Figure 14B) No effect was observed after administration of the antibody isotype control (Figure 14B). 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 (Figure 14B). 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 (Figure 14B).

Recently the small cytokine CXCL12, a known chemoattractant for different cell types, has been reported to be able to induce invasive migration on TCam-2 seminoma cells by activation of the ERK pathway (Gilbert Dc et al., 2009). By comparing the GDNF chemoattractant activity with that of CXCL12, we found that GDNF is more effective (approximately 50%) than CXCL12 (Figure 14C). In conclusion, these data show for the first time, that GDNF can stimulate directional migration of TCam-2 seminoma cell line. Intriguingly, along with the observation that GDNF is an *in vitro* chemoattractant for

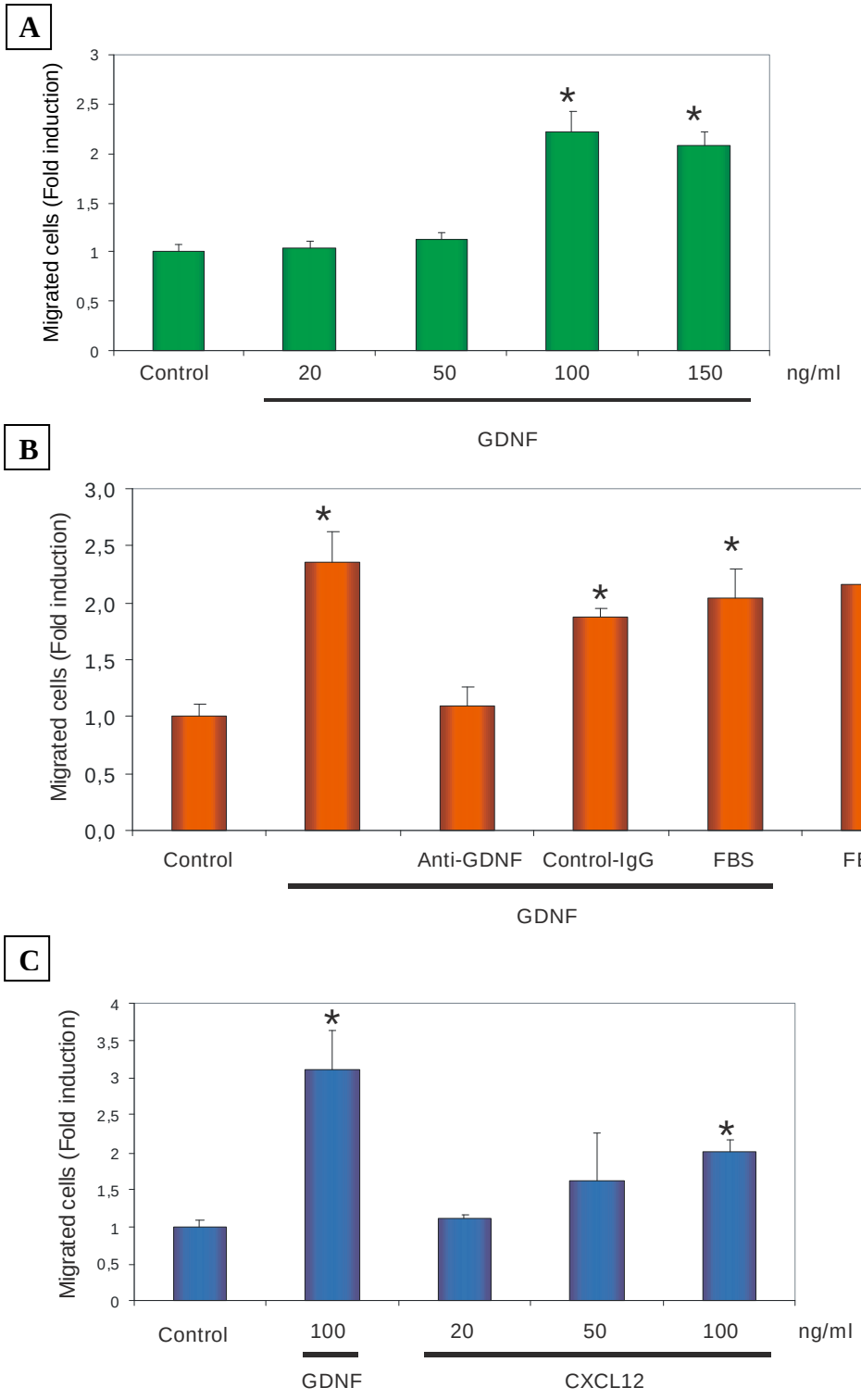

Fig. 14 - GDNF-dependent migration of TCam-2 cells.
Cell migration was evaluated using the Boyden chamber assay as detailed in the Materials and Methods section. (A) TCam-2 cells were treated with increasing concentrations of GDNF. (B) TCam-2 cells were treated as indicated at final concentrations of 100 ng/ml GDNF, 15 µg/ml anti-GDNF antibody, 15 µg/ml control-IgG or fetal 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).

undifferentiated mouse spermatogonia (Dovere L, manuscript in preparation), 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 tumor cell types, GDNF activates the MEK and PI3K pathways (Su CM et al., 2009; Song H and Moon A, 2006; Veit C et al., 2004). Three different inhibitors were tested as follows: PP-2 (a potent Src-inhibitor), LY294002 (a PI3K-inhibitor), and U0126 (a MEK-inhibitor). PP-2 (Figure 15A) and UO126 (Figure 15B), but not LY294002 (Figure 15C), 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.

6.4 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. For this purpose we performed modified Boyden chamber assays with Matrigel-coated filters. Matrigel is a solubilized basement membrane preparation whose major component is laminin, followed by collagen IV, heparan sulfate proteoglycan, and entactin. This substrate is useful to mimic the *in vivo* mechanisms on the base of tissue invasion where cells have to move trough and degrade the ECM components to invade adjacent tissues. Invasion was analyzed at two time points: 24 and 48 hours. At both time points, GDNF administration significantly enhanced cell invasion compared with the control, as well as with CXCL12 (Figure 16A). Tumor cell invasion can occur through two modalities: a proteolytic mesenchymal-like or non-proteolytic amoeboid-like modality (Sahai E and Marshall CJ, 2003; Wolf K et al., 2003). The mesenchymal-like strategy of invasion requires extracellular matrix degradation by proteases, including matrix metalloproteinases (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

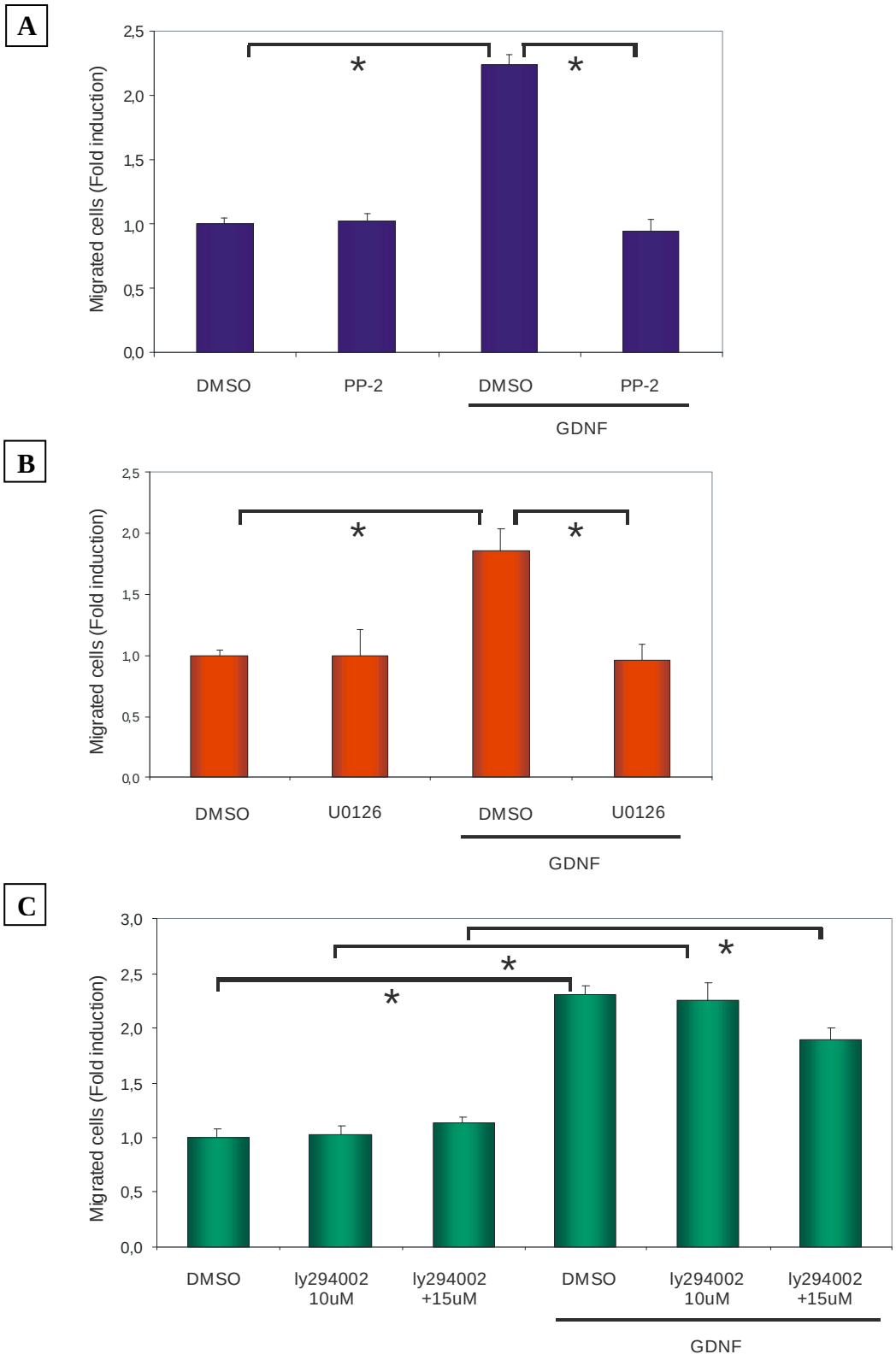

Fig. 15 - The effect of different inhibitors on GDNF-induced TCam-2 cell migration.
Cell migration was evaluated using the Boyden chamber assay as detailed in the Materials and Methods section. TCam-2 cells were pre-treated for 30 min with 10 μm PP-2 (A), 10 μm U0126 (B) or an indicated concentration 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).

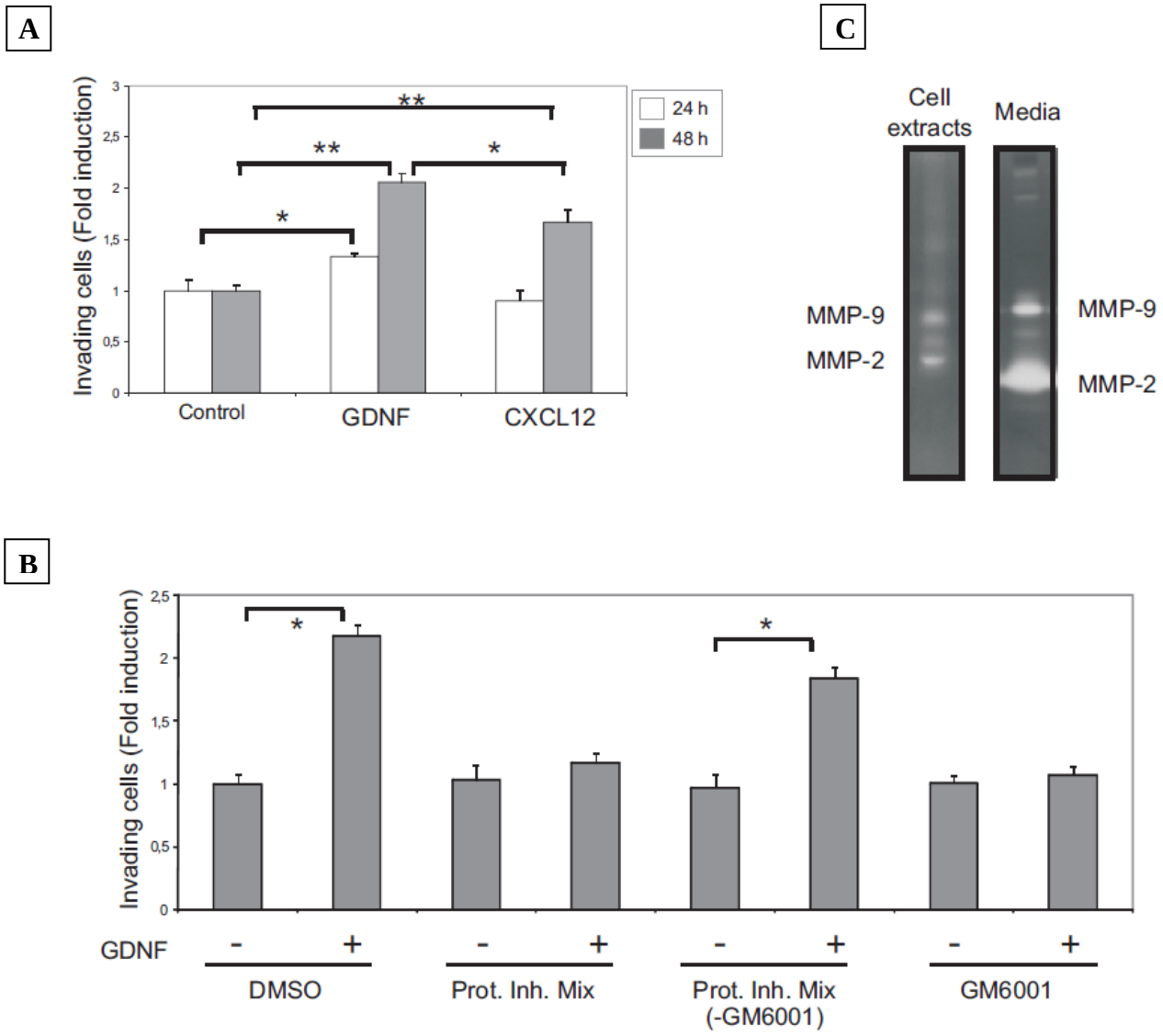

Fig. 16 - GDNF promotes invasion in TCam-2 cells.
Matrigel invasion assays were performed and evaluated as detailed in the Materials and Methods section. (A) TCam-2 cells were treated for 24 and 48 hours with 100 ng/ml GDNF and 100 ng/ml CXCL12; (B) TCam-2 cells were pre-treated for 30 min with indicated protease inhibitors 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.

TCam-2 cells in GDNF-induced cell invasion, we tested a cocktail of protease inhibitors (named prot. inh. mix), which included GM6001 (a broad matrix metalloproteinases 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 (Figure 16B), 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 (Figure 16B). MMP-2 and MMP-9 activity (Figure 16C) was detected in TCam-2 cell extracts and conditioned medium by gelatin zymography.

DISCUSSION

In recent years, a large body of evidence pointed to the importance of GDNF for proper spermatogenesis. In mice, GDNF is secreted by Sertoli cells and promotes spermatogonial stem cell self-renewal (Hofmann MC, 2008). Over-expression of GDNF in adult transgenic mice induces proliferation of clusters of undifferentiated spermatogonia within seminiferous tubules (Meng X et al., 2001). Additionally, older animals develop testicular tumors that mimic human seminoma, suggesting that over-activation of this pathway may lead to the insurgence of TGCTs (Meng X 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 N et al., 2010). An alternative mechanism for driving germ cell tumor progression could be the up-regulation of wild-type GFRA1 and/or Ret proteins. Overexpression of GFRA1 and/or Ret has been found in different cancers (Dawson DM et al., 1998; Nakashima M et al., 2007; Takaya K et al., 1996), and in some instances, Ret over-expression correlates with poor survival (Ito Y et al., 2005) as well as the development of a subgroup of breast tumors (Esseghir S et al., 2007). We demonstrate, for the first time, that GFRA1 is extensively expressed in CIS, and its expression is maintained in invasive seminoma. However, Ret was not detected by immunohistochemistry 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. To date, a number of CIS and seminoma cells markers are known to be expressed in fetal gonocytes 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 ME, 2006). Because GFRA1 is detected in dark and pale type A spermatogonia, GFRA1 lengthens the list of tumor germ cell markers that are expressed in normal germ cells.

To elucidate the biological activity of GDNF in TGCTs, 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 tumor cell types (Okada Y et al. 1999; Paratcha G et al. 2006; Song H and Moon A, 2006; Su CM et al., 2009; Tang MJ et al., 1998; Veit C et al., 2004; Young HM et al., 2001), 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 C 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 tumor dissemination involves proteolytic degradation and remodeling of the extracellular matrix (ECM) barriers, a process dependent upon extracellular proteases, including MMPs (Coussens LM et al., 2002). However, protease inhibition does not completely abolish tumor cell migration and dissemination, suggesting alternative compensating protease-independent mechanisms (Coussens LM et al., 2002; Zucker S et al., 2000). Of note, when proteolysis is blocked, some tumor cell types switch from a protease-dependent mesenchymal type of motility to an ameboid-like rounded mode of motility that requires Rho/ROCK signaling (Sahai E and Marshall CJ, 2003; Wolf K 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

tumorigenesis (Kessenbrock K et al., 2010). The lack of invasion upon inhibition of pericellular proteases suggests that TCam-2 cells are not able to undergo the mesenchymal-ameboid switch, thus suggesting a lack of plasticity compared with more aggressive tumor cell types (Carragher NO et al., 2006).

We demonstrated that GFRA1 expression is upregulated 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 K et al,. 2010). The expression level of GDNF was found to be similar in peritubular cells isolated from patients suffering from azoospermia and fibrotic remodeling of the peritubular wall (Spinnler K 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. In infertile mouse models, GDNF levels are inversely correlated with the germ cell content of the testis, possibly through the regulation of the hypothalamus–pituitary axis (Tadokoro Y et al., 2002). Moreover, GDNF expression in Sertoli cells is induced by inflammatory cytokines (Simon L et al., 2007). Therefore, it could be speculated that, as described in some breast cancers (Esseghir S et al., 2007), local levels of GDNF in the tumor microenvironment may be increased as a consequence of leukocyte 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 tumor 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, i.e., stage I non-seminoma (NS). Patients with moderate to strong expression of CXCL12 have a reduced risk of relapse, suggesting that a high level of chemokines in the tumor microenvironment may

impact tumor germ cell spreading and metastatic potential (Gilbert DC et al., 2009). Following this line of reasoning, it would be interesting to assess whether the expression level of GDNF signaling pathway molecules have a similar prognostic value in patients affected by seminoma tumors.

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Hepatocyte growth factor (HGF) regulates blood–testis barrier (BTB) in adult rats

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ABSTRACT

We have studied the effects of HGF on BTB dynamics in adult rats. We demonstrate that, at stages VII–VIII of the epithelium wave when germ cells traverse the BTB, HGF reduces the levels of occludin and influences its distribution pattern and assembling. Moreover, we report that, at stages VII–VIII, HGF significantly increases the amount of active TGF-β and the amount of uPA present in the tubules. For the first time we report that, in the same stages, HGF reduces the amount of actin present in the BTB region, in which occludin levels are highest, and modifies the morphology of the actin cytoskeleton network. At the level of maximal intensity of occludin fluorescence, we report that HGF also modifies the colocalization of occludin and actin. Lastly, we demonstrate that HGF is maximally expressed at stages VII–VIII, whereas its levels fall in the subsequent stages.

1. Introduction

In adult mammalian testes, Sertoli cells form junctional complexes with the adjacent Sertoli cells that consist of tight junctions (TJs) and anchoring junctions (AJs), including a testis-specific cell–cell actin-based AJ type, which is involved in the formation of the "blood–testis" barrier (BTB). The BTB separates mitotic spermatogonia from meiotic germ cells and is periodically disassembled to allow the passage of preleptotene spermatocytes across the barrier (Dym and Fawcett, 1970; Russell, 1977). The molecular changes in BTB which permit germ cell movement remain largely unknown, although it is widely accepted that a very complex mechanism allows junction restructuring while maintaining barrier integrity (Mruk and Cheng, 2004; Lee and Cheng, 2005; Xia et al., 2005b; Wong and Cheng, 2005).

In recent years, several molecules have been shown to be crucial regulators of BTB dynamics (Siu et al., 2003; Wong and Cheng, 2005; Xia et al., 2005a; Yan and Cheng, 2005; Capaldo and Nusrat, 2009). Testosterone and TGF-β3 are involved in the regulation of the permeability of Sertoli cell tight junctions, possibly by modulating occludin, zonula occludens-1 (ZO-1) and claudin-11 levels (Chung and Cheng, 2001; Lui et al., 2001; Kaitu'u-Lino et al., 2007). The BTB uses both tight and adherens junctions to allow germ cell movement. It has been demonstrated that besides its effect on tight junctions, TGF-β3 also acts on AJs when the ERK signalling pathway is activated (Xia and Cheng, 2005; Xia et al., 2006). BTB disruption, and a concomitant reduction in occludin levels, also occurs when TNF-α is administered to adult rat testes *in vivo*, as reported by Li et al. (2006). Our group has reported that HGF modulates Sertoli–Sertoli tight junction dynamics by reducing the levels and distribution of occludin (Catizone et al., 2008). The effect of interleukin-1α on the localization of occludin has also been reported (Sarkar et al., 2008; Lie et al., 2011) and the effect of gonadotropins on tight junction regulation has recently been demonstrated (McCabe et al., 2010). Although it is widely accepted that cytokines are involved in the regulation of cell junction dynamics, the biochemical mechanisms underlying transient TJ "opening" are not yet fully understood (Lui and Cheng, 2007; Li et al., 2009; Cheng and Mruk, 2010).

Hepatocyte growth factor (HGF) is a cytokine originally identified as a mitogenic factor for hepatocytes (Nakamura et al., 1984, 1989) and separately identified as a scatter factor (SF) due to its ability to disperse sheets of contiguous epithelial cells (Stoker et al., 1987). It is now well known that HGF/SF is a pleiotropic cytokine that regulates various functional activities of numerous cellular types (Matsumoto and Nakamura, 1993, 1996; Zarnegar and Michalopoulos, 1995), including the positioning and activity of tight junctions (Pollack et al., 2004). HGF reduces the level of occludin in endothelial cells (Jiang et al., 1999), and in breast cancer cells modulates tight junction proteins with consequent disruption of the junctions (Martin et al., 2004). We have previously reported

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