CHARACTERIZATION OF DIFFERENT SUBSETS OF SPERMATOGONIAL STEM CELLS: INFLUENCE OF NICHE-DERIVED FACTORS.

Tesi Dottorato di Ricerca
Dott.ssa Lisa Dovere

Relatore
Dott.ssa Elena Vicini

Coordinatore del corso di Dottorato
Prof. Mario Stefanini

ANNO ACCADEMICO
2009-2010
CONTENTS

INTRODUCTION ........................................................................................................................................... 3

1. Spermatogenesis ....................................................................................................................................... 3
   1.2 Molecular phenotype of spermatogonial stem cells .............................................................................. 5
   1.3 Heterogeneity of spermatogonial stem cells: revisiting the “As model” ........................................... 6

3. Spermatogonial stem cells cultures .......................................................................................................... 9

4. Cell migration ........................................................................................................................................... 12
   4.1 The process of cell movement ............................................................................................................ 12
   4.2 The actin-binding families of proteins ............................................................................................... 14
   4.3 ENA/VASP PROTEINS: Regulators of the actin cytoskeleton ...................................................... 15

AIMS OF THE PRESENT STUDY .................................................................................................................. 19

MATERIALS AND METHODS ....................................................................................................................... 21
   5.1 Germ cells preparation ....................................................................................................................... 21
   5.2 Thy1-positive cells isolation .............................................................................................................. 21
   5.4 Quantitative Real-Time PCR ........................................................................................................... 22
   5.6 Western blot analysis ....................................................................................................................... 23
   5.7 Spermatogonial stem cell cultures ................................................................................................... 24
   5.8 Statistical analysis .............................................................................................................................. 25

RESULTS ...................................................................................................................................................... 26
   6.1 GFRA1-positive spermatogonia show a migrating phenotype .......................................................... 26
   6.2 GDNF exert chemoattractant action on undifferentiated spermatogonia ........................................... 27
   6.3 Undifferentiated spermatogonia express VASP .............................................................................. 28
   6.4 GDNF regulates VASP expression levels ......................................................................................... 29

DISCUSSION ................................................................................................................................................. 39

BIBLIOGRAPHY ........................................................................................................................................ 35
INTRODUCTION

1. Spermatogenesis

1.1 Spermatogenesis and spermatogonial stem cells

Spermatogenesis is a complex and highly regulated process that occurs throughout the lifetime of the male mammals. This process takes place in the testes of mammals and proceeds inside the seminiferous tubules. The seminiferous epithelium consists of different cell types: somatic and germinal cells. The wall of the tubules is lined by peritubular myoid cells that with their contractile elements generate peristaltic waves along the tubules. On the basement membrane of the tubules there are Sertoli cells that support and regulate the developing germ cells by the secretion of growth factors. The germ cells are organized in concentric layers, at different stages of development, from the basement membrane to the lumen of seminiferous tubules (Fig. 1). The interstitial tissue between the tubules contains blood and lymph vessels but never penetrate the seminiferous tubules. The blood vessels are surrounded by Leydig cells and other interstitial cells (macrophages and lymphoid epithelial cells) (Fig. 2). Sertoli cells develop an epithelium inside the tubules that harbors prominent tight junctions between the cells; this structure constitutes the anatomical basis of the blood-testis barrier that separates the basal and adluminal compartments.

The entire process of spermatogenesis is composed of three main phases: the first phase, termed the “proliferative phase”, consist of mitotic amplifying divisions of spermatogonia. These cells ultimately became primary spermatocyte, that enter the second phase of spermatogenesis, termed the “meiotic phase”. After two meiotic divisions, haploid spermatids are produced that undergo the third phase, termed “spermiogenesis”, consisting of transformation from round germ cells to specialized spermatozoa (Fig. 3). The basal compartment, the space between the tight junction and the basement membrane, is
Figure 1 - Schematic drawing illustrating cross-section of a single seminiferous tubule and the contribute of different niche factors to the SSC behavior. Abbreviations: SSC, spermatogonial stem cells; PTM, peritubular myoid cells. Modified from Caires et al. (2010) *Journal of Endocrinology*.

Figure 2 - Schematic representation of a testis cross section that highlight the relationship between seminiferous tubules and the vascular/interstitial tissue consisting of macrofages, leydig cells and blood vessels. Modified from Shetty and Meistrich (2007) *Cell*. 
Figure 3 – Schematic representation of spermatogenesis. The stem cell, spermatogonial, meiotic and post-meiotic compartments of spermatogenesis are shown. Modified from Yoshida et al. (2010) Develop Growth Differ
occupied by spermatogonia that represent the mitotic stage of spermatogenic cells (1-2). On entering the meiotic prophase, the cells translocate to the adluminal compartment across the tight junctions, followed by subsequent movement toward the lumen, and are eventually released as mature spermatozoa.

Spermatogenesis is supported by the spermatogonial stem cells, which are defined as possessing the ability to self-renew and to differentiate into spermatozoa. The dual capacity of these cells ensures the long-lasting ability of the testes to produce spermatozoa. In the widely accepted model spermatogonial stem cells (SSCs) are single cells located on the basal membrane of the seminiferous tubules and are called A-single spermatogonia (As). These cells either divide into two single cells (self-renewing division) or into a pair of spermatogonia (Apr) that do not complete cytokinesis and stay connect by an intercellular bridge. Until now the transition between As and Apr spermatogonia was considered the first differentiative step of germ cells. However, as it will be discussed later, this model has been recently questioned by recent evidences that have shown that the model is more complex than previously appreciated.

The Apr spermatogonia divide further to form chains of 4, 8 occasionally up to 32 A-aligned spermatogonia (Aal). Collectively, the As, Apr and Aal germ cells are referred to as undifferentiated spermatogonia. As mentioned above, the isolated spermatogonia (A-single or As spermatogonia) are considered to be the most primitive cells, and the length of the cell cysts parallels the differentiation status (1). Based on morphological analysis it has been estimated that As are very rare cells constituting only the 0.03% of the total germ cells in an adult mouse testis and only 1% of the spermatogonia (3).

The Aal spermatogonia can go through a differentiation step and give rise to differentiating spermatogonia called A1 through A4 spermatogonia in the mouse, indicating the number of mitotic amplifying division they undergo. The differentiating A4 spermatogonia are capable of further maturation into intermediate and type B spermatogonia that finally enter meiosis, becoming primary spermatocytes. These cells proceed along the lengthy prophase of first
meiotic division and are indicated as preleptotene, leptotene, zygotene and pachitene spermatocytes.

1.2 Molecular phenotype of spermatogonial stem cells

Several genes such as PLZF, NGN3, OCT4, SOX3, GFRA1, NANOS2, NANOS3, TEX14, THY-1 were found to be expressed by undifferentiated spermatogonia (Fig. 4). The promyelocytic leukemia zinc-finger (PLZF or Zfp145), is a transcriptional repressor that plays a critical role during embryogenesis in the limb and axial skeletal patterning. In the testis PLZF is expressed in As, Apr and Aal spermatogonia and it has been shown that it plays an important role in the regulation of the self-renewal of spermatogonial stem cells by maintaining an undifferentiated state (4-5). Strikingly, its ablation induces a progressive testicular phenotype that culminates in male sterility. GFRA1 is the co-receptor along with Ret for the growth factor GDNF (glial cell-line derived factor). GDNF, produced by Sertoli cells, regulates the fate of undifferentiated spermatogonia and the correct balance between self-renewal and differentiation of spermatogonial stem cells (6). It has been reported that GDNF-/+ mice show depletion of spermatogonial stem cells, whereas mice overexpressing GDNF show an accumulation of spermatogonial stem cells (6). Moreover, it has been demonstrated that a combination of GDNF and soluble GFRA1 allows the self-renewal of SSCs in vitro (7). NGN3 (Neurogenin3), a helix-loop-helix transcriptional factor is expressed in As, Apr and Aal spermatogonia and is predominantly expressed in the c-Kit negative spermatogonia (8). Oct4 (POU5F1), a homeobox transcription factor that is required for the maintenance of totipotency of embryonic stem cells (9), is expressed also in undifferentiated spermatogonia (10-11). Another marker used to purify spermatogonial stem cell population is Thy-1, the glycosil phosphatidylinositol (GPI)-anchored glycoprotein. Kubota et al. (2003) determined that almost all (about 95%) of the SSCs in adult mouse testis are present in the THY-1 positive fraction, however the function of THY-1 in the testis is unknown. Nanos genes encode evolutionarily conserved proteins that play important roles during germ cell development.
**Figure 4 - Expression profile of spermatogonia**

The expression profiles of indicated genes are delineated for the different stages of spermatogonial development.
Nanos2 is involved in maintaining the stem cell state during spermatogenesis and is expressed in As to Apr cells, whereas Nanos3 is expressed not only in undifferentiated spermatogonia but also in differentiating spermatogonia (13,14). Interestingly, some of these markers can be used to purify spermatogonial stem cells. As a matter of fact the development of transplantation technique combined with fluorescence-activated cell sorting (FACS) has represented an excellent tool to study and characterize cell surface molecules of SSCs. It is possible, in this way to isolate a population from the testis that is enriched for spermatogonial stem cells.

1.3 Heterogeneity of spermatogonial stem cells: revisiting the “As model”

It has been established that the spermatogenic stem cells activity resides in the undifferentiated spermatogonia compartment (1). Biological examination of these cells is difficult because, as mentioned above, they are extremely rare and there are no phenotypic, biochemicals or molecular features that distinguish unequivocally the stem cells from their cell progeny. The only way to definitively identify SSCs in adult testis is by observing their capacity to produce and maintain colonies of spermatogenesis in a functional assay (15).

The current rodent stem cell model assumes that the stem cell population resides in the As population and that cyst length reflects the extent of differentiation in a linear manner. In this model the interconnected spermatogonia are committed to differentiation and irreversibly lose the stem cell potential (1). A consequence of this “As model” is that all As cells are stem cells therefore they are suppose to be functionally and phenotypically homogeneous (Fig 5A).

In the last years several reports from our and other laboratories challenged the As model. The analysis of the expression profile has demonstrated that As and more in general undifferentiated spermatogonia are indeed heterogeneous. In our laboratory we recently described, for the first time, that As spermatogonia do not uniformly express GFRA1 (16). GFRA1 negative As spermatogonia are less abundant, representing about 10% of the total number of As spermatogonia and were colonogenic, as shown by transplantation assay. They
were found isolated or intermingled with other undifferentiated spermatogonia. We reported, also, the presence (5%) of Apr spermatogonia that express GFRA1 asymmetrically. We therefore proposed that subsets of As spermatogonia may originate from asymmetric stem cell division (16). More recently it has been reported that NANOS2 and NANOS3 are also expressed in subset of undifferentiated spermatogonia (As to Aal) (17). In addition comparison of expression patterns of genes that mark the As, Apr, and/or Aal population, revealed that while genes such as PLZF and E-CAD are found expressed in all the undifferentiated spermatogonia, other genes such as GFRA1 and NGN3, were expressed only in subsets of the PLZF/E-CAD-positive cells. Interestingly, all the PLZF/E-CAD-positive cysts expressed either or both of these genes. Thus, spermatogonial cysts are heterogeneous for the expression of GFRA1 and NGN3 (18). Hence, the As, Apr, and Aal populations appear different not only morphologically (cyst length) but also for their gene expression.

In the last years several reports from Yoshida’s group have described the presence of functionally heterogeneous subset of stem cells. By using transgenic animal models they have shown that two different populations of stem cells exist: the actual and the potential stem cells (19). The first population is active during the steady-state spermatogenesis whereas the second type may function during regeneration that follows tissue injury. Therefore, the potential stem cells are able to revert to actual stem cells both in function and in gene expression. It is commonly considered that during differentiation cells make a transition from GFRA1-positive to NGN3-positive and finally to A1 spermatogonia, because GFRA1-expressing cells are mostly As or Apr, whereas NGN3-expressing cells are generally Aal (18). Yoshida’s group has demonstrated that by clone fragmentation NGN3-positive cells can revert to a GFRA1-positive status. The frequency of clone fragmentation increases when tissue is injured and regeneration takes place (18).

All these new evidence were recently integrated in a new model to describe the behavior of the stem cell compartment. This model proposes a number of important extensions to the “old As model”. Firstly, not all As cells act regularly as stem cells and secondly Apr and Aal
spermatogonia are not committed unidirectionally to differentiation but are capable to revert to As by clone fragmentation (18) (Fig. 5B). To summarize the latest findings suggest that the As model needs to be revised to fit the new experimental data.

2. The spermatogonial stem cells niche

Stem cells are affected by the special microenvironment in which they reside, called niche. The niche comprises cells, extracellular matrix components, and local soluble factors present very close to the stem cell that regulates cell fate. In the testis, the cellular environment in the niche will support spermatogonial stem cell renewal, whereas spermatogonial stem cells that leave the niche will most likely get into an environment promoting their differentiation (20). The structural basis for the SSC niche in the mammalian testis is the basal compartment of the seminiferous tubules that is composed of Sertoli cells and peritubular myoid cells (21). Recently the localization of undifferentiated spermatogonia on the basement membrane of seminiferous tubules has been analyzed. These findings indicate that the As, Apr, and Aal spermatogonia are not distributed at random over the basal membrane of the seminiferous tubules but they are preferentially located in specific areas (22, 23). In the mature mouse testis it has been observed that undifferentiated spermatogonia are preferentially located in restricted portions of the basal compartment within the seminiferous tubules adjacent to the blood vessels and the interstitium that contains Leydig cells, macrophages and other cells surrounding the blood vessels (Fig. 6) (22, 23). In vivo time-laps analysis of NGN3-expressing cells showed that during the differentiation these cells migrate out of these restricted areas and diffuse over the entire basal compartment of the seminiferous epithelium. Therefore these limited regions can be considered the niche for undifferentiated spermatogonia (23).

A key regulator of the SSC niche is GDNF a factor produced and secreted by Sertoli cells. This cell type play an important role in the regulation of SSC behaviour because they produce also a number of growth factors that have an effect on SSCs. Particularly GDNF acts through Ret
Figure 5 - The behavior of unidifferentiated spermatogonial according to the traditional “As” model (A) and the modified “As” model (B). From Yoshida et al. (2010) Science
Figure 6 - The hypothetic niche for the As/Apr/Aal spermatogonia. The region adjacent to the surrounding blood vessels and interstitial cells is considered important for the activity of undifferentiated spermatogonia population. From Yoshida et al. (2010) Science
receptor tyrosine kinase and GFRA1 co-receptor, which form a receptor complex on the surface of As, Apr and Aal spermatogonia (16). GDNF signaling is essential for stem cell maintenance. Mutants in Gdnf locus, similar to Plzf, show gradual loss of spermatogonial integrity (6). Moreover, in vitro, the addition of GDNF to the culture media is essential for the maintenance of spermatogonia with stem cell potential (7, 24).

By using in vitro cultures, a list of candidate niche-derived soluble factors has been obtained. Among these, bFGF (basic fibroblast growth factor, also known as FGF2) secreted by Sertoli cells also regulates SSC proliferation and self-renewal, although its in vivo role is not clear (7, 24). Other Sertoli cell-derived factors may be involved in the regulation of self-renewal and/or differentiation of SSC. It has been demonstrated that Activin A and bone morphogenic protein 4 (BMP4) when added to cultures of spermatogonial stem cells reduce the maintenance of these stem cells, thus suggesting that these factors promote stem cells differentiation (25). Sertoli cells are in turn influenced by different cell types present outside the seminiferous epithelium, i.e. myoid peritubular cells, or Leydig cells that influence Sertoli cells by producing testosterone (20). Therefore, Sertoli cell activity on SSC may be also modulated by components of the interstitial compartment. Moreover, besides Sertoli cells, peritubular myoid cells interstitial cells and blood factors may have direct effects on SSCs too (20). It appears essential to further investigate the niche and the factors the regulate the fate of spermatogonial stem cells, in order to better understand the behavior of these cells, during the regeneration after damage and to overcome spermatogenic defects in infertile men.

3. Spermatogonial stem cells cultures

The in vitro culture of SSC is a key tool for studying regulatory mechanisms that regulate their functions such as their self-renewal and differentiation. Historically, methods to culture spermatogonial stem cells were very difficult to establish. During the past years, however, remarkable progress in SSC cultures has been reported (7,24). Rodent SSCs can now be
maintained for a very long time with a significant amplification in their numbers. Stem cell activity in these cultures was confirmed by SSC transplantation (24).

There are several factors crucial to the establishment of long-term SSC cultures. An important prerequisite, is the purity of the starting cell population (7,24). If the concentration of the contaminating somatic cells is too high they outgrow SSCs. Thus, it is essential begin with an enriched SSC population. The purest fraction of SSCs can be obtained by FACS (26), MACS sorting (27) or differential attachment and replating (24). These methods produce the enrichment of SSCs and an effective removal of somatic cells favoring expansion of germ cells.

A second critical component is the appropriate cocktail of growth factors that are essential to support SSC self-renewal and survival. A serum-free defined medium, containing many growth factors (i.e EGF, LIF, etc.) has been tested to facilitate SSC expansion in vitro. Specifically, GDNF is necessary and crucial to maintain and expand SSCs in culture (7, 24). The positive effects of GDNF in both mice- and rats-derived cultures is enhanced by the addition of soluble GFRA1 (the co-receptor for GDNF) and FGF2 (7, 28).

Finally, STO or mouse embryonic fibroblast (MEF) feeder cells are usually required even though Shinohara’s group demonstrated that mouse SSCs can also be maintained in feeder-free conditions (29).

The role of GDNF on SSC functions is mediated by several target genes that are upregulated or/and downregulated by this growth factor. Recently Brinster’s group has identified some of these genes by microarray analysis (30). The significance of three of these genes (Bcl6b, Erm and Lhx1) was established by transfecting SSC cultures with siRNAs specific for each gene. siRNA treatment caused decreased clump formation in vitro and decreased colonization of recipient testes after transplantation (30). Thus, through genetic manipulation, siRNA and transplantation assay, it is now possible to investigate the molecular pathways that underlying self-renewal and/or differentiation of spermatogonial stem cells in vitro.

A hallmark of stem cells is to divide infinitely by self-renewal division. Numerous studies
have shown that cells with a high replicative potential in vitro often display many abnormalities. In fact, serial propagation in culture induces chromosomal abnormalities and other degenerative cellular changes, including changes in metabolism, replicative efficiency or growth rate that culminates in apoptosis or tumorigenic behavior (31). Shinohara’s group in 2005 have shown that spermatogonial stem cells have a remarkable stability and proliferative capacity during long term culture. This study revealed that the growth rate of the GS cells was constant during the 2 years in culture and their functional stability was confirmed by production of fertile offspring from cells kept in culture continuously for 2 years. This demonstrates that the functional characteristics of spermatogonial stem cells are fully retained despite their long-term in vitro expansion (32). In normal cells telomeric DNA erodes progressively in the absence of telomerase activity. On the other hand, telomerase activity is associated with immortality in tumor and ES cells (33). In spermatogonial stem cells it has been reported that despite the presence of telomerase activity telomeres shortening occurs during long-term culture (32) Therefore these studies suggested that spermatogonial stem cells have a great stability in culture although the shortening telomeres suggests that these cells have limited proliferative potential (32). Assuming a constant rate of telomere loss, GS cells should continue to proliferate for 34 months before they undergo crisis. This should provide an enough time for in vitro investigation and genetic modification of GS cells for various purposes (32).
4. Cell migration

4.1 The process of cell movement

Cell migration is a highly complex process during many phases of development and adult life. Cells can either migrate as individuals or move in the context of tissues. Movement is controlled both in response to external as well as internal signals, which activate complex signal transduction cascades resulting in highly dynamic and localized remodeling of the actin–myosin cytoskeleton, cell–cell and cell–substrate interactions (34). The actin cytoskeleton is significantly dynamic and the actin structures can be readily reorganized by the cell to adjust their behavior for movement according to the surrounding environment. The regular reorganization of the actin cytoskeleton and the transition from one actin structure to another, enabling the cell to change its properties rapidly, and this dynamic response is essential for cell movement (35). There are different kinds of cell motility, such as the swimming of sperm cells, cell crawling and the movement of some bacteria by the rotation of flagellar motors. Cell crawling, however, is the common mechanism employed by most motile eukaryotic animal cells (36). To better understand these processes, is necessary to analyze the crucial structural cytoskeletal components and their spatio-temporal dynamics (34). The cytoskeleton is a polymer network, composed of three distinct biopolymer types: actin, microtubules and intermediate filaments. Among these, the actin cytoskeleton is the essential machinery that drives cell protrusion, the first step of movement. However the other polymers also help in cell movement and powering translocation (35).

Cell movement, which depends in particular by the constant reorganization of the actin cytoskeleton under the plasma membrane, can be divided into three stages (Fig. 7). First, a cell propels the membrane forward by orienting and reorganizing (growing) the actin network at its leading edge. Second, it adheres to the substrate at the leading edge. Third it deadheres (releases) at the cell body and rear of the cell. Finally, contractile forces, generated
by the acto-myosin network, push the cell forward (35).

At the leading edge of the cells, it can assemble different structures: lamellipodia and filopodia. Lamellipodia are flat sheet-like membrane protrusions, contain a dense network of interconnected actin filaments that form a two-dimensional mesh, with shorter and longer branched filaments, at the front of motile cells. Filopodia, (also microspikes) are finger-like membrane protrusions, which extend from the leading edge of migrating cells. These cytoplasmic projections contain a compact, linear bundle of long, unbranched filaments (37). Together these structures can be used by migrating cells to move forward in its surrounding environment.

It is important to distinguish random cell migration, in which cells migrate in all directions in a no coordinated manner, from oriented cell migration, in which cells respond to polarizing cues to migrate in a given direction (38).

An external signal, that induces oriented cell migration, can be a physical, chemical, diffusible or non-diffusible signal that is detected by receptor proteins located on the cell membrane, and transmitted by them via signaling cascades to the cell interior (39). A cell, such as a white blood cell, or yeast cell, is believed to sense the signal direction by spatially recognizing external gradients (receptor proteins become more concentrated on the side of the cell where the signal is present) (39).

After sensing the signal, the cell starts moving in response to the signal by polymerizing actin. If the signal is a chemoattractant, actin polymerizes in the region of the cell closest to the signal, whereas if the signal is a chemorepellant, the cell moves away by polymerizing actin in the opposite side (35). The continuous formation of new actin network at the leading edge of migrating cells is considered to be essential for pushing the cell forward.
4.2 The actin-binding families of proteins

The actin network can be remodeled with the help of numerous families of proteins. Nucleating proteins (e.g. WASp, arp2/3 complex, ENA/VASP) act to initiate the polymerization and assembly of new actin filaments. Actin depolymerization promoting proteins (e.g. cofilin) can also help network growth. Cofilin (also known as Actin Depolymerizing Factor ADF) severs actin filaments and creates new plus ends for the growth of new actin filaments. Actin binding proteins (e.g. profilin, thymosineβ-4) maintain a stable actin monomer pool for polymerization, while crosslinking and bundling proteins (e.g. filamin, α-actinin, fascin) help form connected actin networks. Capping proteins (e.g. CapZ) control filament length by attaching to actin filament ends and stopping further polymerization, while severing and fragmenting proteins (e.g. gelsolin, severin) cut actin filaments and networks. All these proteins work together to coordinate actin network formation and bring about leading edge motility in several steps, as described previously (Fig. 8) (40).

Another group of proteins seems to play an important role inducing the initial signals leading to the polarization of migrating cells: small G-proteins, Cdc42, Rac and Rho (41). These proteins participate in cell movement by regulating cytoskeleton organization balancing the activities between the front and the rear of the cells (38). Cdc42 and Rac are gradually activated at the front of the cells where they control actin and microtubule reorganization, by the contrast Rho is active at the rear of the cells inducing and controlling rear retraction that allows forward movement (38). The role of Cdc42 is dual because it controls not only the protrusive process but it’s also implicated in cell orientation during directed cell migration in response to exogenous cues such as a chemotactic signal (38).

Cells undergo chemotaxis in response to a diversity of extracellular signals, for example, soluble chemoattractants, cell-matrix and cell-cell interactions and by intracellular signals...
generated by the cell-cycle machinery. This requires polarization of the actin cytoskeleton, which allows cells to protrude at the front and retract at the back (42). The variety of extra- and intracellular signals received by the cell need to be integrated in order to select a single appropriate polarity axis (42). Cdc42 seem to play a role in this integration since is able to interact with different target proteins to control many essential cellular functions (42). An important challenge is to determine how a given stimulus applied to a given cell can direct Cdc42 or other proteins involved in cell movement to recruit and activate the appropriate effectors (42).

4.3 ENA/VASP PROTEINS: regulators of the actin cytoskeleton

ENA/VASP family is one of the several families of actin-binding proteins. One of its members is Vasodilator-stimulated phosphoprotein (VASP). This is a 40 kDa protein, related with cell adhesion, migration and areas of dynamic membrane activity. It was first characterized in 1989 as a major phosphorylated protein in platelets and endothelial cells, following stimulation with vasodilators such as prostaglandins. Evidence is now emerging to suggest VASP, and related proteins, are important components in the cellular machinery responsible for actin filament assembly (43). By SDS-PAGE analysis, VASP migrates with an apparent molecular mass of 46 kDa. Three phosphorylation sites have been identified in human protein, serine-157, serine-239 and threonine-278. Phosphorylation of serine-157 results in a marked conformational change in VASP that decreases its mobility in SDS-PAGE (43). VASP is a major substrate of PKA (protein kinase A) and PKG (protein kinase G). PKG phosphorylates both Ser157 and Ser239 with similar kinetics. Thr278 is phosphorylated by both PKA and PKG. Only phosphorylation of VASP on Ser157, but not on Ser239 or Thr278, results in an apparent mobility shift from 46 to 50 kDa on SDS-PAGE (43, 44).

VASP belongs to the Ena/VASP family, which also consists of Drosophila Enabled (Ena),
**Figure 7** - Schematic representation illustrating the different phases of cell migration

**Figure 8** - Schematic diagram illustrating regulatory proteins involved in actin-cytoskeleton remodeling
mammalian Enabled (Mena), and Ena/ VASP-like proteins (EVL) (45). Members of the Ena/VASP family share a conserved domain structure with an amino-terminal Ena/VASP homology 1 (EVH1) domain and a carboxyl-terminal Ena/VASP homology 2 (EVH2) domain separated by a more variable proline-rich region (PRD) (45). Three conserved motifs are located in the EVH2 domain: a G-actin binding motif (GAB), a F-actin-binding site (FAB), and a coiled-coil motif (CO) essential for tetramerization (46). The EVH1 domain binds to specific poly-Pro sequences in a number of target proteins, including zyxin, vinculin, helping to recruit Ena/VASP at the leading edge of the cell (47). The Pro-rich region binds domains of various signaling proteins as well as profilin, which is the most abundant actin-monomer carrier in the cell (Fig. 9A) (48).

Growing evidence has demonstrated that the Ena/VASP proteins play crucial roles in actin-based cellular processes (47). VASP localizes to region with dynamic actin reorganization, such as leading edge, focal adhesions, cell-cell contacts, actin stress fiber and at the tips of filopodia and lamellipodia (49), where it has been proposed to promote actin polymerization. The proposed model of VASP-mediated elongation suggest that a molecule of VASP attached at the barbed-end of the actin filament, via its F-actin binding FAB domain, this interaction is enhanced by the presence of the CC tetramerization domain. Both the FAB and CC domains are required for Ena/VASP to remain attached at the barbed-end of the filament while actin subunits are being incorporated (50, 47). Than VASP mediates the transition of a profilin–actin complex from the poly-Pro region to the EVH2 (or GAB) domain and then to the barbed-end of the actin filament. At this point, profilin detach from the filament, allowing for the processive stepping of VASP and continued elongation (47). It is commonly assumed that the poly-Pro region of VASP contributes to filament elongation by increasing the local concentration of profilin–actin complexes (Fig. 9B) (51, 47). However, processive filament elongation implies that VASP must remain bound at the barbed end of the filament, compete with capping proteins, and step forward with each addition of an actin subunit (52, 47).

VASP functions as a tetramer, VASP monomers have poor nucleating and elongating
activities (53). The four subunits of VASP probably work in parallel, nucleating the two helical strands of a filament and neighboring filaments to form actin bundles (Fig. 9C) (50, 47).

4.4 Role of Vasp in cell motility and other cellular processes

In mammals, member of the Ena/VASP family have overlapping functions and expression patterns, therefore knock out of individual member results in a minor phenotypes in mouse model. Thus to further investigate the role of these proteins it has been generated an Ena/VASP triple null mouse (54).

Studies on Ena/VASP triple null mice revealed the essential role of the Ena/VASP proteins in endothelial structural integrity and neuritogenesis in the developing cortex (54, 55). These mice exhibit neuronal defect, including exencephaly (55), severe vascular defects, profound edema and hemorrhage which lead to embryonic lethality (54). Ena/VASP proteins are also important in the regulation of cell motility. Studies showed that Ena/VASP proteins are critical in efficient movement of the pathogenic bacterium *Listeria monocytogenes*. It has been demonstrated that listeria use the host cell’s actin cytoskeleton to move within and from cell to cell (56). Within living cells, listeria speeds are reduced in absence of Ena/VASP proteins. In contrast, Ena/VASP negatively regulates fibroblast motility by producing longer and less-branched actin filaments in lamellipodia (52). Therefore it is important to consider that Ena/VASP proteins probably have different role in different contest.

It is well-established that VASP is phosphorylated by both PKA and PKG, and it has been suggested that phosphorylation at either Ser157 or Ser239 serves as an important regulatory mechanism. As previously described, VASP has been demonstrated to regulate cell-morphology change, filopodia formation, cell motility, and chemotaxis (46). Yet, it is not clear how VASP phosphorylation regulates these events.
In a recent paper it has been demonstrated the VASP phosphorylation plays a negative role in filopodia protrusion, in particular in VASP null cells decreases the number of filopodia while it does not affect the length of filopodia (46). Furthermore VASP phosphorylation is important for its localization to the membrane cortex and interaction with different proteins as well as Wasp (Wiskott Aldrich Syndrome Protein, nucleating factor) and WIPa (Wasp interactive proteins) (46). As recently shown phosphorylation of VASP increases its binding to WIPa (46), it is more probably that this interaction plays an important role for the translocation of VASP in specific area of cortical membrane. On the contrary VASP may be recruited by several proteins such as spectrin, AbI and src, that interact directly with VASP, but phosphorylation abolishes the interaction between VASP and these proteins (46, 57).

Ena/VASP proteins have been proposed to act as anti-capping proteins. This activity is necessary to allow the formation of long actin filaments that contribute to structures such as filopodia. The anti-capping hypothesis of Ena/VASP function assumes that these proteins, associate with elongating actin filaments, block the action of capping proteins that terminate the elongation (58). In vitro assays demonstrated that VASP phosphorylation at Ser239 or Thr278 decreases its anti-capping and filament building activity and its ability to bind F- and G-actin. By contrast phosphorylation at Ser 157 does not affect interaction with actin, but is involved in activities such as filopodia formation (59).

Thus, it is clear that differential phosphorylation of VASP, at each of the residues, modulates its function and its specific role in different processes.

Recently it has been demonstrated that Vasp interact directly with a chemokine CXCR2 implicated in the recruitment of the leucocytes during inflammation (60). The interaction between VASP and CXCR2 is directed and enhanced by CXCL8 stimulation that induces VASP phosphorylation on Ser 157. The interaction of VASP and CXCR2 represents a direct link between the receptor and the actin cytoskeleton. This establishes a novel and important role for VASP in translating the extracellular chemokine gradient to intracellular polarization cues (60).
Figure 9 - Schematic illustration of VASP structure and function.
A) Domain organization of VASP (EVH1, Ena/VASP homology domains 1; poly-Pro sequence; GAB and FAB, globular- and filamentous-actin binding domain; CC, coiled-coil). B) Schematic representation of the proposed model of VASP-mediated elongation. VASP attaches at the barbed-end of the actin-filament, via its FAB domain, then profilin mediates the transition from a poly-Pro region to the GAB domain and then to the barbed-end of the filament. Finally, profilin, detach from the filament, and VASP continued elongation. C) VASP acts as tetramer, probably the four subunits of VASP work in parallel, nucleating the two helical strands of actin-filaments.
AIMS OF THE PRESENT STUDY

Recently it has been demonstrated, in our laboratory and by other group that undifferentiated spermatogonia are a heterogeneous population. Analysis of whole-mounted seminiferous tubules from mouse adult testis, in which we employed an anti-GFRA1 antibody and an anti-Plzf antibody in double-staining, showed that not all the As were positive for GFRA1 expression. Consistently a small fraction (around 10%) of all As cells were negative for GFRA1 expression. We also found that GFRA1-negative cells are colonogenic in vivo, whereas GFRA1-positive cells are not colonogenic (16). Furthermore others group have shown that As spermatogonia are heterogeneous also for expression of NGN3, NANOS2 and NANOS3 (17, 18).

Thus, the aim of the present project is to further characterize the As GFRA1-positive and negative subsets. During our analysis of whole mounted seminiferous tubules, we noticed that GFRA1 positive As and Apr spermatogonia show a particular morphology, with lamellipodia and filopodia-like structures, typical of migrating cells. Several evidence indicate that GDNF stimulates migration and chemotaxis in different cell types, such as epithelial cells (61) and enteric neural cells (62). Therefore, we have hypothesized that GDNF acts as a chemoattractant for undifferentiated spermatogonia, stimulating directional migration of these cells. Cell morphology is largely determined by the organization of internal structural elements, including the filamentous structures of cytoskeleton and variations in cell morphology are consequences of changes in cytoskeletal organization and dynamics. For instance, cell migration is a polarized process where remodeling of the actin cytoskeleton plays a central role. The actin cytoskeleton can be remodeled by many different families of proteins, including the enabled/vasodilator stimulated phosphoprotein Ena/Vasp family and Wiskott-aldrich syndrome protein (Wasp). Therefore to gain more insight into the migratory morphology of GFRA1 expressing spermatogonia, we investigate the expression pattern of proteins involved in actin remodeling in undifferentiated spermatogonia. To further analyze
cell heterogeneity and the migrating phenotype of GFRA1-positive cells in a more amenable model we will develop spermatogonial stem cell cultures.
MATERIALS AND METHODS

5.1 Germ cells preparation

In all of the experiments, germ cells were obtained from adult C57BL/6 mice. Briefly, testis were removed, decapsulated and subjected to enzymatic digestion under shaking at 37°C. Two sequential digestion steps were performed in D-MEM containing 1mg/ml collagenase type IV, 0,2mg/ml Dnase I and Trypsin 0,25% for 15 and 5 min, respectively. Trypsin was blocked with 20% FBS. The cells were then centrifuged at 1000rpm for 5’ resuspended in DMEM and counted.

5.2 Thy1-positive cells isolation

Cell sorting was performed by Magnetic Activated Cell Sorter (MACS, Miltenyi Biotech). Germ cells were selected by Macs according to manufacturer’s instructions. Cells were incubated for 30minutes at 4°C with rat anti-mouse thy-1-PE antibody. After washing the cells were incubated 20 minutes at 4°C with beads anti-PE. After washing, cells were separated on a column placed in the magnetic field of a Macs separator. Thy-1 positive and negative cell fractions were collected, centrifuged at 1000rpm for 5min and resuspended in D-MEM for counting. Then Thy-1 positive cells were used for migration assays.

5.3 Cell Migration assay

Migration assays were carried out using a modified Boyden chambers, to assess cell migration through a polyvinilpirrolidone filter (pore size 5-μm, Whatman International Ltd.). To evaluate the migration potential, Thy-1 positive cells (about 50 000/well) were plated into the upper chamber in D-MEM + 0,1% BSA, while the bottom chamber was filled with D-MEM
+ 0,1% BSA containing 0,1μg/ml GDNF. Cultures were carried out in an humidified incubator at 37°C and 5% CO₂ for 5 h. After a 5h-incubation all cells were carefully removed from the top of the filter using a cotton swab and the remaining cells on the bottom side of the filter, were fixed with 4% paraformaldeide, stained with hematoxylin and then counted. About 50 fields were counted at 40x. Since inspection of medium in the bottom chamber didn’t reveal the presence of any cells, the number of stained cells on the underside of the filter was considered as migrated Thy-1 positive cells.

5.4 Quantitative Real-Time PCR

Tubules from an adult mouse were seeded in 12-well culture plates with DMEM supplemented with antibiotics, Lglutammin, non essential amminoacid, Hepes pH 7.7, gentamicin and GDNF (20, 50 and 100 ng/ml) for 20h. In order to quantify relative expression levels of selected genes, total RNA was isolated, purified and quantified from the tubules using TRIzol reagent (Sigma, St. Louis, MO). Then gene expression was evaluated by Real-Time PCR analysis. 1 μg of total RNA/sample were used for cDNA synthesis, with primer random and Transcriptor Reverse Transcriptase (Roche Applied Science). Then, cDNA produced in each reaction was used for each Real-Time reaction; analysis was performed in triplicate for each sample. cDNA was mixed with 0.3 μM of both forward and reverse primers mix and 10 μl of 2x Master mix (FluoCycleTMII SYBR Green Mix, Euroclone) to a final reaction volume of 20 μl. Reactions were performed on Opticon2 DNA Engine (MJ Research). cDNA levels were standardized by normalising to the β-actin control. The primers used to detect VASP were ordered from PrimerDesign (Eppendorf), while primers to detect Bcl6b were designed and evaluated using the PRIMER3 program and ordered from MWG Oligo Synthesis. Data were presented as fold increase compared to basal level of expression.
5.5 Whole mount immunofluorescence

Seminiferous tubules from adult C57BL/6 mice were fixed in 4% paraformaldehyde at 4°C for 3h. Briefly, tubules were washed in PBS +1%BSA + 0.1% triton X-100(PBT) and incubated 10min at RT with glycine 1M to reduced aldehyde-caused fluorescent background. Unspecific antibody binding was prevent by incubating tubules with 5% normal donkey serum in PBT for 2h at RT, then incubated O/N at 4°C with mouse monoclonal anti-PLZF antibody, goat anti-mouse GFRA1 (Calbiochem, Neuromics, respectively), rabbit anti-VASP, anti-mDIA, anti-profilin, respectively. After washing, tubules were incubated in PBT with FITC-conjugated donkey anti-mouse, cy5-conjugated donkey anti-goat and Cy3-conjugated donkey anti-rabbit secondary antibodies (Jackson Laboratories), for 1h at RT. After washing, nuclear staining was performed with Hoechst 33342 (0,1μg/ml) or TOTO3(0,2μg/ml) for 10 min at RT. Tubules have been then washed in PBS and mounted with Vectashield medium (Vector). Finally whole mounted tubules have been observed on a confocal microscope (Leica).

5.6 Western blot analysis

Seminiferous tubules were plated on 12 well-plate with D-MEM supplemented with antibiotics, L-glutamin, non essential amminoacid, Hepes pH 7.7 , gentamicin and GDNF 100 and 50ng/ml at 37°C for 24 h. After that tubules were spinned and protein extracts were prepared by homogenizing the pellet in Lysis buffer ( 50 mM Hepes pH 7.4, 2 mM EGTA, 15 mM MgCl₂, 1% TritonX-100, 120 mM NaCl, 12% glycerol, 1mM dithiothreitol and a protease inhibitor mixture). Proteins from cell extracts (50 μg) were resolved on 10% SDS-PAGE and transferred to a nitrocellulose membrane. For immunoblotting, nitrocellulose membranes were blocked with TBS containing 0.05% Tween-20 and 5% BSA incubated with rabbit anti-VASP antibody (1:1000) or mouse anti-tubuline antibody (1:1000). Membranes were washed
three times for 10 min with TBS containing 0.05% Tween20 and then incubated with anti-rabbit IgG or anti-mouse antibodies peroxidase conjugate (1:4000; Santa Cruz Biotechnology). After three washes, staining was revealed by enhanced chemiluminescence (ECL; Santa Cruz Biotechnology). Aida 2.0 software were used to measure the band intensity of phosphorylated and total VASP.

5.7 Spermatogonial stem cell cultures

To obtain spermatogonial stem cells cultures, testes were collected from 7-old mice DBA and EGFP/DBA. Dissociated testis cells were plated on 0.2% gelatin-coated 12well- plate. Culture medium for the testis cells was StemPro-34 SFM (Invitrogen) supplemented with StemPro supplement (Invitrogen), 25 µg/ml insulin, 100 µg/ml transferrin, 60 µM putrescine, 30 nM sodium selenite, 6 mg/ml D-(1)-glucose, 30 µg/ml pyruvic acid, 1 µl/ml DL-lactic acid (Sigma), 5 mg/ml bovine albumin (Biomedicals), 2mM L-glutamine, 5 x10⁻⁵ M 2-mercaptoethanol, minimal essential medium (MEM) vitamin solution (Invitrogen), MEM nonessential amino acid solution (Invitrogen), 10⁻⁴ M ascorbic acid, 10 µg/ml d-biotin, 30 ng/ml β-estradiol, 60 ng/ml progesterone (Sigma), 20 ng/ml mouse epidermal growth factor (Becton Dickinson, Bedford, MA), 10 ng/ml human basic fibroblast growth factor (Becton Dickinson), 10⁻³ U/ml ESGRO (murine leukemia inhibitory factor), 10 ng/ml recombinant rat glial cell line-derived neurotrophic factor (GDNF) (R&D Systems,) and 1% fetal calf serum. The cells were maintained at 37°C in an atmosphere of 7.5% carbon dioxide in air (24). Cells were cultured on mitomycin C-inactivated mouse embryonic fibroblasts(MEF) and passaged every 5–7 days to fresh MEF at a one-one to one-two dilution. Next we cultured these cells also on laminin (Sigma) feeder plated on 12-well plate (2μg/µl), to further enrich this population.
5.8 Statistical analysis

The migratory assays were performed in triplicate and repeated three times. All results are reported as means ± standard error (SE). Statistical significance of difference between mean values was assessed using Sigma plot. A p value ≤0.05 was considered significant.
6.1 GFRA1-positive spermatogonia show a migrating phenotype

In order to get more insight on the physiological meaning of the different subsets of As spermatogonia we decided to further investigate morphology of the different subsets. To this end we performed whole mounted immunofluorescence on isolated tubules from mouse adult testis in which we employed an anti-GFRA1 antibody (Fig. 1). By confocal microscopy analysis, we noticed that GFRA1 positive As, Apr and some Aal spermatogonia showed a particular polarized morphology, with lamellipodia and filopodia-like structures (Fig.1A). Lamellipodia and filopodia are present at the front, leading edge, of motile cells, and are considered to be the motor which pulls the cells forward during cell migration. This phenotype is typical of migrating cells, in which a front-rear polarization occurs. Confocal analysis of whole-mounted seminiferous tubules, revealed that GFRA1 positive spermatogonia have particular shapes in which, sometimes, it is possible recognize a front-rear organization, with long protrusions and extended membrane ruffling (Fig. 1E-I). Among all GFRA1-expressing cells we observed this phenotype particularly in As and in Apr spermatogonia. GFRA1 is the co-receptor for GDNF, the growth factor produced by Sertoli cells. Intriguingly it has been demonstrated that GDNF is able to induce directional migration in different cell types such as enteric neural and epithelial cells (61, 62). We therefore next hypothesized that GFRA1-positive spermatogonia may migrate in response to a GDNF gradient.
Figure 1 - Localization of GFRA1 reveals a polarized morphology in undifferentiated spermatogonia

Whole-mount seminiferous tubules isolated from adult testis were stained with anti-PLZF (green), anti-GFRA1 (red) antibodies and TOPRO-3 nuclear staining (blue). Confocal analysis show that GFRA1 positive spermatogonia have particular shapes in which it is possible recognize a front-rear organization, with long protrusions (1E) and extended membrane ruffling (1I).
6.2 GDNF exert chemoattractant action on undifferentiated spermatogonia

To test the hypothesis that undifferentiated spermatogonia respond to a GDNF gradient we used two different experimental approaches: 1) freshly selected Thy-1 spermatogonia from adult testis and 2) spermatogonial stem cell culture. In the first approach undifferentiated spermatogonia were selected using Thy-1 antibody in MACS experiments. The Thy-1-expressing cell population includes GFRA1-positive cells (16). Selected cells were employed in directional migration assays using a modified Boyden chambers. To create a positive gradient for the putative chemoattractant, the upper compartment was filled with D-MEM+0.1% BSA only, while the lower chamber contained D-MEM+0,1% BSA with GDNF (0,1μg/ml). The results showed that, consistent with our hypothesis, after 5h of incubation, a significant higher fraction of Thy-1 positive cells were able to migrate in the presence of GDNF when compared to controls without GDNF. As a control, we tested if the anti-GDNF antibody was able to block GDNF activity on Thy-1-positive spermatogonia (Fig. 2). Addition of the blocking antibody along with GDNF inhibited the migration of cells at a level comparable to the control. We also tested the activity of SDF1 a small cytokine that acts as a chemoattractant for different cell types and is involved in hematopoietic progenitor cell trafficking to the bone marrow (63). We found that cell migration in the presence of SDF1 was not significantly different from controls.

In a second series of experiments we derived spermatogonial stem cell culture from neonatal mice using published protocols (Fig. 3A,B) (24). Cells were routinely cultured on mouse embryonic fibroblasts (MEF) in presence of GDNF, bFGF, EGF, LIF and FBS (24, 7). However, for migration experiments cells were kept two weeks on laminin-treated plate, in order to obtain a pure population of undifferentiated spermatogonia, without contaminating MEF (Fig.3B). Cells were used in migration experiments in the same conditions described above. Again, we observed that a significant higher fraction of spermatogonial stem cells were able
to migrate across the membrane pores in presence of GDNF when compared to controls in the absence of GDNF. Addition of the blocking antibody along with GDNF inhibited the migration of cells at a level comparable to the control (Fig. 3C).

These data shown for the first time, that GDNF can stimulate directional migration of undifferentiated spermatogonia. This indicates that besides controlling the rate of self-renewal and differentiation, GDNF may play alternative role in the undifferentiated spermatogonia cell compartment.

6.3 Undifferentiated spermatogonia express VASP.

Cell movement is controlled by internal and external signals, which activate intricate signal transduction cascades resulting in greatly dynamic and localised remodeling of the cytoskeleton, cell–cell and cell–substrate interactions (34).

Since Boyden chamber experiments demonstrated that GDNF treatment induces cell migration it could be speculated that GDNF causes an active remodeling of the cytoskeleton in target cells. To analyze the connection between GDNF treatment and cytoskeleton rearrangements, we sought to determine which proteins, involved in the cellular migration and leading to reorganization of actin cytoskeleton, are expressed by undifferentiated spermatogonia. The expression profile of candidate genes were studied by whole mount immunofluorescence experiments on isolated tubules. Tubules were double immunostained with anti-PLZF and with anti-profilin, anti- mDia or anti-VASP antibodies respectively and were analyzed by confocal microscopy (Fig. 4). We found that profilin and mDia were homogeneously expressed by all seminiferous tubule cells (Fig. 4A,B), whereas, surprisingly VASP expression was restricted to undifferentiated spermatogonia (Fig. 4C). VASP is a member of ENA/ VASP family implicated in many cellular functions, including cell adhesion, neuronal and fibroblast migration.
Figure 2 - GDNF stimulation induces migration of freshly selected Thy-1-positive spermatogonia

Histogram show the number of migrated Thy-positive spermatogonia in the absence or presence of GDNF, GDNF plus blocking antibody and SDF1. A significant higher fraction of Thy-1 positive cells is able to migrate in presence of GDNF when compared to controls or SDF1.
Figure 3 - GDNF induces spermatogonial stem cells migration

(A-B) Morphological appearance of spermatogonial stem cell cultures. Morula-like clusters growing on MEF feeder layer in (A) and chained-clusters growing on laminin in (B). (C) Histogram show number of migrated in the absence or in the presence of GDNF and GDNF plus blocking antibody. A significant higher fraction of spermatogonial stem cells is able to migrate in presence of GDNF when compared to controls.
Figure 4 - Expression pattern of mDia, VASP and profilin in adult seminiferous tubules

Whole-mounted adult seminiferous tubules stained with anti-PLZF (green), anti-mDia (red in A), anti-PROFILIN1 (red in B), anti-VASP (red in C) antibodies and TOPRO-3 nuclear staining (blue). Confocal analysis reveals that mDia and profilin are homogeneously expressed by all cells in seminiferous tubules, whereas VASP is expressed only by undifferentiated spermatogonia.
Western blot analysis of proteins extracted from seminiferous tubules and separated by SDS-PAGE, revealed a doublet of bands at 46-50 kDa confirming VASP expression, both in phosphorilated and unphosphorilated forms (Fig. 5A). We next investigated VASP expression during post-natal testis development. Western blot analysis revealed that VASP is expressed throughout testis development, but its expression levels decrease in adult testis when compared to immature testis (Fig. 5B,C). We next characterize the expression profile of VASP within the undifferentiated spermatogonia compartment by double immunostaining with PLZF and GFRA1. Morphological analysis of double stained tubules revealed that VASP staining decorated large lamellae structures and also long protrusion compatible with retraction fibers (Fig. 6A). In most of the polarized cells, cell arrangement revealed a clear front-rear organization (Fig. 6B,C). We found that VASP expression levels positively correlate with those of GFRA1 particularly in As and Apr spermatogonia (Fig. 7). Next, we investigated VASP expression in spermatogonial stem cell cultures (Fig. 8). We observed that VASP was expressed in all cells and localized in the cytoplasm.

6.4 GDNF regulates VASP expression levels

Immunolocalitation experiments indicated that VASP expression positively correlated with GFRA1. Since GFRA1 is the co-receptor for GDNF we hypothesized that VASP transcriptional levels are regulated by GDNF itself. To test this hypothesis we isolated seminiferous tubules from adult testis. In intact tubules cell-cell interactions are preserved and undifferentiated spermatogonia are kept in their natural microenvironment. Tubules isolated from adult testis were cultured with increasing concentrations of GDNF (20, 50 and 100 ng/ml) for 18-20h. At the end total RNA was isolated and gene expression was evaluated by real-time PCR (Fig. 9). The results obtained indicated that VASP mRNA was up-regulated by GDNF in a dose-dependent fashion. We next asked whether GDNF may regulate VASP in a post-transcriptional fashion. Tubules were treated for 18 hrs with increasing concentration of
GDNF and extracted proteins were analyzed by Western blot analysis. A protein doublet was detected in all the samples and densitometry revealed that long-term GDNF stimulation increased VASP levels, both in unphosphorylated and phosphorylated form (Fig. 10). We next performed a short-term time-course using GDNF at 100 ng/ml. As positive control for VASP phosphorylation tubules were treated with forskolin, a cAMP-elevating agent that cause VASP phosphorylation through PKA. We found that in intact tubules VASP levels rapidly increased after GDNF treatment reaching a maximum at 10 min and decreased thereafter reaching control levels at 30 min (Fig. 11). Altogether, these data indicate that VASP is regulated by GDNF both at transcriptional and post-transcriptional level.
Figure 5 - Western blot analysis of VASP
Western blot analysis for VASP expression in isolated adult seminiferous tubules (A) and in testis at different ages of postnatal development (B). Fifty micrograms of proteins for each sample were subjected to SDS-PAGE, western blotted, and probed with anti-VASP antibody that recognize both VASP and P-VASP. (C) Densitometric quantification of the blot from (B). OD values were obtained by scanning densitometry and data were normalized against tubulin values. DPP, day post partum.
Figure 6 - Expression pattern of VASP, PLZF and GFRA1 in undifferentiated spermatogonia

Whole-mounted adult seminiferous tubules stained with anti-PLZF (green), anti-VASP (red in A; green in B and C) and anti-GFRA1 (cyan) antibodies. A) Morphological analysis reveals that VASP staining decorated large lamellae structures (white arrow) and also long protrusion compatible with retraction fibers (dashed arrow). (B-C) Cells are highly polarized with a clear front-rear organization (dashed arrow).
Figure 7 - Expression pattern of VASP and GFRA1 in undifferentiated spermatogonia
Whole-mounted adult seminiferous tubules stained with anti-VASP (green) and anti-GFRA1 (cyan) antibodies. Morphological analysis reveals that VASP expression levels positively correlate with those of GFRA1 particularly in As and Apr spermatogonia.

Figure 8 - Expression pattern of VASP and GFRA1 in spermatogonial stem cell cultures
Stem cell cultures were stained with anti-VASP (green), anti-GFRA1 (red) antibodies and TOPRO-3 nuclear staining (blue). Morphological analysis reveals that VASP is expressed in all cells and localizes in the cytoplasm.
Figure 9 - Regulation of VASP expression by GDNF
Real Time PCR analysis shows that GDNF stimulates dose-responsive increase in VASP mRNA levels at doses from 0 to 100 ng/ml. Expression of Bcl6b was included as positive internal control. Data are expressed as mean ± SD.
Figure 10 - Long-term post-transcriptional regulation by GDNF

(A) Western blot analysis of proteins extracted from seminiferous tubules treated in vitro with increasing concentration of GDNF for 18h (B) Densitometric quantification of the blot presented in (A). OD values were obtained by scanning densitometry and data were normalized against tubulin values. Long-term GDNF stimulation increases VASP levels, both in unphosphorylated and phosphorylated forms. Data are expressed as mean ± SEM from n=4 experiments.
Fig 11 - Short-term post-trascriptional regulation by GDNF

(A) Western blot analysis of proteins extracted from seminiferous tubules treated for increasing time with GDNF at 100 ng/ml (B) Densitometric quantification of the blot presented in (A). OD values were obtained by scanning densitometry and data were normalized against tubulin values. VASP levels rapidly increases after GDNF treatment reaching a maximum at 10’ and decreases thereafter reaching control levels at 30’. FSK, a cAMP-elevating agent that cause VASP phosphorylation, was included as positive internal control. Data are expressed as mean ± SEM from n=2 experiments.
DISCUSSION

In this study we have obtained morphological and functional evidence that the subset of GFRA1-expressing As and Apr express a migrating phenotype. A large body of evidence demonstrated that GDNF is important to regulate self-renewal and differentiation of spermatogonial stem cells both in vivo and in vitro. Disruption of one GDNF allele in the mouse causes depletion of SSCs reserves, resulting in testes that contain only the supporting Sertoli cells. On the other hand the testis-specific overexpression of GDNF causes accumulation of clusters of undifferentiated spermatogonia (6). Shinohara’s group showed that the presence of GDNF in culture medium is necessary to maintain spermatogonial stem cell activity in vitro (29, 7). Despite GDNF represent the most analyzed factor regulating the fate and the behavior of spermatogonial stem cells, until now its functions are incompletely understood. By analyzing the expression pattern of GFRA1 (the co-receptor for GDNF) expression we found positive and negative subsets of spermatogonial stem cells. During our analysis of whole mounted seminiferous tubules we noticed that GFRA1 expressing As and Apr spermatogonia, showed a particular morphology reminiscent of migrating cells. Interestingly, several evidence in the literature indicated that GDNF stimulates directional migration of different cell types. Dressler et al. in 1998 demonstrated that epithelial RET-expressing cells migrated towards a source of GDNF and that the activation of RET pathways resulted in increased cell motility (61). GDNF induced directional migration of enteric neural cells (62). Thus we investigated whether GDNF acted as a chemoattractant for undifferentiated spermatogonia, stimulating directional migration of these cells. Our results indicate that GDNF is able to significantly induce migration of freshly isolated Thy-1-positive spermatogonia that includes GFRA1-expressing spermatogonia. This effect was specific because it was completely reverted by a blocking anti-GDNF antibody. We show also that GDNF is also able to induce migration of spermatogonial stem cells maintained in culture.
Therefore, these new evidence, suggest for the first time that GFRA1-positive spermatogonia migrate in response to GDNF.

The migrating behaviors of undifferentiated spermatogonia was first proposed and observed by other groups (23, 22) When the position occupied by undifferentiating spermatogonia on the basal compartment of seminiferous epithelium was investigated, Russel’s group, firstly, and Yoshida’s group subsequently, observed that during their differentiation undifferentiated spermatogonia migrate away from areas of the seminiferous epithelium closer to the intestitium and the blood vessels (23,22). Based on this observation it was proposed that these areas could represent the niches for the spermatogonial stem cells and therefore that cell migration could be necessary for the differentiating spermatogonia to reach different areas of the seminiferous tubule. A recent study has addressed the spermatogonial stem cells behavior in order to understand how the stem cells compartment ensures tissues homeostasis (64). This study indicates that during the mouse life span, SSCs frequently and stochastically loses their self-renewal capacity and are replaced from neighboring stem cells. This behavior implies that stem cells migrate to repopulate the empty niches in the seminiferous tubule and therefore stem cells compartment must not be a static population (64). Thus, if the conclusions of this study are correct, stem cell migration is fundamental to maintain stem cells number necessary to sustain spermatogenesis. In this line of reasoning GNDF may maintain self-renewal of the stem cell compartment allowing migration of GFRA1-expressing stem/progenitor cells to replenish depleted niches. This fascinating hypothesis deserves further investigations.

Since cellular movement involves cytoskeleton remodeling necessary to arrange lamellipodia and filopodia structures, in the present study we have investigated which actin-binding regulating protein could be specifically expressed by GFRA1-positive spermatogonia. We found that undifferentiated spermatogonia expressed VASP (Vasodilator stimulated phosphoprotein) a member of ENA/VASP family implicated in many cellular processes such as cell adhesion, neuronal and fibroblast migration. Whole mount immunofluorescence
revealed that in the seminiferous tubules VASP was expressed only by undifferentiated spermatogonia where it decorated large lamellipodia-like structures. Interestingly, among differentiated spermatogonia, VASP expression levels directly correlated with those of GFRA1. Moreover, we found that As and Apr spermatogonia showed higher VASP expression levels compared to Aal spermatogonia whose VASP expression progressively decreased and finally disappeared in differentiated spermatogonia. Consistent with these observations, Western Blot analysis performed on young and adult testes revealed that VASP (both phosphorilated and unphosphorilated forms) was higher in immature testes compared to adult testes. In fact during postnatal testis development, the undifferentiated spermatogonia are outnumbered by more differentiated germ cells and therefore any protein specific for the undifferentiated spermatogonia compartment result less expressed in adult compared to immature testis.

The direct correlation of VASP and GFRA1 expression levels brought us to hypothesize a direct regulation of VASP by GDNF. We found that VASP (both phosphorilated and unphosphorilated) was regulated by GDNF both at transcriptional and post-transcriptional level as revealed by qRT-PCR and Western Blot analysis. It should be pointed out that at the present we do not have experimental evidence for a VASP involvement in the GDNF-induced spermatogonia migration. However, since GDNF directly regulates VASP in target cells we proposed a possible mechanistic link between GDNF and VASP. VASP could be implicated in actin assembly necessary to organize lamellipodia structures in GDNF-target-GFRA1-positive spermatogonia (49). VASP activation in target cells by GDNF could be also implicated in cell-cell adhesion or in cell-substrate interaction. To gain insight in the functional link among GDNF, VASP and spermatogonia migration we are currently exploring the impact of VASP knockdown by RNAi in spermatogonial stem cells on different parameters such as cell migration, in vivo colonogenic activity, etc.

In conclusion, we found for the first time that GFRA1-expressing spermatogonia express a migrating phenotype. GDNF is able to induce directional migration in both freshly isolated
and in vitro maintained spermatogonia. Among candidate actin-binding proteins we found that VASP is specifically expressed in undifferentiated spermatogonia including As and Apr. Interestingly, GDNF regulates VASP at transcriptional and post-transcriptional levels and we propose that VASP may mechanistic mediate GDNF-induced migration. These data suggest that GDNF chemoattractant function may impinge on SSCs self-renewal/differentiation in vivo.
BIBLIOGRAPHY


cells. The journal of cell biology 142, 1337-45.

