Genes involved in spermatogonial stem cell self-renewal and differentiation

Maaike Petronella Antonia van Bragt

Promotiecommissie

Promotor	Prof. dr. D.G. de Rooij
Co-promotor	Dr. A.M.M. van Pelt
Overige leden	Prof. dr. B. Colenbrander Dr. B.J.L. Eggen Prof. dr. S.J.L. van den Heuvel Prof. dr. L.H.J. Looijenga Prof. dr. C.L. Mummery

Paranimfen

W. van Dijk
Dr. H. de Jonge

ISBN: 978-90-393-4827-7

Printed by: PrintPartners Ipskamp, Enschede

Layout & cover: Yuri J. van Overmeeren en Maaike P.A. van Bragt

The research described in this thesis was performed at the department of endocrinology, university of Utrecht, the Netherlands and at the Center for Reproductive Medicine, Academic Medical Center, Amsterdam, the Netherlands. Under supervision of Dr. A.M.M. van Pelt and Prof. Dr. D.G. de Rooij.

Genes involved in spermatogonial stem cell self-renewal and differentiation

Genen betrokken bij de zelfvernieuwing en differentiatie van spermatogoniale stamcellen

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van
de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. J.C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op maandag 16 juni 2008 des ochtends te 10.30 uur

door

Maaike Petronella Antonia van Bragt

geboren op 27 maart 1976
te Helmond

Promotor:	Prof.dr. D.G. de Rooij
Co-promotor:	Dr. A.M.M. van Pelt

Table of Contents

Chapter 1 7
General introduction
Partly published in APMIS. 2005 Nov-Dec; 113(11-12):727-42

Chapter 2 31
LY6A/E (SCA-1) expression in the mouse testis
Biol Reprod. 2005 Oct; 73(4):634-8

Chapter 3 45
Expression of pluripotency marker, UTF1, is restricted to a subpopulation of early A spermatogonia in rat testis
Accepted reprod. Apr 2008

Chapter 4 63
Controlled differentiation of spermatogonial stem cell line reveals genes involved in early steps of spermatogonial differentiation
Manuscript in preparation

Chapter 5 79
Activin A induced differentiation of a spermatogonial stem cell line indicates role for CXCL12 in regulating spermatogonial stem cell fate
Manuscript in preparation

Chapter 6 93
Summarizing discussion

Summary & Samenvatting 111

Color figures, acknowledgement, publications & curriculum vitae . 117

Chapter 1: General Introduction

Parts of this chapter were published in:
Spermatogonial stem cells: characteristics and experimental possibilities
Aponte P.M.*, van Bragt M.P.A.*, de Rooij D.G. and van Pelt A.M.M.
APMIS. 2005 Nov-Dec; 113(11-12):727-42.

* These authors contributed equally to this review

Part 1: Spermatogenesis

As early as 1677, Antoni van Leeuwenhoek (1632-1723) described the presence of small tadpole-like creatures in human semen which were named "animalcules" or "spermatick worms" [1, 2]. A few years later, in 1695, Nicolas Hartsoeker (1656-1725) suggested that within the head of these animalcules a preformed fetus was present, the homunculus (Fig.1) [1]. We now know that these "animalcules" are the haploid spermatozoa and that these cells can only form a new life after fusing with a female haploid egg.

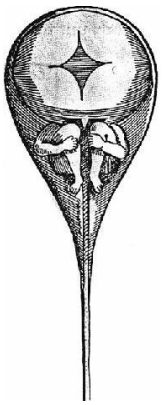


Figure 1: Homunculus.

Male fertility requires the production of large numbers of these highly specialized cells and in order to maintain this production throughout adult life, new spermatozoa are constantly formed by a process called spermatogenesis. At the base of this process is a pool of stem cells, the spermatogonial stem cells [3, 4].

A stem cell is an undifferentiated cell that is defined by its ability to proliferate indefinitely and its ability to produce daughter cells which can terminally differentiate into particular cell types [5]. Totipotent, pluripotent, multipotent and unipotent stem cells are distinguished depending on the ability to differentiate in different cell types [6, 7]. The zygote (fertilized egg) is totipotent as it can give rise to all embryonic and extra-embryonic cell types. An embryonic stem cell is a pluripotent stem cell as it can differentiate into all cell types present within the body. An example of a multipotent stem cell is the hematopoietic stem cell which can differentiate in all different types of blood cells. The spermatogonial stem cells are unipotent stem cells as they can only differentiate into spermatozoa [5-7].

The process of spermatogenesis starts with the mitotic division of a spermatogonial stem cell. This mitotic division results either in the formation of two new spermatogonial stem cells (self-renewal) or in the formation of two daughter cells that are committed to differentiate into spermatozoa eventually (spermatogonial stem cell differentiation) [8]. These committed spermatogonia thereafter continue the process of spermatogenesis which can be roughly divided into three phases. 1) The proliferative phase, during which spermatogonia undergo rapid successive divisions to increase the number of germ cells. 2) The meiotic phase, in which the DNA is recombined and segregated, and 3) the differentiation or spermiogenic phase in which the germ cells transform into cells that are able to reach and fertilize the egg [9]. The whole process of spermatogenesis is a tightly regulated and complex process of which, 330 years after the first report by van Leeuwenhoek, still many aspects are not fully understood.

The testis

Spermatogenesis takes place in the testis (plural: testes). The testis is surrounded by a capsule of connective tissue, called tunica albuginea. Unlike in the human, where testes are partitioned by so-called septa, testes of rat and mouse contain very little intertubular connective tissue [9]. Inside the testis two major compartments can be found: the interstitium or interstitial tissue and the seminiferous tubules (Fig.2a). The interstitium contains macrophages, blood and lymphatic vessels, innervation, Leydig cell precursors and adult Leydig cells. The seminiferous tubules contain the germ cells and the Sertoli cells [9].

Seminiferous tubules, first described in 1669 by Reinier de Graaf (1641-1673), are coiled and at both ends connected to the rete testis [9, 10]. On average one mouse testis contains about 2 meters of seminiferous tubules, a rat testis about 17,4 meters [11, 12]. Seminiferous tubules are limited by complex membranes, which consist of two concentric lamellae of non-cellular material which enclose the peritubular myoid cells [13]. These peritubular myoid cells are smooth muscle cell-like. Contractions of these cells induce frequently recurring movements of the seminiferous epithelium, which are thought to play a role in the release of spermatozoa from the epithelium and their transport to the rete testis [13, 14].

Within the seminiferous tubules the germ cells are supported by Sertoli cells (Fig.2b). The Sertoli cells are named after Enrico Sertoli (1842-1910) who in 1865 described the possibility of a functional relationship between the "branched cells" and the production of spermatozoa [15]. Sertoli cells are situated on the basal membrane

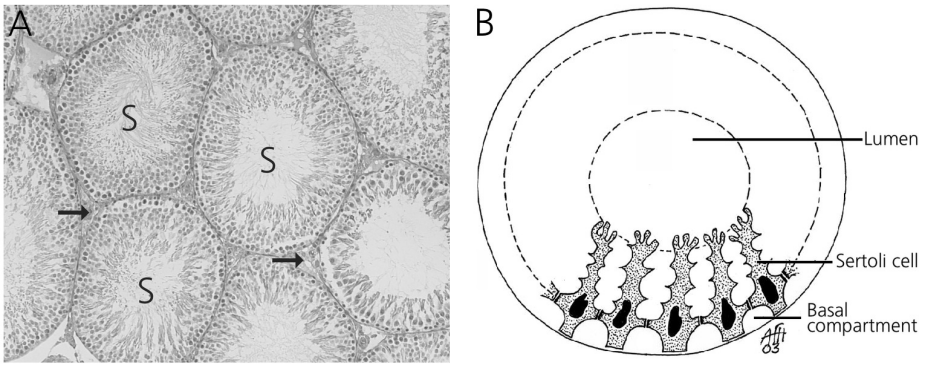


Figure 2. A. Part of a cross-section of an adult rat testis. Two compartments can be distinguished, the seminiferous tubules (S) and the space in between the tubules, called interstitium or interstitial compartment (arrows). Testis was fixed in diluted Bouin's fluid, magnification 100x. B. Schematic drawing of Sertoli cells within a seminiferous tubule. Together the Sertoli cells enclose several germ cells in different stages during spermatogenesis. Drawing adapted from Holstein et al. [20].

of the tubules but their cytoplasmic ramifications reach up to the lumen of the seminiferous tubules and can therefore support germ cells during all stages of development (Fig.2b) [16, 17]. These somatic cells express and secrete growth factors and other factors which regulate spermatogonial stem cell self-renewal and germ cell differentiation [16, 17]. At 12 dpp (days post partum) and 14-16 dpp for mice and rats respectively, Sertoli cells cease to divide and become terminally differentiated [18, 19]. Thereafter the number of Sertoli cells remains constant per unit length of seminiferous tubule at all stages of the epithelial cycle throughout the lifespan of the animal [12].

The germ cells

As described above, the formation of spermatozoa is a multi-step process in which spermatozoa are formed through a cascade of proliferation and differentiation steps starting with the mitotic divisions of the spermatogonial stem cells (Fig.3).

Spermatogonial stem cells are thought to be single cells located on the basal membrane of the seminiferous tubules and are called A-single (A_s) spermatogonia [4]. These cells either divide into two new single cells or into a pair of spermatogonia (A_{pr}) that do not complete cytokinesis and stay connected by an intercellular bridge [21, 22]. The A_{pr} spermatogonia are believed to be committed to differentiation. Starting with the A_{pr} spermatogonia, cytokinesis in all further divisions will also be incomplete, leading to the formation of increasingly large syncytia of germ cells (Fig.4) [23].

A_s A_{pr} spermatogonia are morphologically similar to A_s spermatogonia, the intercellular bridge can be taken as the first visible sign of the entrance of the cells into the differentiation pathway. The A_{pr} spermatogonia divide further to form chains of 4, 8, 16 or sporadically 32 aligned A spermatogonia (A_{al}). Together, the A_s, A_{pr} and Aal spermatogonia are called early or undifferentiated spermatogonia. At a set time the Aal spermatogonia differentiate, without mitosis, into A1 spermatogonia. This differentiation step involves slight morphological changes and brings about changes in cell cycle characteristics of the spermatogonia [8, 24]. The transition of early A spermatogonia into A1 spermatogonia is also accompanied by the up-regulation of c-KIT [25, 26].

In most non-primate mammals there are six mitotic divisions following the formation of A1 spermatogonia, giving rise to successively A2, A3, A4, intermediate (In) and B spermatogonia. The B spermatogonia divide into spermatocytes. In total, there are about 10 spermatogonial divisions between the spermatogonial stem cell and the formation of spermatocytes [4]. The spermatocytes undergo two meiotic divisions, after which the haploid spermatids start the process of spermiogenesis [4, 9].

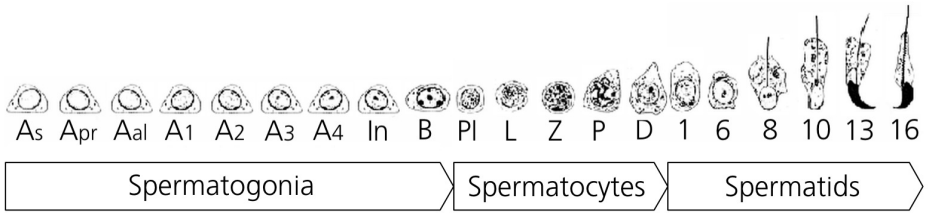


Figure 3. The various differentiation steps of spermatogenesis in mice. Adapted from Russell et al. [9]. Spermatogonia: (As) single, (Apr) paired, (Aal) aligned, (In) intermediate. Spermatocytes: (Pl) preleptotene, (L) leptotene, (Z) zygotene, (P) pachytene, (D) diplotene.

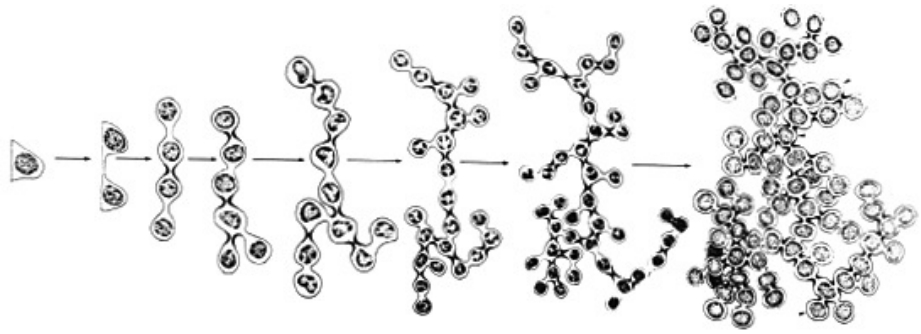


Figure 4. Formation of increasingly large syncytia of germ cells. Adapted from Moens et al. [23].

Stages of the seminiferous epithelial cycle

In a cross-section of a seminiferous tubule, germ cells in various phases of differentiation can be observed. In 1952, Charles Leblond and Yves Clermont observed that various germ cells of different developmental phases are always present in specific combinations [27]. The number and composition of these combinations, which are called epithelial stages, differ per species [27-30]. The epithelial stages are designated by roman numbers. In mice and rats, epithelial stages I-XII and I-XIV are designated, respectively (Fig.5).

By determining the developmental step of the spermatids the composition of the spermatogonial compartment can be identified. Unfortunately, early type A spermatogonia (A_s, A_{pr} and A_{al}) are not specifically present in a particular epithelial stage but are present throughout the epithelial cycle (Fig.5) [8, 31, 32]. However, the numbers of the different early A spermatogonia differ per epithelial stage [8]. For example, in the rat testis, the total number of early A spermatogonia reaches it maximum in epithelial stage VII. As at the transition of epithelial stage VII into VIII most A_{al} spermatogonia differentiate into A1 spermatogonia there is a sharp decrease in the total number of early A spermatogonia [8].The number very slowly increases again during stages X-XIV after which the proliferation continuous throughout

epithelial stages I-V, which leads to a gradual expansion in the number of early A spermatogonia. A plateau is reached in epithelial stage V, after which the mitotic activity of the early A spermatogonia ceases. The fluctuation in the number of early A spermatogonia is largely caused by the fluctuation in the number of A_{al} spermatogonia as they form the largest group within the population of early A spermatogonia. The number of A_s and A_{pr} spermatogonia stays more or less constant throughout the epithelial cycle [8].

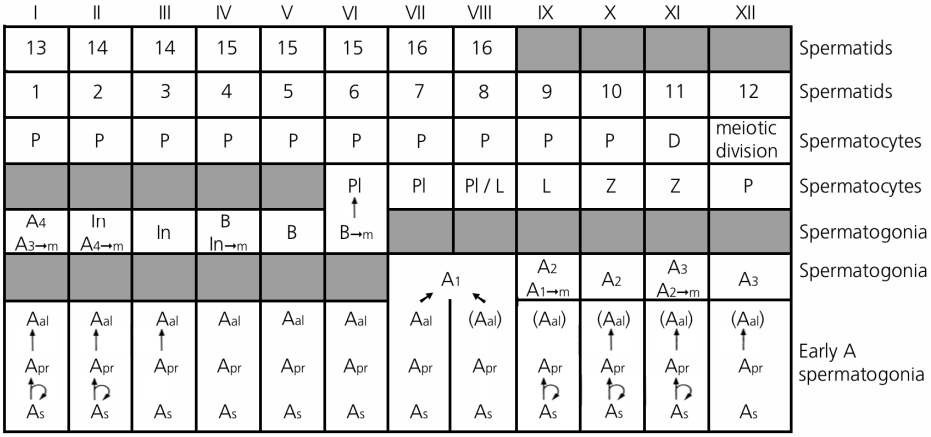


Figure 5. Stages of the seminiferous epithelial cycle in mouse [3, 9]. For example, in a cross-section of a seminiferous tubule in epithelial stage V of a mouse testis, we can observe B spermatogonia, pachytene spermatocytes, round spermatids in differentiation step 5 and elongated spermatids in step 15. Sertoli cells and early spermatogonia are present in all epithelial stages. Development of the germ cell can be followed by starting in the left bottom corner and reading the lines from left to right, from bottom to top.

Part 2: The spermatogonial stem cell

To maintain spermatogenesis throughout adult life, new germ cells are constantly produced. Spermatogonial stem cells can only supply this constant need for germ cells by maintaining a precise balance between self-renewal and differentiation. The mechanisms by which this balance is maintained are largely unknown. Unfortunately, these mechanisms are difficult to study because of the lack of methods to purify germ cells from the testis. Research on spermatogonial stem cells is also hampered by the low number of spermatogonial stem cells present in the testis. In the mouse it was found that stem cells comprise 0.03% of all germ cells, 1.25% of all spermatogonia, amounting to approximately 35.000 spermatogonial stem cells per mouse testis [33]. In a rat testis, weighing about 10 times more, one can speculate that there will be about 350.000 stem cells per rat testis [4, 33].

Morphology of the spermatogonial stem cell

Articles describing the morphology of spermatogonia are rare and most only describe the morphology of spermatogonia in a more or less superficial way not allowing their precise identification in every instance. However, using high resolution light microscopy and specifically fixed, plastic-embedded tissue, Helio Chiarini-Garcia and Lonnie Russell have observed more subtle differences [24]. In contrast to more advanced A spermatogonia, A_s to A_{al} spermatogonia contain no heterochromatin at the rim of the nucleus, but have a fine mottled appearance throughout the nucleus (Fig.6) [24]. Nevertheless, even with good morphology and a well-trained eye, identification of spermatogonial stem cells is extremely difficult in sections.

Fortunately, spermatogonial stem cells can be recognized morphologically using a special technique. To this end, pieces of seminiferous tubules are prepared in their entirety to produce whole mounts [34]. Whole mounts of seminiferous tubules enable one to study the topography of early A spermatogonia lying on the basal membrane and to distinguish singles (A_s), pairs (A_{pr}) and chains (A_{al}) of these cells.

Figure 6. Morphology of an A spermatogonium compared to intermediate and B spermatogonia [4]. Notice the lack of heterochromatin at the edge of the nucleus in the A spermatogonium and the increase of heterochromatin in intermediate and B spermatogonia.

Spermatogonial stem cell transplantation

In 1994, the Brinster group published the first and until now only functional assay for spermatogonial stem cells [35, 36]. A mixed population of testicular cells was isolated from testes of 4 and 12 dpp old donor mice. These cells were injected into seminiferous tubules of recipient mice via the efferent ductules and the rete testis. W/W mice were used as recipients because endogenous spermatogenesis is absent in testes of these mice as a result of a mutation in the dominant-white spotting locus, which encodes the transmembrane tyrosine kinase receptor c-KIT [37]. Upon transplantation of donor cells in recipient mice testes complete spermatogenesis was observed in recipient testes. As the donor cell population was obtained from young animals, the observed spermatozoa must have been produced *de novo* in the recipient testis. These findings indicated that the spermatogonial stem cells migrated from the lumen of the seminiferous tubules to the basal membrane, that the spermatogonial

stem cells passed the blood testis barrier and that they were capable to restart spermatogenesis from their new found niches [35, 36]. A remarkable finding and a major breakthrough for the research on spermatogonial stem cells.

With this technique, populations of cells can now be checked for the presence of spermatogonial stem cells, allowing for example, the development of purification protocols [38-41]. Furthermore, as with this technique also the increase or decrease in the number of spermatogonial stem cells in a population can be observed, the effect of different growth factors on spermatogonial stem cells in *in vitro* experiments can be determined [42, 43]. The transplantation assay can also be used to detect whether fertility defects in transgenic mice are caused by a defect in the somatic cells or in the germ cells [44]. For example, Nakamura *et al.* reported that mice lacking *Cnot7* are infertile due to impaired maturation of spermatids. Fertility was restored upon transplantation of *Cnot7*$^{-/-}$ spermatogonial stem cells in W/W recipient mice, suggesting that the function of the testicular somatic cells is disturbed in the *Cnot7*$^{-/-}$ mice [45].

Since, the first report of the spermatogonial stem cell transplantation technique, various modifications and many new approaches have been described in different species [46-48]. Xeno-transplantations of spermatogonial stem cells of other species (e.g. rabbit, dog, monkey, bull and human) into recipient mouse testis revealed that spermatogonial stem cells were able to migrate to the basal membrane of the recipient mouse testis, however no differentiation of spermatogonia occurred [49-52]. Surprisingly, transplantation of rat and hamster spermatogonial stem cells resulted in full spermatogenesis in the recipient mouse testes [53, 54]. However, defects were observed in the hamster spermatids and acrosomes within the seminiferous tubules of the recipient mouse [53].

Spermatogonial stem cell markers

Since the establishment of the transplantation technique several new markers have been identified that could be used to isolate populations of cells that are (highly) enriched for spermatogonial stem cells (table 1). Also by immunohistochemistry on testis sections, several new genes expressed by early spermatogonia have been identified (table 2). However, until now no genes have been identified of which expression in the testis is restricted to the spermatogonial stem cells (A_S).

Table 1. Overview of markers that have been used successfully to isolate enriched populations of spermatogonial stem cells from testes, either by positive or negative selection.

Proteins with which an enriched population of spermatogonial stem cells can be isolated by positive selection	CD9 [55], integrin α6 [41], integrin β1 [41], integrin αV [40], THY-1 [39], CD24 [39]
Proteins with which an enriched population of spermatogonial stem cells can be isolated by negative selection	c-kit [39], MHC1 [39], CD34 [39]
Promotor (region)s used to direct expression of a label (GFP, LacZ) to a subpopulation of germ cells including the spermatogonial stem cells	Stra8 [56], OCT4 [57]

Table 2. Overview of markers expressed specifically by the indicated populations of germ cells.

A_s and A_{pr}	GFRα1 [58, 59]
A_s, A_{pr} and A_{al}	PLZF [60, 61], OCT4 [62], NGN3*[63], NOTCH1 [58], SOX3 [64], c-Ret [65]
A spermatogonia	RBM [66]
Spermatogonia	EP-CAM [67]
Pre-meiotic germ cells	STRA8 [68], EE2 [69]
Cells on the basal membrane and the interstutium	CD9 [70]
Spermatogonia, spermatocytes and round spermatids	GCNA1 [71]
Pre-meiotic germ cells and post-meiotic spermatids	TAF4B [72]
All germ cells	Hsp90α [73]

* It was found by Raverot *et al.* that NGN3 is also expressed in some spermatocytes [64].

Other stem cells and their specific markers

Many, if not all, tissues of the body contain stem cells or early progenitor cells that like spermatogonial stem cells express proteins that can be used to distinguish them from their surrounding tissue. As research on, for example the hematopoietic or embryonic stem cells is more advanced compared to the research on spermatogonial stem cells, much can be learned from looking at other stem cell. Studying the markers that are used for enrichment and/or identification of other stem cells could help to identify and enrich spermatogonial stem cells. Tables 3 and 4 list markers commonly used to identify murine hematopoietic and embryonic stem cells, respectively.

Side population

Besides the molecular markers that can be used to isolate enriched populations of spermatogonial stem cells from the testis, spermatogonial stem cells also posses other specific characteristics which were used to isolate enriched populations of spermatogonial stem cells from the testis [82-84].
The side population technique was first described in 1996 by Goodell *et al.* [84]. The technique is based on the differential efflux of the fluorescent DNA-binding dye Hoechst 33342 from stem cells relative to non-stem cells. The stem cells are thought to be efficient in expelling the fluorescent dye because they express Breast Cancer Resistance Protein 1 (Bcrp1) and multidrug resistance-1 (Mdr1). The stem cells can therefore easily be isolated, as the side population, with FACS [85, 86]. The protocol for isolation of side population cells was originally established for murine bone marrow hematopoietic stem cells, but since its first report the technique has been

Table 3. List of proteins used to enrich murine hematopoietic stem cells (HSC) from the total population of bone marrow [74, 75]. The right column indicates if the protein is expressed by spermatogonial stem cells (SSC) [39, 40].

protein	HSC	SSC
THY-1	low	+
CD24	+	+
SCA-1	+	?
c-KIT	+	-
Lin*	-	
MHC-1	+	-

*The hematopoietic stem cells are negative for markers which are used for detection of lineage commitment. For purification of hematopoietic stem cells by FACS up to 14 different mature blood-lineage markers (eg. CD45, Mac1, CD18, Gr-1, Ter119, Il7Ra, CD3, CD4, CD5 and CD8) are used as a mixture to deplete the lineage$^+$ cells. (+) expressed, (-) not expressed, (?) unknown.

Table 4. List of proteins expressed by murine embryonic stem cells (ES) isolated from the inner cell mass (ICM) [76, 77]. Right column indicates if the protein is expressed by spermatogonial stem cells (SSC) [62, 70, 78-80].

Protein	ES	SSC
SSEA-1	+	-
SSEA-3	-	?
SSEA-4	-	?
TRA-1-60	-	?
TRA-1-81	-	?
CD9	+	+
Osteopontin	+	+
OCT3/4 (pou5f1)	+	+
REX-1 (Zfp42)	+	?
NANOG	+	-
Fbx15	+	?*
FGF4	+	?
UTF1	+	?
SOX2	+	+
ZFP57	+	?*

* ZFP57 and Fbx15 are expressed in testis, however localization in testes is unknown [77, 81]. (+) expressed, (-) not expressed, (?)unknow

adapted for other species and tissues [87-89]. About four years ago, four articles reported the presence of a side population in the testis [39, 90-92]. However, it is not clear whether the side population also contains the spermatogonial stem cells. Kubota *et al.* reported that no colonization of recipient mouse testes occurred upon transplantation of side population cells isolated from adult cryptorchid mice [39]. Surface markers found on these side population cells also differed from those found on spermatogonial stem cells [39]. In contrast, Lassalle *et al.* showed that the side population cells isolated from W/W or cryptorchid mice were able to colonize recipient mouse testes and that their side population cells expressed *Stra8* and integrin α6, two genes known to be expressed by spermatogonial stem cells (table 1) [90]. Falciatori *et al.* also showed that the side population cells they isolated from 20-day-old mice contained spermatogonial stem cells [91]. Interestingly, Lo *et al.* reported restoration of Leydig cell numbers and testosterone levels in LH receptor-knockout (LhrKO) recipients when side population cells were transplanted into the interstitial tissue of testes of these mice. LhrKO mice are infertile due to targeted disruption of LH/hCG [93]. Restoration of Leydig cell numbers and testosterone levels in LhrKO mice upon transplantation of side population cells indicated that testicular side population cells included Leydig cell progenitors.

Spermatogonial stem cell lines GC-5spg and GC-6spg

The isolation of spermatogonial stem cells is difficult as only low numbers are present within the testis and isolation methods are not efficient. Furthermore, culture of a pure population of spermatogonial stem cells without feeder cells is until now only possible after immortalization and single cell cloning. A number of immortalized germ cell lines have been established using transfection of SV40 large T to immortalize testicular germ cells [94-98].

In 2002, two rat spermatogonial cell lines were established in our lab [96]. A mixed population of A_s, A_{pr} and A_{al} spermatogonia was isolated from testes of so-called vitamin A deficient (VAD) rats. As the seminiferous tubules of these VAD rats only contain Sertoli cells, sporadic spermatocytes and early A spermatogonia, a highly enriched population of early A spermatogonia could be obtained from these testes [83, 99]. The isolated early A spermatogonia were transfected with a pSV3-neo vector containing the intact SV40 early region. To obtain pure cell lines, the cells were single cell cloned and two stable cell lines were established, GC-5spg and GC-6spg. Both cell lines exhibited molecular characteristics of spermatogonial stem cells. They were c-KIT low, OCT-4 and HSP90α positive and upon transplantation in mouse testes, these cells were able to migrate to the basal membranes of the seminiferous tubules and colonization of recipient mouse testes was observed. Unfortunately, no differentiation

of the transplanted germ cell was observed [96]. Nevertheless, both cell lines possess characteristics of spermatogonial stem cells and can therefore be used to study certain aspects of spermatogonial stem cells. For example, the effects of drugs or environmental toxicants on spermatogonial stem cells could be studied, thereby possibly replacing experiments on laboratory animals. Even though, *in vivo* differentiation of the cell lines was not observed and studies on *in vitro* differentiation were not yet performed the cell lines could potentially be used to study the mechanisms of spermatogonial stem cell differentiation *in vitro*.

Part 3: Mechanisms of spermatogonial stem cell self-renewal and differentiation

Since spermatogonial stem cell divisions occur in a symmetric manner, the ratio between self-renewal and differentiation should be 1. If the balance shifts to self-renewal, reduced numbers of spermatozoa are formed, spermatogonial stem cells accumulate and even tumors will arise. On the other hand if the balance shifts towards differentiation, the spermatogonial stem cell population will deplete eventually, resulting in seminiferous tubules that only contain Sertoli cells.

The mechanisms that induce differentiation and/or regulate self-renewal of spermatogonial stem cells are not yet fully understood. However, in recent years some growth factors and transcription factors have been identified that play a role in the self-renewal and/or differentiation of spermatogonial stem cells (Fig. 8).

TGFβ family members

The transforming growth factor beta (TGFβ) superfamily of ligands, includes more than 40 proteins, e.g. TGFβs, activins and inhibins, bone morphogenetic proteins (BMPs), growth differentiation factors (GDFs), nodal, anti müllarian hormone (AMH) and the distant related glial cell line derived neurothropic factor (GDNF) [100]. TGFβ family members have been found to play a role in a variety of cellular processes, including cell-cycle progression, cell differentiation, development, cell motility, reproductive function, adhesion, bone morphogenesis and neural growth [101]. With the exception of GDNF, TGFβ superfamily members bind to specific transmembrane receptors with serine/threonine kinase activity [100]. Thereafter the signal is transduced via phosphorylation of Smad 1, 2, 3, 5 or 8 depending on the ligand. The activated Smads bind to Smad4, translocates to the nucleus where gene transcription can be effected upon binding to smad binding elements [102]. Three members of the TGFβ superfamily, GDNF, BMP4 and activin A, were found to play a role in spermatogonial stem cell self-renewal and differentiation (Fig.7) [42, 103].

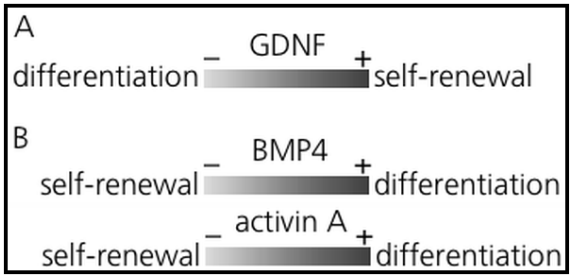


Figure 7. Effects of TGFβ superfamily members on spermatogonial stem cell self-renewal and/or differentiation. A: GDNF, low concentrations allow differentiation while high levels induce self-renewal B: BMP4 and Activin A are both reported to induce differentiation.

GDNF was found to have a dosage dependent effect on the fate of the spermatogonial stem cell (Fig.7a) [103]. Overexpression of GDNF in testes of mice resulted in the accumulation of spermatogonial stem cells, whereas in testes of $GDNF^{+/-}$ mice depletion of spermatogonial stem cells was observed [103]. In the testis, GDNF is secreted by the Sertoli cells.

The regulation of GDNF expression is still poorly understood, but follicle-stimulating hormone (FSH) was found to control the GDNF levels in Sertoli cells [104]. GDNF binds to the GDNF family receptor α1 (GFRα1), which is known to be expressed by single and paired spermatogonia [58, 59]. In turn this receptor-ligand complex activates RET tyrosine kinase. Silencing of GFRα1 also leads to the differentiation of spermatogonial stem cells via the inactivation of RET tyrosine kinase [105].

In order to identify down-stream targets of GDNF, a micro-array experiment was performed [106]. One of the genes found to be down-regulated upon the withdrawal of GDNF from the culture medium was B-cell CLL/lymphoma 6, member B (*Bcl6b*). The importance of *Bcl6b* in spermatogonial stem cell maintenance was confirmed by siRNA experiments which showed a reduction in both size and number of germ cell clumps after 7 days of culture while the percentage of apoptotic germ cells increased in these cultures [106]. Furthermore, the *Bcl6b* $^{-/-}$ mice are capable of siring offspring, but mating of *Bcl6b* $^{-/-}$ mice produced lower numbers of pups per litter compared to mating with wild-types. Testis weight was reduced and histological examination of testes of *Bcl6b* $^{-/-}$ mice revealed that 7% of the round seminiferous tubules had degenerative spermatogenesis or a Sertoli cell only phenotype [106].

Both, activin A and BMP4 were found to have a negative effect on stem cell maintenance *in vitro* (Fig.7b) [42]. Germ cells cultured for 7 days in the presence of activin A or BMP4 gave rise to lower numbers of colonies upon transplantation in a depleted recipient testis compared to cells that were cultured in the absence of activin A or BMP4 [42]. These results suggested that BMP4 and activin A induced differentiation of spermatogonial stem cells [42].

Furthermore, up-regulation of c-KIT was observed when spermatogonia isolated from 4ddp old mice were *in vitro* exposed to BMP4 [107]. As c-KIT is expressed by

differentiating spermatogonia, but not by spermatogonial stem cells, upregulation of c-KIT is commonly used as a marker for spermatogonial stem cell differentiation [25, 26]. *Bmp4* heterozygous mice on a C57BL/6 background showed reduced sperm counts and motility. Histological examination of the testis of these mice showed an increased occurrence of meiotic germ cell degeneration [108].

The expression pattern of BMP4 in the testis of adult mice is unclear. Different research groups reported different expression patterns in mice. Hu *et al.* showed high levels of *Bmp4* mRNA in pachytene spermatocytes whereas lower levels were detected in other cell types, including Sertoli cells [108]. Baleato *et al.* described that BMP4 at both the protein and the mRNA level was expressed in germ cells, mainly in spermatogonia and early spermatocytes and was not expressed by Sertoli cells [109]. Pellegrini *et al.* did not detect *Bmp4* in spermatogonia, spermatocytes or spermatids but did observe expression of *Bmp4* in Sertoli cells isolated from 4dpp testis [107].

Activins were originally identified as gonadal proteins capable of inducing the secretion of pituitary FSH [110, 111]. Activin proteins are generated from two gene products, activin βA and activin βB that dimerise to form activin A (βAβA), activin B (βBβB) or activin AB (βAβB) [111]. The Sertoli cells are the major side of activin A production in adult rats [112]. A recent report shows that activin A is secreted in a cyclic manner with a peak secretion at epithelial stage VIII [113]. The activins signal via cell surface serine-threonine kinase receptors, like most TGFβ superfamily members. Activins bind to type II ligand binding receptors (ActRIIa/ ActRIIb) whereafter the type II receptor heterodimerises with a type I (ActRIa/ ActRIb) receptor, which is then phosphorylated and that in turn leads to phosphorylation of Smad2/3, translocation of the signal to the nucleus and regulation of transcription of specific target genes [114, 115].

Unfortunately, activin βA knockout mice die from multiple abnormalities within the first 24 h after birth [116]. Mice that overexpress the βA subunit in the testis are infertile, however the mechanisms that underlie this infertility are unclear as the βA subunit is also a part of inhibin A, which is a functional antagonist of activin A [111, 113].

FGFs and FGFRs

Basic fibroblast growth factor (bFGF or FGF2) was found to promote maintenance and proliferation of primordial germ cells (PGC's) *in vitro* [117, 118]. Culture of spermatogonial stem cells in the presence of both GDNF and FGF2 showed a synergistic effect on the number of stem cells maintained in culture compared to cultures with only GDNF [119]. Including FGF2, currently 22 FGFs are known, which all interact with various affinity to the different FGF receptors (FGFRs) [120]. Four

members of the FGFR family (FGFR1, FGFR2, FGFR3 and FGFR4) are encoded by distinct genes and their structural variability is increased by alternative splicing [121]. The various FGFs show different FGFR specificity, FGF2 binds to distinct splice variants of the different FGFRs. The FGFR1c, FGFR3c and FGFR4 isoforms were found to be the preferential targets of FGF2. Lower affinity of FGF2 was observed for FGFR1b and FGFR2c [122]. A role of FGF signaling in the process of spermatogonial stem cell self-renewal was furthermore supported by a study which showed that a specific mutation of FGFR2 in spermatogonial stem cells resulted in a selective advantage for these cells compared to spermatogonial stem cells that were not mutated [123].

Besides the direct effect of FGF2 on spermatogonial stem cells, FGF2 was found to effect the expression levels of the transcription factor Ets related molecule (ERM) or Ets variant gene 5 (ETV5) in the Sertoli cells that form the spermatogonial stem cell niche [124]. ERM was found to be essential for spermatogonial stem cell self-renewal. Targeted disruption of this protein was found to result in the loss of spermatogonial stem cell self-renewal without a block in normal spermatogenic differentiation. Seminiferous tubules of these mice become depleted with time [125]

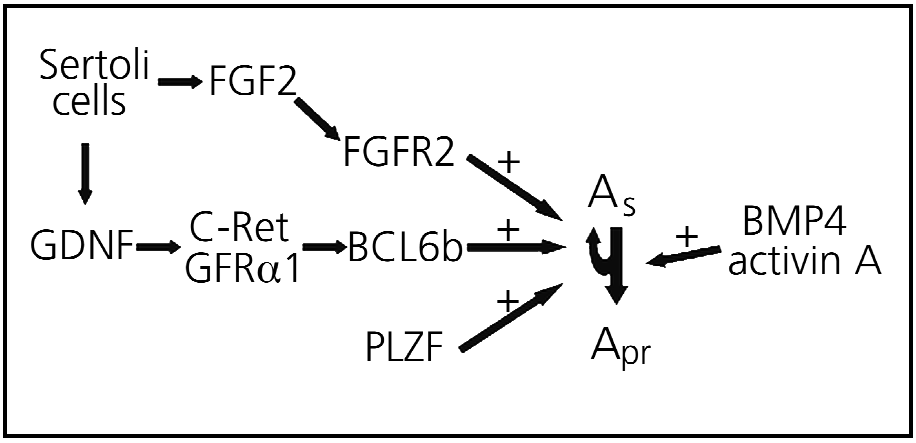


Figure 8. Overview of the presently known factors involved in the regulation of self-renewal and differentiation of spermatogonial stem cells. From de Rooij [130].

Transcription factors

During the last decade, a number of transcription factors have been identified that play an important role during spermatogenesis. These transcription factors were identified by chance when knockout mice, generated to study the role of these transcription factors in other organs, appeared to be infertile. Until now, the up- and downstream signals of these transcription factors are mostly unknown [126]. The two

transcription factors described below were found to be essential for the regulation of spermatogonial stem cell self-renewal.

Luxoid mice, which arose spontaneously in 1950, show skeletal abnormalities of especially the hind limb and homeotic transformations of anterior skeletal elements into posterior structures. Luxoid males developed infertility due to the loss of germ cells with age [127, 128]. Recently, the luxoid testis phenotype was reassessed and a mutation in the first codon of zinc finger protein 145 (*Zfp145*) was found to be responsible for the luxoid phenotype [60]. Around the same time, Zpf145$^{-/-}$ mice were generated and testes of these mice showed the same loss of spermatogonia with age [61]. Furthermore, expression of promeolytic zinc finger (PLZF), the protein encoded by *Zfp145*, was found to be restricted to early A spermatogonia (A_s, A_{pr} and A_{al}) in the testis [60, 61]. Recently, it was reported that PLZF is a direct transcriptional repressor of *c-Kit* transcription. Within the promoter region of *c-Kit* two PLZF responsive elements were found, of which only the second was found to have an inhibitory effect on the expression of c-KIT *in vitro* upon binding of PLZF [129]. Together, these findings thus suggest that PLZF is required for the process of self-renewal and/or inhibition differentiation of spermatogonial stem cells and maintaining a stem cell pool in the testis throughout life [60, 61].

Expression of TATA-binding protein associated factor 4b (TAF4B) is restricted to gonocytes, spermatogonia and spermatids [72]. Targeted knock-down of *Taf4b* resulted in a rapid loss of germ cells in the testis. Thus TAF4b, a component of the TFIID complex of RNA polymerase II basal transcription machinery, appeared to be essential for the proliferation of spermatogonial stem cells [72].

Part 4: Aim and outline of this thesis

The aim of the work presented in this thesis was to obtain more knowledge about the mechanisms by which self-renewal and differentiation of spermatogonial stem cells are regulated. In order to gain more insight into these mechanisms the research was approached from two directions.

- A "candidate approach", in which genes known to be important for the regulation of self-renewal or differentiation of other stem cells, e.g. embryonic stem cells or hematopoietic stem cells, were studied to investigate if these genes also play a role in the regulation of self-renewal or differentiation of spermatogonial stem cells. The work on two of these genes is described in the chapters two and three.
- A "genomics approach" in which the gene expression profile of spermatogonial stem cells was compared to the expression profile of differentiated spermatogonia. For this approach, experiments were performed to investigate

how differentiation of the spermatogonial stem cell line GC-6spg could be induced *in vitro*. Thereafter micro-array studies were performed to identify genes involved in spermatogonial stem cell differentiation. Results that were obtained using this approach are described in chapters four and five.

Chapter 2 shows the localization of LY6A/E (SCA-1) in testes of adult mice of different strains by immunohistochemistry and describes the morphological study of the testis of LY6A/E$^{-/-}$ mice. Stem cell antigen 1 (SCA-1) was selected because it is frequently used for the isolation of HSCs. Skeletal muscle stem cells and mammary epithelium stem cells also express SCA-1. Furthermore, SCA-1 was found to play a role in the repopulating capacity of hematopoietic stem cells and the development of committed progenitor cells, megakaryocytes, and platelets. In the testis, both Kubota *et al.* and Falciatori *et al.* found that SCA-1 is expressed by the side population cells. However, as described above, these research groups disagreed about the cell types that could be isolated with the side population technique.

Chapter 3 shows, the expression pattern of UTF1 in the rat testis at different developmental stages during pre- and postnatal life. This chapter furthermore describes the co-localization experiment of UTF1 with PLZF and the distribution of UTF1 expressing cells over the different epithelial stages. UTF1 was selected as its expression was reported to be restricted to embryonic stem cells and in the adult mouse to the ovary and testis. Furthermore, expression of UTF1 in embryonic stem cells was rapidly down-regulated upon induction of differentiation of these cells, suggesting that expression is restricted to the pluripotent stem cells.

Chapter 4 reports that spermatogonial stem cell line GC-6spg can be induced to differentiate *in vitro* by addition of BMP4 to the culture medium. It is shown that BMP4 receptors are expressed by the GC-6spg cells, Smads are phosphorylated and c-KIT expression is upregulated. This chapter also reports the results of the micro-array experiments in which GC-6spg cells cultured in standard medium are compared with GC-6spg cells cultured in the presence of BMP4 at different time points. Analysis and confirmation of the micro-array results are reported in this chapter as well.

Chapter 5 shows that differentiation of spermatogonial stem cell line GC-6spg can also be induced by addition of activin A to the culture medium. Experiments were performed to study if genes found in chapter 4 are general markers of spermatogonial stem cell differentiation.

Chapter 6 summarizes and discusses the data described within this thesis.

References

1. Clarke GN. A.R.T. and history, 1678-1978. Hum Reprod 2006; 21: 1645-1650.
2. Leeuwenhoek A. The Collected Letters, Volume II 1676-1679. Swets and Zeitlinger Ltd, Amsterdam; 1941.
3. de Rooij DG. Stem cells in the testis. Int J Exp Pathol 1998; 79: 67-80.
4. de Rooij DG, Russell LD. All you wanted to know about spermatogonia but were afraid to ask. J Androl 2000; 21: 776-798.
5. Smith A. A glossary for stem-cell biology. nature 2006; 441: 1060.
6. Wagers AJ, Weissman IL. Plasticity of adult stem cells. Cell 2004; 116: 639-648.
7. Alison MR, Poulsom R, Forbes S, Wright NA. An introduction to stem cells. J Pathol 2002; 197: 419-423.
8. Huckins C. The spermatogonial stem cell population in adult rats. I. Their morphology, proliferation and maturation. Anat Rec 1971; 169: 533-557.
9. Russell LD, Ettlin R, Sinha-Hikim AP, Clegg E. Histological and Histopathological evaluation of the testis. cache river press.; 1990.
10. Jay V. A portrait in history. The legacy of Reinier de Graaf. Arch Pathol Lab Med 2000; 124: 1115-1116.
11. Bascom KF, Osterud HL. Quantitative studies of the testicule. II. Pattern and total tubule length in the testicules of certain common mammals. Anat. Rec. 1925; 31: 159-169.
12. Wing TY, Christensen AK. Morphometric studies on rat seminiferous tubules. Am J Anat 1982; 165: 13-25.
13. Maekawa M, Kamimura K, Nagano T. Peritubular myoid cells in the testis: their structure and function. Arch Histol Cytol 1996; 59: 1-13.
14. Clermont Y. Contractile elements in the limiting membrane of the seminiferous tubules of the rat. Exp Cell Res 1958; 15: 438-440.
15. Baratelli GM, Lanzani A, Sacco RN. Biography of Enrico Sertoli. Urology 2002; 60: 196-198.
16. Petersen C, Soder O. The sertoli cell--a hormonal target and 'super' nurse for germ cells that determines testicular size. Horm Res 2006; 66: 153-161.
17. Skinner MK, Griswold MD. Sertoli cell biology. San Diego: Elsevier academic press; 2005.
18. Kluin PM, Kramer MF, de Rooij DG. Proliferation of spermatogonia and Sertoli cells in maturing mice. Anat Embryol (Berl) 1984; 169: 73-78.
19. Steinberger A, Steinberger E. Replication pattern of Sertoli cells in maturing rat testis in vivo and in organ culture. Biol Reprod 1971; 4: 84-87.
20. Holstein AF, Schulze W, Davidoff M. Understanding spermatogenesis is a prerequisite for treatment. Reprod Biol Endocrinol 2003; 1: 107.
21. Fawcett DW, Ito S, Slautterback D. The occurrence of intercellular bridges in groups of cells exhibiting synchronous differentiation. J Biophys Biochem Cytol 1959; 5: 453-460.
22. Hamer G, Roepers-Gajadien HL, Gademan IS, Kal HB, De Rooij DG. Intercellular bridges and apoptosis in clones of male germ cells. Int J Androl 2003; 26: 348-353.
23. Moens PB, Hugenholtz AD. The arrangement of germ cells in the rat seminiferous tubule: an electron-microscope study. J Cell Sci 1975; 19: 487-507.
24. Chiarini-Garcia H, Russell LD. High-resolution light microscopic characterization of mouse spermatogonia. Biol Reprod 2001; 65: 1170-1178.
25. Rossi P, Sette C, Dolci S, Geremia R. Role of c-kit in mammalian spermatogenesis. J Endocrinol Invest 2000; 23: 609-615.
26. Schrans-Stassen BH, van de Kant HJ, de Rooij DG, van Pelt AM. Differential expression of c-kit in mouse undifferentiated and differentiating type A spermatogonia. Endocrinology 1999; 140: 5894-5900.
27. Leblond CP, Clermont Y. Definition of the stages of the cycle of the seminiferous epithelium in the rat. Ann N Y Acad Sci 1952; 55: 548-573.

28. Clermont Y. The cycle of the seminiferous epithelium in man. Am J Anat 1963; 112: 35-51.
29. Oud JL, de Rooij DG. Spermatogenesis in the Chinese hamster. Anat Rec 1977; 187: 113-124.
30. Oakberg EF. Duration of spermatogenesis in the mouse and timing of stages of the cycle of the seminiferous epithelium. Am J Anat 1956; 99: 507-516.
31. Huckins C. Cell cycle properties of differentiating spermatogonia in adult Sprague-Dawley rats. Cell Tissue Kinet 1971; 4: 139-154.
32. Huckins C. The spermatogonial stem cell population in adult rats. II. A radioautographic analysis of their cell cycle properties. Cell Tissue Kinet 1971; 4: 313-334.
33. Tegelenbosch RA, de Rooij DG. A quantitative study of spermatogonial multiplication and stem cell renewal in the C3H/101 F1 hybrid mouse. Mutat Res 1993; 290: 193-200.
34. Clermont Y, Bustos-Obregon E. Re-examination of spermatogonial renewal in the rat by means of seminiferous tubules mounted "in toto". Am J Anat 1968; 122: 237-247.
35. Brinster RL, Avarbock MR. Germline transmission of donor haplotype following spermatogonial transplantation. Proc Natl Acad Sci U S A 1994; 91: 11303-11307.
36. Brinster RL, Zimmermann JW. Spermatogenesis following male germ-cell transplantation. Proc Natl Acad Sci U S A 1994; 91: 11298-11302.
37. Ohta H, Tohda A, Nishimune Y. Proliferation and Differentiation of Spermatogonial Stem Cells in the W/Wv Mutant Mouse Testis. Biol Reprod 2003; 69: 1815-1821.
38. Griswold MD. What can spermatogonial transplants teach us about male reproductive biology? Endocrinology 2000; 141: 857-858.
39. Kubota H, Avarbock MR, Brinster RL. Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells. Proc Natl Acad Sci USA 2003; 100: 6487-6492.
40. Shinohara T, Orwig KE, Avarbock MR, Brinster RL. Spermatogonial stem cell enrichment by multiparameter selection of mouse testis cells. Proc Natl Acad Sci USA 2000; 97: 8346-8351.
41. Shinohara T, Avarbock MR, Brinster RL. beta1- and alpha6-integrin are surface markers on mouse spermatogonial stem cells. Proc Natl Acad Sci U S A 1999; 96: 5504-5509.
42. Nagano M, Ryu BY, Brinster CJ, Avarbock MR, Brinster RL. Maintenance of mouse male germ line stem cells in vitro. Biol Reprod 2003; 68: 2207-2214.
43. Kubota H, Avarbock MR, Brinster RL. Culture conditions and single growth factors affect fate determination of mouse spermatogonial stem cells. Biol Reprod 2004; 71: 722-731.
44. Ogawa T, Dobrinski I, Avarbock MR, Brinster RL. Transplantation of male germ line stem cells restores fertility in infertile mice. Nat Med 2000; 6: 29-34.
45. Nakamura T, Yao R, Ogawa T, Suzuki T, Ito C, Tsunekawa N, Inoue K, Ajima R, Miyasaka T, Yoshida Y, Ogura A, Toshimori K, Noce T, Yamamoto T, Noda T. Oligo-astheno-teratozoospermia in mice lacking Cnot7, a regulator of retinoid X receptor beta. Nat Genet 2004; 36: 528-533.
46. Izadyar F, Creemers LB, van Dissel-Emiliani FM, van Pelt AM, de Rooij DG. Spermatogonial stem cell transplantation. Mol Cell Endocrinol 2000; 169: 21-26.
47. Dobrinski I, Ogawa T, Avarbock MR, Brinster RL. Computer assisted image analysis to assess colonization of recipient seminiferous tubules by spermatogonial stem cells from transgenic donor mice. Mol Reprod Dev 1999; 53: 142-148.
48. Izadyar F, Den Ouden K, Stout TA, Stout J, Coret J, Lankveld DP, Spoormakers TJ, Colenbrander B, Oldenbroek JK, Van der Ploeg KD, Woelders H, Kal HB, De Rooij DG. Autologous and homologous transplantation of bovine spermatogonial stem cells. Reproduction 2003; 126: 765-774.
49. Dobrinski I, Avarbock MR, Brinster RL. Transplantation of germ cells from rabbits and dogs into mouse testes. Biol Reprod 1999; 61: 1331-1339.
50. Nagano M, McCarrey JR, Brinster RL. Primate spermatogonial stem cells colonize mouse testes. Biol Reprod 2001; 64: 1409-1416.

51. Nagano M, Patrizio P, Brinster RL. Long-term survival of human spermatogonial stem cells in mouse testes. Fertil Steril 2002; 78: 1225-1233.
52. Dobrinski I, Avarbock MR, Brinster RL. Germ cell transplantation from large domestic animals into mouse testes. Mol Reprod Dev 2000; 57: 270-279.
53. Ogawa T, Dobrinski I, Avarbock MR, Brinster RL. Xenogeneic spermatogenesis following transplantation of hamster germ cells to mouse testes. Biol Reprod 1999; 60: 515-521.
54. Clouthier DE, Avarbock MR, Maika SD, Hammer RE, Brinster RL. Rat spermatogenesis in mouse testis. Nature 1996; 381: 418-421.
55. Kanatsu-Shinohara M, Toyokuni S, Shinohara T. CD9 Is a Surface Marker on Mouse and Rat Male Germline Stem Cells. Biol Reprod 2004; 70: 70-75.
56. Giuili G, Tomljenovic A, Labrecque N, Oulad-Abdelghani M, Rassoulzadegan M, Cuzin F. Murine spermatogonial stem cells: targeted transgene expression and purification in an active state. EMBO Rep 2002; 3: 753-759.
57. Ohmura M, Yoshida S, Ide Y, Nagamatsu G, Suda T, Ohbo K. Spatial analysis of germ stem cell development in Oct-4/EGFP transgenic mice. Arch Histol Cytol 2004; 67: 285-296.
58. von Schonfeldt V, Wistuba J, Schlatt S. Notch-1, c-kit and GFRalpha-1 are developmentally regulated markers for premeiotic germ cells. 2004: 235-239.
59. Hofmann MC, Braydich-Stolle L, Dym M. Isolation of male germ-line stem cells; influence of GDNF. Dev Biol 2005; 279: 114-124.
60. Buaas FW, Kirsh AL, Sharma M, McLean DJ, Morris JL, Griswold MD, de Rooij DG, Braun RE. Plzf is required in adult male germ cells for stem cell self-renewal. Nat Genet 2004; 36: 647-652
61. Costoya JA, Hobbs RM, Barna M, Cattoretti G, Manova K, Sukhwani M, Orwig KE, Wolgemuth DJ, Pandolfi PP. Essential role of Plzf in maintenance of spermatogonial stem cells. Nat Genet 2004; 36: 653-659.
62. Pesce M, Wang X, Wolgemuth DJ, Scholer H. Differential expression of the Oct-4 transcription factor during mouse germ cell differentiation. Mech Dev 1998; 71: 89-98.
63. Yoshida S, Takakura A, Ohbo K, Abe K, Wakabayashi J, Yamamoto M, Suda T, Nabeshima Y. Neurogenin3 delineates the earliest stages of spermatogenesis in the mouse testis. Dev Biol 2004; 269: 447-458.
64. Raverot G, Weiss J, Park SY, Hurley L, Jameson JL. Sox3 expression in undifferentiated spermatogonia is required for the progression of spermatogenesis. Dev Biol 2005.
65. Meng X, de Rooij DG, Westerdahl K, Saarma M, Sariola H. Promotion of seminomatous tumors by targeted overexpression of glial cell line-derived neurotrophic factor in mouse testis. Cancer Res 2001; 61: 3267-3271.
66. Jarvis S, Elliott DJ, Morgan D, Winston R, Readhead C. Molecular markers for the assessment of postnatal male germ cell development in the mouse. Hum Reprod 2005; 20: 108-116.
67. Gaskell TL, Esnal A, Robinson LL, Anderson RA, Saunders PT. Immunohistochemical profiling of germ cells within the human fetal testis: identification of three subpopulations. Biol Reprod 2004; 71: 2012-2021.
68. Oulad-Abdelghani M, Bouillet P, Decimo D, Gansmuller A, Heyberger S, Dolle P, Bronner S, Lutz Y, Chambon P. Characterization of a premeiotic germ cell-specific cytoplasmic protein encoded by Stra8, a novel retinoic acid-responsive gene. J Cell Biol 1996; 135: 469-477.
69. Koshimizu U, Nishioka H, Watanabe D, Dohmae K, Nishimune Y. Characterization of a novel spermatogenic cell antigen specific for early stages of germ cells in mouse testis. Mol Reprod Dev 1995; 40: 221-227.
70. Kanatsu-Shinohara M, Toyokuni S, Shinohara T. CD9 Is a Surface Marker on Mouse and Rat Male Germline Stem Cells. Biol Reprod 2003; 3: 3.
71. Enders GC, May JJ, 2nd. Developmentally regulated expression of a mouse germ cell nuclear antigen examined from embryonic day 11 to adult in male and female mice. Dev Biol 1994; 163: 331-340.

72. Falender AE, Freiman RN, Geles KG, Lo KC, Hwang K, Lamb DJ, Morris PL, Tjian R, Richards JS. Maintenance of spermatogenesis requires TAF4b, a gonad-specific subunit of TFIID. Genes Dev 2005; 19: 794-803.
73. Lee SJ. Expression of HSP86 in male germ cells. Mol Cell Biol 1990; 10: 3239-3242.
74. Spangrude GJ, Heimfeld S, Weissman IL. Purification and characterization of mouse hematopoietic stem cells. Science 1988; 241: 58-62.
75. Li CL, Johnson GR. Murine hematopoietic stem and progenitor cells: I. Enrichment and biologic characterization. Blood 1995; 85: 1472-1479.
76. Koestenbauer S, Zech NH, Juch H, Vanderzwalmen P, Schoonjans L, Dohr G. Embryonic stem cells: similarities and differences between human and murine embryonic stem cells. Am J Reprod Immunol 2006; 55: 169-180.
77. Okazaki S, Tanase S, Choudhury BK, Setoyama K, Miura R, Ogawa M, Setoyama C. A novel nuclear protein with zinc fingers down-regulated during early mammalian cell differentiation. J Biol Chem 1994; 269: 6900-6907.
78. Luedtke CC, McKee MD, Cyr DG, Gregory M, Kaartinen MT, Mui J, Hermo L. Osteopontin expression and regulation in the testis, efferent ducts, and epididymis of rats during postnatal development through to adulthood. Biol Reprod 2002; 66: 1437-1448.
79. Shi YQ, Wang QZ, Liao SY, Zhang Y, Liu YX, Han CS. In vitro propagation of spermatogonial stem cells from KM mice. Front Biosci 2006; 11: 2614-2622.
80. Kanatsu-Shinohara M, Miki H, Inoue K, Ogonuki N, Toyokuni S, Ogura A, Shinohara T. Long-term culture of mouse male germline stem cells under serum-or feeder-free conditions. Biol Reprod 2005; 72: 985-991.
81. Tokuzawa Y, Kaiho E, Maruyama M, Takahashi K, Mitsui K, Maeda M, Niwa H, Yamanaka S. Fbx15 is a novel target of Oct3/4 but is dispensable for embryonic stem cell self-renewal and mouse development. Mol Cell Biol 2003; 23: 2699-2708.
82. Davis JC, Schuetz AW. Separation of germinal cells from immature rat testes by sedimentation at unit gravity. Exp Cell Res 1975; 91: 79-86.
83. van Pelt AM, Morena AR, van Dissel-Emiliani FM, Boitani C, Gaemers IC, de Rooij DG, Stefanini M. Isolation of the synchronized A spermatogonia from adult vitamin A-deficient rat testes. Biol Reprod 1996; 55: 439-444.
84. Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med 1996; 183: 1797-1806.
85. Zhou S, Morris JJ, Barnes Y, Lan L, Schuetz JD, Sorrentino BP. Bcrp1 gene expression is required for normal numbers of side population stem cells in mice, and confers relative protection to mitoxantrone in hematopoietic cells in vivo. Proc Natl Acad Sci U S A 2002; 99: 12339-12344.
86. Bunting KD. ABC transporters as phenotypic markers and functional regulators of stem cells. Stem Cells 2002; 20: 11-20.
87. Summer R, Kotton DN, Sun X, Ma B, Fitzsimmons K, Fine A. Side population cells and Bcrp1 expression in lung. Am J Physiol Lung Cell Mol Physiol 2003; 285: L97-104.
88. Triel C, Vestergaard ME, Bolund L, Jensen TG, Jensen UB. Side population cells in human and mouse epidermis lack stem cell characteristics. Exp Cell Res 2004; 295: 79-90.
89. Welm BE, Tepera SB, Venezia T, Graubert TA, Rosen JM, Goodell MA. Sca-1(pos) cells in the mouse mammary gland represent an enriched progenitor cell population. Dev Biol 2002; 245: 42-56.
90. Lassalle B, Bastos H, Louis JP, Riou L, Testart J, Dutrillaux B, Fouchet P, Allemand I. 'Side Population' cells in adult mouse testis express Bcrp1 gene and are enriched in spermatogonia and germinal stem cells. Development 2004; 131: 479-487.

91. Falciatori I, Borsellino G, Haliassos N, Boitani C, Corallini S, Battistini L, Bernardi G, Stefanini M, Vicini E. Identification and enrichment of spermatogonial stem cells displaying side-population phenotype in immature mouse testis. FASEB J 2004; 18: 376-378.
92. Lo KC, Lei Z, Rao CV, Beck J, Lamb DJ. De Novo Testosterone Production in Luteinizing Hormone Receptor Knockout Mice After Transplantation of Leydig Stem Cells. Endocrinology 2004; 145: 4011-4015.
93. Lei ZM, Mishra S, Zou W, Xu B, Foltz M, Li X, Rao CV. Targeted disruption of luteinizing hormone/human chorionic gonadotropin receptor gene. Mol Endocrinol 2001; 15: 184-200.
94. Hofmann MC, Hess RA, Goldberg E, Millan JL. Immortalized germ cells undergo meiosis in vitro. Proc Natl Acad Sci U S A 1994; 91: 5533-5537.
95. Hofmann MC, Narisawa S, Hess RA, Millan JL. Immortalization of germ cells and somatic testicular cells using the SV40 large T antigen. Exp Cell Res 1992; 201: 417-435.
96. van Pelt AM, Roepers-Gajadien HL, Gademan IS, Creemers LB, de Rooij DG, van Dissel-Emiliani FM. Establishment of cell lines with rat spermatogonial stem cell characteristics. Endocrinology 2002; 143: 1845-1850.
97. Tascou S, Nayernia K, Samani A, Schmidtke J, Vogel T, Engel W, Burfeind P. Immortalization of murine male germ cells at a discrete stage of differentiation by a novel directed promoter-based selection strategy. Biol Reprod 2000; 63: 1555-1561.
98. Hofmann MC, Braydich-Stolle L, Dettin L, Johnson E, Dym M. Immortalization of mouse germ line stem cells. Stem Cells 2005; 23: 200-210.
99. Mitranond V, Sobhon P, Tosukhowong P, Chindaduangrat W. Cytological changes in the testes of vitamin-A-deficient rats. I. Quantitation of germinal cells in the seminiferous tubules. Acta Anat (Basel) 1979; 103: 159-168.
100. Loveland KL, Hime G. TGFbeta superfamily members in spermatogenesis: setting the stage for fertility in mouse and Drosophila. Cell Tissue Res 2005; 322: 141-146.
101. Lin SJ, Lerch TF, Cook RW, Jardetzky TS, Woodruff TK. The structural basis of TGF-beta, bone morphogenetic protein, and activin ligand binding. Reproduction 2006; 132: 179-190.
102. Itman C, Mendis S, Barakat B, Loveland KL. All in the family: TGF-beta family action in testis development. Reproduction 2006; 132: 233-246.
103. Meng X, Lindahl M, Hyvonen ME, Parvinen M, de Rooij DG, Hess MW, Raatikainen-Ahokas A, Sainio K, Rauvala H, Lakso M, Pichel JG, Westphal H, Saarma M, Sariola H. Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. Science 2000; 287: 1489-1493.
104. Tadokoro Y, Yomogida K, Ohta H, Tohda A, Nishimune Y. Homeostatic regulation of germinal stem cell proliferation by the GDNF/FSH pathway. Mech Dev 2002; 113: 29-39.
105. He Z, Jiang J, Hofmann MC, Dym M. Gfra1 Silencing in Mouse Spermatogonial Stem Cells Results in Their Differentiation via the Inactivation of RET Tyrosine Kinase. Biol Reprod 2007.
106. Oatley JM, Avarbock MR, Telaranta AI, Fearon DT, Brinster RL. Identifying genes important for spermatogonial stem cell self-renewal and survival. Proc Natl Acad Sci U S A 2006; 103: 9524-9529.
107. Pellegrini M, Grimaldi P, Rossi P, Geremia R, Dolci S. Developmental expression of BMP4/ALK3/SMAD5 signaling pathway in the mouse testis: a potential role of BMP4 in spermatogonia differentiation. J Cell Sci 2003; 116: 3363-3372.
108. Hu J, Chen YX, Wang D, Qi X, Li TG, Hao J, Mishina Y, Garbers DL, Zhao GQ. Developmental expression and function of Bmp4 in spermatogenesis and in maintaining epididymal integrity. Dev Biol 2004; 276: 158-171.
109. Baleato RM, Aitken RJ, Roman SD. Vitamin A regulation of BMP4 expression in the male germ line. Dev Biol 2005; 286: 78-90.
110. Vale W, Rivier J, Vaughan J, McClintock R, Corrigan A, Woo W, Karr D, Spiess J. Purification and characterization of an FSH releasing protein from porcine ovarian follicular fluid. Nature 1986; 321: 776-779.

111. Mathews LS. Activin receptors and cellular signaling by the receptor serine kinase family. Endocr Rev 1994; 15: 310-325.
112. Roberts V, Meunier H, Sawchenko PE, Vale W. Differential production and regulation of inhibin subunits in rat testicular cell types. Endocrinology 1989; 125: 2350-2359.
113. Tanimoto Y, Tanimoto K, Sugiyama F, Horiguchi H, Murakami K, Yagami K, Fukamizu A. Male sterility in transgenic mice expressing activin betaA subunit gene in testis. Biochem Biophys Res Commun 1999; 259: 699-705.
114. Ferguson CA, Tucker AS, Heikinheimo K, Nomura M, Oh P, Li E, Sharpe PT. The role of effectors of the activin signalling pathway, activin receptors IIA and IIB, and Smad2, in patterning of tooth development. Development 2001; 128: 4605-4613.
115. Massague J, Chen YG. Controlling TGF-beta signaling. Genes Dev 2000; 14: 627-644.
116. Matzuk MM, Kumar TR, Vassalli A, Bickenbach JR, Roop DR, Jaenisch R, Bradley A. Functional analysis of activins during mammalian development. Nature 1995; 374: 354-356.
117. Matsui Y, Zsebo K, Hogan BL. Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. Cell 1992; 70: 841-847.
118. Resnick JL, Bixler LS, Cheng L, Donovan PJ. Long-term proliferation of mouse primordial germ cells in culture. Nature 1992; 359: 550-551.
119. Kubota H, Avarbock MR, Brinster RL. Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. Proc Natl Acad Sci U S A 2004; 101: 16489-16494.
120. Ribatti D, Vacca A, Rusnati M, Presta M. The discovery of basic fibroblast growth factor/fibroblast growth factor-2 and its role in haematological malignancies. Cytokine Growth Factor Rev 2007; 18: 327-334.
121. Johnson DE, Williams LT. Structural and functional diversity in the FGF receptor multigene family. Adv Cancer Res 1993; 60: 1-41.
122. Ornitz DM, Xu J, Colvin JS, McEwen DG, MacArthur CA, Coulier F, Gao G, Goldfarb M. Receptor specificity of the fibroblast growth factor family. J Biol Chem 1996; 271: 15292-15297.
123. Goriely A, McVean GA, Rojmyr M, Ingemarsson B, Wilkie AO. Evidence for selective advantage of pathogenic FGFR2 mutations in the male germ line. Science 2003; 301: 643-646.
124. Simon L, Ekman GC, Tyagi G, Hess RA, Murphy KM, Cooke PS. Common and distinct factors regulate expression of mRNA for ETV5 and GDNF, Sertoli cell proteins essential for spermatogonial stem cell maintenance. Exp Cell Res 2007; 313: 3090-3099.
125. Chen C, Ouyang W, Grigura V, Zhou Q, Carnes K, Lim H, Zhao GQ, Arber S, Kurpios N, Murphy TL, Cheng AM, Hassell JA, Chandrashekar V, Hofmann MC, Hess RA, Murphy KM. ERM is required for transcriptional control of the spermatogonial stem cell niche. Nature 2005; 436: 1030-1034.
126. de Rooij DG, de Boer P. Specific arrests of spermatogenesis in genetically modified and mutant mice. Cytogenet Genome Res 2003; 103: 267-276.
127. green mc. Luxoid - a new hereditary leg and foor abnormality: in the house mouse. J of Hered 1955; 46: 91-99.
128. Forsthoefel PF. The skeletal effects of the luxoid gene in the mouse, including its interactions with the luxate gene. J Morphol 1958; 102: 247-287.
129. Filipponi D, Hobbs RM, Ottolenghi S, Rossi P, Jannini EA, Pandolfi PP, Dolci S. Repression of kit expression by Plzf in germ cells. Mol Cell Biol 2007.
130. De Rooij D. Regulation of spermatogonial stem cell behavior. Anim. Reprod. 2006; 3: 130-134.

Chapter 2: LY6A/E (SCA-1) Expression in the Mouse Testis

Maaike P.A. van Bragt[1], Nadia Ciliberti[2], William L. Stanford[2], Dirk G. de Rooij[1,3] and Ans M.M. van Pelt[1]

1 Department of Endocrinology, Faculty of Biology, Utrecht University, 3584 CH Utrecht, The Netherlands
2 Institute of Biomaterials and Bioengineering, Department of Chemical Engineering and Applied Chemistry, Institute of Medical Science, University of Toronto, Toronto, Canada M5S 1A8
3 Department of Cell Biology, University Medical Center Utrecht, 3584 CH Utrecht, The Netherlands

BIOLOGY OF REPRODUCTION 73, 634–638 (2005)

Abstract

Recently, it was found by two research groups that LY6A, known widely in the stem cell community as stem cell antigen-1 or SCA-1, is expressed on testicular side population (SP) cells. Whether these SP cells are spermatogonial stem cells is a point of disagreement and, therefore, the identity of the LY6A-positive cells as well. We studied the expression pattern of LY6A in testis by immunohistochemistry and found it to be expressed in the interstitial tissue on peritubular myoid, endothelial and spherical-shaped peritubular mesenchymal cells. To address the question whether LY6A has a function in spermatogenesis or testis development, we studied the testis of $Ly6a^{-/-}$ mice (allele Ly6atm1Pmf). We found no morphological abnormalities or differences in numbers of spermatogonia, spermatocytes, Leydig cells, or macrophages in relation to the number of Sertoli cells. Therefore, we conclude that LY6A expression does not influence testis development or spermatogenesis and that spermatogonial stem cells are LY6A negative.

Introduction

Spermatogenesis, the process during which spermatozoa are formed after several steps of mitosis, meiosis, and differentiation, begins with the spermatogonial stem cells. Spermatogonial stem cells must self-renew to keep spermatogenesis going throughout life, and, on the other hand, must give rise to differentiating cells to ultimately produce spermatozoa [1]. The mechanisms by which stem cell fate is regulated are still largely unknown. Research in this field is hampered by the fact that there are only 35 000 spermatogonial stem cells in a mouse testis [2] and by the fact that spermatogonial stem cells cannot yet be isolated from the testis as a pure population.

Attempts to isolate spermatogonial stem cells were made and considerable enrichment was reached by using surface markers, such as integrin alpha 6, KIT, and integrin alpha v [3]. Progress in the purification protocol has been possible by the use of the spermatogonial stem cell transplantation technique as an assay for these cells. Only true spermatogonial stem cells will be able to colonize and repopulate depleted seminiferous tubules of recipient mice [4].

Goodell *et al.* [5] were the first to discover that a small, distinct subset of bone-marrow cells can easily be isolated with fluorescence activated cell sorting (FACS) when making use of the fact that these cells are able to quickly expel the fluorescent dye, Hoechst 33342 (Hoechst). The isolated cells were called side population (SP) cells, and they were also found in other tissues, i.e., skeletal muscle [6], mammary gland

[7], brain [8], and liver [9]. It was suggested that the SP cells are the most primitive and true stem cells in each of these tissues.

The testis also contains SP cells [10–13]. Kubota *et al.* [10] were the first to report the existence of SP cells in the testis. Intriguingly, these authors did not find colonization of recipient mouse testes with SP cells from adult cryptorchid mice. The surface markers they found on the SP cells were also different from those present on spermatogonial stem cells. In contrast, Lassalle *et al.* [11] reported that their isolated testicular SP cells were able to colonize recipient mouse testes upon transplantation and hence were spermatogonial stem cells. In addition, they showed that the SP cells expressed Stra8 and integrin alpha 6, two germinal cell markers [3, 14]. Similar results were obtained by Falciatori *et al.* [12] by isolating SP cells from 20-day-old mice. Interestingly, Lo *et al.* [13] found restoration of Leydig cell numbers and testosterone levels in LH receptor-knockout (LhrKO) recipients when SP cells were transplanted into the interstitial tissue of the testis. LhrKO mice are infertile due to targeted disruption of LH/hCG [15]. Restoration of Leydig cell numbers and testosterone levels in LhrKO mice after transplantation with SP cells indicates that testicular SP cells include progenitors of Leydig cells.

Both Kubota *et al.* [10] and Falciatori *et al.* [12] looked for expression of surface markers on testicular SP cells. The marker they both found to be present on testicular SP cells was the glycosyl phosphatidylinositol-linked cell-surface glycoprotein, LY6A (also known as SCA-1). LY6A is frequently used for isolation of hematopoietic stem cells. Sorting with the specific E13-161.7 antibody yields a 100-fold enrichment of hematopoietic stem cells out of bone marrow [16]. Expression of LY6A is not restricted to hematopoietic stem cells, it is also found on skeletal muscle stem cells, mammary epithelium stem cells, kidney epithelial cells, osteoblasts, and vasculature of brain, heart, and liver [6, 7, 17, 18]. LY6A is encoded by the prototypic member of the Ly-6 gene family, Ly6a, encoding two alleles (Ly6a.1 or Ly6e and Ly6a.2) in a mouse strain-specific manner [17]. Thus, LY6A has also been referred to as Ly6A/E. In addition to differing by two amino acids, the two alleles demonstrate differential tissue distribution due to differential transcriptional or posttranscriptional regulation [19, 20]. In Ly6a.2 mouse strains, including C57BL/6, essentially all bone marrow-repopulating cells are LY6A-positive compared with 25% in Ly6a.1/Ly6e strains, such as BALB/c [21]. Also, the number of LY6A-positive thymocytes and peripheral T lymphocytes is different between different mouse strains [21, 22]. In $Ly6a^{-/-}$ mice, LY6A was found to have important functions in regulating the repopulating capacity of hematopoietic stem cells and the development of committed progenitor cells, megakaryocytes, and platelets [23]. $Ly6a^{-/-}$ mice also exhibit defects in T cell signaling [24] and self-renewal capacity of early mesenchymal precursors [25], that give rise to adipocytes, osteoblasts, chondrocytes, and muscle cells [26, 27].

In this paper, for the first time, the localization of LY6A in the testis is described. Furthermore, to determine a possible role of LY6A in spermatogenesis or testis development, we studied the morphology and numbers of various testicular cell types in Ly6a$^{-/-}$ mice.

Material and methods

Animals

Ly6a$^{-/-}$ mice (allele Ly6atm1Pmf) on the BALB/c background and BALB/c wild-type littermates were generated as described before and maintained at Mount Sinai Hospital, Toronto [24, 25]. FVB and C57BL/ 6 mice were used and maintained according to regulations provided by the animal ethical committee of the University of Utrecht, which also approved the experiments.

Immunohistochemistry

For immunolocalization of LY6A, testes from adult mice of different strains, FVB, C57BL/6, and BALB/c, were fixed in Bouin fluid. Five-micrometer paraffin sections were mounted on 3-aminopropyl triethoxysilane (TESPA, Sigma, St. Louis, MO)-coated glass slides and dried overnight at 37˚C. Endogenous peroxidase was blocked with 0.35% H_2O_2 in PBS for 15 min. After blocking in 5% normal rabbit serum (Vector Laboratories, Burlingame, CA), slides were incubated overnight at 4˚C with 1:200 LY6A/E antibody (E13.161.7 no. 553333 or D7 no. 557403; BD Biosciences, Franklin Lakes, NJ) in 1% BSA in PBS (Sigma, St. Louis, MO). Secondary biotinylated rabbit anti-rat antibody (BA-4000, Vector Laboratories) was used 1:200 in 1% BSA in PBS for 1 h at room temperature. Horseradish peroxidase avidin-biotin complex reaction was performed according to the manufacturer's protocol (Vector Laboratories). Antibody was finally detected by diaminobenzidine (DAB; Sigma) in 50 mM Tris-HCl, pH 7.6. The reaction was amplified with cobalt-chloride (0.025%) and nickel ammonium sulfate (0.02%). Sections were counterstained with nuclear fast red, dehydrated, and mounted with Pertex (Cellpath Ltd., Hemel Hempstead, U.K.). For positive and negative controls, Bouin-fixed kidney [28] and Ly6a$^{-/-}$ mice testis were used, respectively.

Immunofluorescence

Immunolocalization of LY6A in adult mice testis was performed on Bouin-fixed testes of different mice strains, FVB, C57BL/6, and BALB/ c. Five-micrometer paraffin

sections were mounted on TESPA-coated glass slides and dried overnight at 37˚C. Slides were incubated overnight at 4˚C with 1:50 LY6A/E antibody (E13.161.7 no. 553333 or D7 no. 557403; BD Biosciences) in 5% normal rabbit serum in Coons buffer (10 mM 5,5-diethylbarbituric acid sodium salt, 145 mM NaCl, pH 7.4, 0.01% BSAc, 0.2% Triton x-100). Second, rabbit anti-rat (BA-4000; Vector Laboratories), and third antibody, goat anti-rabbit Texas red, were used 1:100 in coons and incubated for 1 h at room temperature. Sections were mounted with VECTAshield (Vector Laboratories) and viewed on a Leitz DMIRB fluorescence microscope (Leica, Voorburg, The Netherlands) interfaced with a Leica TCS4D confocal laser scanning microscope (Leica, Heidelberg, Germany). Images were recorded digitally. For positive and negative controls, Bouin-fixed kidney and Ly6a$^{-/-}$ mice testis were used, respectively.

Cell Counts

Five-micrometer sections were stained with periodic acid Schiff (PAS) and counterstained with hematoxylin. Numbers of A spermatogonia, preleptotene spermatocytes, Leydig cells, and macrophages were counted in 10- and 19-wk-old Ly6a$^{-/-}$ and wild-type littermate testes. For 10- and 19-wk-old animals, four and three testes of either genotype were counted, respectively. Cell numbers were expressed per 1000 Sertoli cells according to the method of Heller *et al.* [29, 30]. Cell counts were performed until at least 300 Sertoli cells were scored. All cell numbers were expressed in mean ± SEM, and statistical analysis was performed using the unpaired t-test.

Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine 5-Triphosphate-Biotin Nick End Labeling on Ly6a$^{-/-}$ Mice Testis

Detection of apoptotic cells was performed on 10- and 19-wk-old Ly6a$^{-/-}$ mice and wild-type littermates. Five-micrometer Bouin-fixed, paraffin-embedded testis sections were boiled for 5 min in 10 mM citric buffer (pH 6.0) at 98˚C and slowly cooled to room temperature. Endogenous peroxidase was blocked with 3% H_2O_2 in MilliQ (MQ) for 5 min. Sections were washed three times with PBS before 60 min incubation in 5-triphosphate-biotin nick end labeling (TUNEL) mix at 37˚C. TUNEL mix consists of 0.3 U/µl calf thymus terminal deoxynucleotidyl transferase (Amersham Biosciences, Freiburg, Germany), 6.66 µM/µl biotin dUTP (Roche, Basel, Switzerland) in terminal transferase buffer (Amersham Biosciences). TUNEL reaction was stopped by incubation in 300 mM NaCl, 30 mM sodium citrate in MQ for 15 min at room temperature. After washing with PBS, sections were blocked with 2% BSA (Sigma) in PBS at room temperature for 10 min. Sections were treated for 30 min at 37˚C in a

moist chamber with a 1:20 dilution of ExtrAvidin peroxidase antibody. After three washes in PBS, detection was performed with DAB+ (Dako, Glostrup, Denmark). Sections were counterstained with Mayer hematoxylin, dehydrated, and mounted with Pertex (Cellpath Ltd., Hemel Hempstead, U.K.). The number of TUNEL-positive cells was counted in four and three testes of 10- and 19-wk old-animals, respectively, and calculated as number per 100 tubule cross sections. At least 100 tubules were counted. Numbers were expressed as mean ± SEM, and statistical analysis was performed using the unpaired t-test.

Results

Immunolocalization of LY6A in the Testis

To investigate the localization of LY6A in the testis, we performed both immunohistochemical and immunofluorescence studies with the monoclonal antibody, E13.161.7, that recognizes LY6A in both Ly6a and Ly6e mouse strains. With both techniques, a similar expression pattern was observed. In C57BL/6 (not shown) and FVB (Fig. 1, A, D, and G) wild-type mice, the cell membrane of most peritubular myoid cells as well as endothelial cells (Fig. 1G) were stained. In BALB/c mice some peritubular myoid, but no endothelial cells were stained (Fig. 1, B, E, and H).

In addition, staining was often observed in spindle-shaped peritubular mesenchymal cells (Fig. 1H) in the interstitial tissue of all three mouse strains. No staining was present in seminiferous tubules in any of the strains tested. Also, in Leydig cells and macrophages, no staining could be observed in any of the strains. As a negative control, testes of Ly6a$^{-/-}$ mice were used. No staining was observed in these control sections (Fig. 1, C and F). For positive control, we showed expression of LY6A in the renal vessels of the kidney (Fig. 1I). Furthermore, using the D7 monoclonal antibody for both immunohistochemistry and immunofluorescence, the same expression pattern was observed as with the E13.161.7 antibody (data not shown).

Morphology and Cell Counting in Ly6a$^{-/-}$ Mice Testes

To address the question whether LY6A has a function in spermatogenesis, we studied testes of 10- and 19-wk-old Ly6a$^{-/-}$ mice and wild-type littermates. PAS-stained tubules cross sections of Ly6a$^{-/-}$ mice appear normal, i.e., a lumen is formed and all spermatogenic cell types are present (Fig. 2A). To study this in more detail we counted the numbers of A spermatogonia and preleptotene spermatocytes in epithelial stages VII and VIII in 10- and 19-wk- old animals and found no significant differences between Ly6a$^{-/-}$ and their wild-type littermates (Table 1). In the interstitial tissue, we

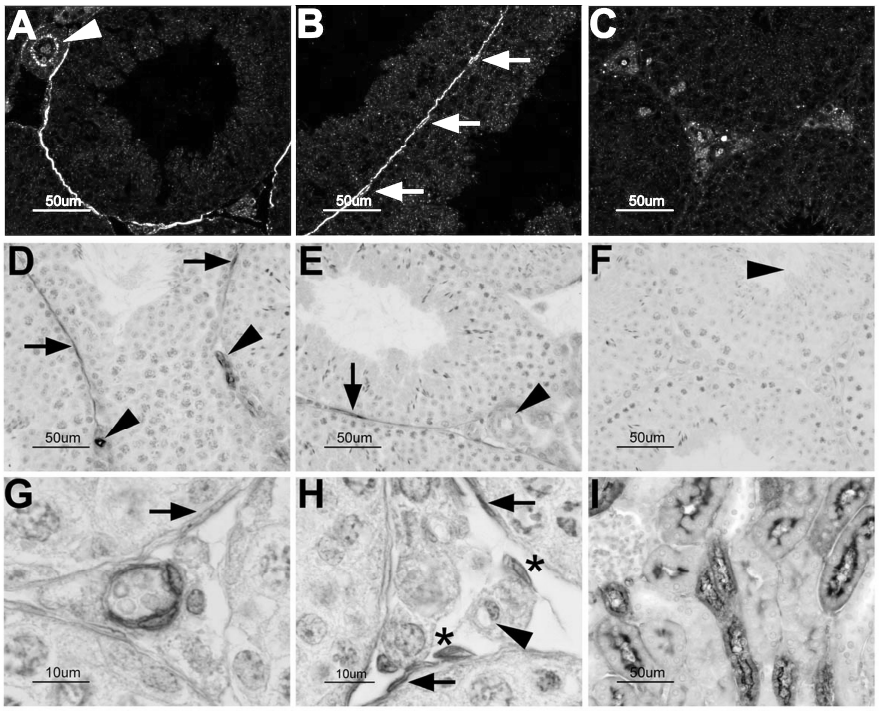


Figure 1. Immunolocalization of LY6A in the testis of different mice strains. A–C) Immunofluorescence staining of LY6A in FVB (A), BALB/c (B), and Ly6a$^{-/-}$ mouse testis (C). (D–I) Immunohistochemical staining of LY6A in FVB (D), BALB/c (E), and Ly6a$^{-/-}$ mouse testis (F). (G–H) Higher magnification of FVB (G) and BALB/c testis (H). Indicated are endothelial cells (arrowheads), peritubular myoid cells (arrow), and spherical-shaped peritubular mesenchymal cells (asterisk). No staining is observed in Leydig cells nor in seminiferous tubules. I) Kidney; staining is observed in the renal vessels [28.]

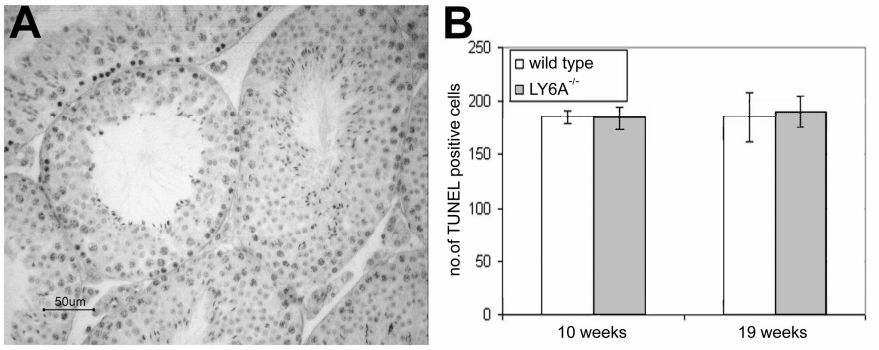


Figure 2. Morphology and apoptosis in Ly6a$^{-/-}$ testis. A) Ly6a$^{-/-}$ mice testis. B) The number of TUNEL-positive germ cells ± SEM per 100 tubular cross sections of Ly6a$^{-/-}$ mice and wild-type littermates testis of 10 and 19 weeks.

counted the numbers of Leydig cells and macrophages in 10- and 19-wk-old mice. No significant differences in the numbers of Leydig cells or macrophages were observed between Ly6a$^{-/-}$ mice and their wild-type littermates (Table 1).

Apoptosis in Ly6a$^{-/-}$ Mice Testes

To determine if LY6A deficiency causes abnormal apoptosis of germ cells, we performed a TUNEL assay on Ly6a$^{-/-}$ mice and wild-type testes. No significant differences were found between the numbers of apoptotic germ cells in Ly6a$^{-/-}$ mice and wild-type littermates (Fig. 2B). In the interstitial tissue of both Ly6a$^{-/-}$ and wild-type testes, we sporadically observed a TUNEL-positive Leydig cell. No TUNEL staining was observed in the peritubular myoid, endothelial, or peritubular mesenchymal cells.

Table 1. Number of A spermatogonia, spermatocytes, Leydig cells, and macrophages in Ly6a$^{-/-}$ and wt littermates of 10 and 19 wk[*].

Cell type	10 weeks		19 weeks	
	Wt	*Sca-1/Ly6A*-null	Wt	*Sca-1/Ly6A*-null
A spermatogonia	343 ± 14	337 ± 37	363 ± 13	387 ± 60
preleptotene spermatocytes	5478 ± 217	5963 ± 196	5603 ± 420	5857 ± 89
Leydig cells	1148 ± 15	1079 ± 32	1197 ± 34	1059 ± 62
macrophages	115 ± 14	117 ± 3	158 ± 30	161 ± 11

[*]*Numbers are expressed as mean ± SEM number per 1000 Sertoli cells.*

Discussion

The present data, for the first time, show that, in the testis, LY6A is solely expressed in the interstitial tissue and not in seminiferous tubules. In the three mouse strains tested, we found LY6A to be expressed in peritubular myoid cells and in spindle-shaped peritubular mesenchymal cells. Furthermore, we found in both FVB and C57BL/6 but not in BALB/c mice testis, LY6A to be expressed in endothelial cells.

Difference in expression pattern of LY6A between different mouse strains is a known phenomenon. For both lymphocytes and hematopoietic stem cells, LY6A expression was found to differ between haplotypes [21, 22]. There are two allelic variants of Ly6a, Ly6e, and Ly6a.2, which are under distinct genetic control, resulting in two haplotypes, Ly6a and Ly6b, respectively. BALB/c encodes the Ly6a haplotype, while C57BL/6 encodes the Ly6b haplotype [21]. This may well explain the difference in expression of LY6A in endothelial cells in C57BL/6 versus BALB/c mice. Expression of

LY6A was shown before in vasculature of heart, brain, and liver in C57BL/6 mice [17]. The haplotype of FVB mice is not known, but our results would now suggest that FVB mice have the Ly6b haplotype.

Testicular peritubular myoid [31], endothelial [32], and peritubular mesenchymal cells [31] all originate from the mesonephros. It was shown that the aorta-gonads-mesonephros (AGM) region is largely LY6A positive [33]. The hematopoietic stem cells as well as endothelial cells also arise from the AGM region [34, 35] and show LY6A expression [16, 17], suggesting a relationship between LY6A expression and the embryonic origin. However, there are also LY6A-positive cells that may not arise from the AGM region.

The germ cells and thus the spermatogonial stem cells originate from extraembryonic regions [36]. In this article, we show that LY6A expression is absent in all germ cells, including the spermatogonial stem cells. This is surprising because stem cells of many other tissues are found to be LY6A positive. However, satellite cells of striated muscle tissue are also LY6A negative [37]. Furthermore, it was already suggested by Kubota *et al.* [10] that spermatogonial stem cells do not express LY6A on their surface.

LY6A is known to be expressed on SP cells of many tissues, including hematopoietic system [16], mammary gland [7], and skeletal muscle [6, 37]. Recently, four groups showed the presence of SP cells in the testis [10–13]. While Kubota *et al.* [10] found SP cells not be able to colonize the testis of busulfan-treated recipient mice, the opposite was found by Falciatori *et al.* [12] and Lassalle *et al.* [11], indicating disagreement about the question whether or not testicular SP cells are spermatogonial stem cells. The differences between these findings may have various causes. First, the groups use different types of donor mice, adult versus immature, and cryptorchid versus noncryptorchid. Second, for the isolation of SP cells, different protocols were used with respect to tissue dissociation, Hoechst concentration, time and temperature of Hoechst incubation, and stringency in the selection of SP cells (FACS gating). Recently, it was described that these parameters should be determined for each tissue tested because these factors all affect yield, viability, and homogeneity of SP cells and differ per tissue [38]. Therefore, different cell populations can be isolated by using different isolation protocols. Further research should be performed to optimize the SP protocol and to investigate whether there is a testicular SP that harbors spermatogonial stem cells.

Nevertheless Kubota *et al.* [10] and Lassalle *et al.* [11] agree that testicular SP cells express LY6A brightly and therefore it was important to identify the LY6A-positive cells to unravel the confusion about the identity of testicular SP cells.

Our results support the findings of Kubota *et al.* [10] that LY6A-positive SP cells are not spermatogonial stem cells because no staining for LY6A was observed in the

seminiferous tubules, including spermatogonial stem cells. Interestingly, the present data support and extend the results obtained by Lo *et al.* [13, 39], who showed restoration of Leydig cell numbers and testosterone levels in LhrKO mice after transplantation of SP cells into the interstitial tissue. They conclude that Leydig cell progenitors are among the SP cells. The present results indicate that spindle-shaped peritubular mesenchymal cells, which are thought to be progenitors of Leydig cells [40], express LY6A on their cell membranes.

LY6A is not only useful as a surface marker, it also plays an important role in the regulation of hematopoietic stem cells [23] and mesenchymal progenitors [25]. Ly6a$^{-/-}$ mice exhibit an age-dependent osteoporosis phenotype due to a primary defect in the self-renewal capacity of mesenchymal progenitors [25]. Furthermore, they have defects in the repopulating ability of hematopoietic stem cells upon competitive and serial transplantation of hematopoietic stem cells in lethally irradiated mice [23]. In contrast, we found that development and morphological appearance of the Ly6a$^{-/-}$ testes were normal in 10- or 19-wk-old animals. Also, the numbers of spermatogonia and preleptotene spermatocytes in stages VII/VIII of the epithelial cycle were similar to those in wild-type mice. This suggests that spermatogonial proliferation in wild-type and Ly6a$^{-/-}$ mice is similar with respect to kinetics as well as the numbers of spermatocytes produced. Furthermore, Leydig cell numbers were not significantly different in 10- or 19-wk-old Ly6a$^{-/-}$ mice and age-matched wild-type mice, indicating that Leydig cells are normally formed and do not deplete until at least 19 wk of age. However, turnover of interstitial cells, including progenitors as well as adult Leydig cells, is low [41]. Therefore, there might be depletion of peritubular mesenchymal progenitors and Leydig cells in older mice. Challenging of the Ly6a$^{-/-}$ peritubular mesenchymal progenitors, by transplanting them into the interstitium of LhrKO recipients testis, might, however, reveal a function of LY6A in the repopulating capacity of these cells.

Based on our results, we can conclude that LY6A-positive cells in the testis are not spermatogonial stem cells but do include cells with morphological appearance of Leydig cell progenitors. Furthermore, LY6A deficiency does not influence testis development, spermatogenesis, or peritubular mesenchymal cell and Leydig cell development. The present results also indicate the importance of performing localization analysis in situ rather then just relying on cell sorting and FACS analysis.

References

1 De Rooij DG. Stem cells in the testis. Int J Exp Pathol 1998 79:67-80

2 Tegelenbosch RA, de Rooij DG. A quantitative study of spermatogonial multiplication and stem cell renewal in the C3H/101 F1 hybrid mouse. Mutat Res 1993 290:193-200

3 Shinohara T, Orwig KE, Avarbock MR, Brinster RL. Spermatogonial stem cell enrichment by multiparameter selection of mouse testis cells. Proc Natl Acad Sci U S A 2000 97:8346-8351

4 Ogawa T, Arechaga JM, Avarbock MR, Brinster RL. Transplantation of testis germinal cells into mouse seminiferous tubules. Int J Dev Biol 1997 41:111-122

5 Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med 1996 183:1797-1806

6 Gussoni E, Soneoka Y, Strickland CD, Buzney EA, Khan MK, Flint AF, Kunkel LM, Mulligan RC. Dystrophin expression in the mdx mouse restored by stem cell transplantation. Nature 1999 401:390-394

7 Welm BE, Tepera SB, Venezia T, Graubert TA, Rosen JM, Goodell MA. Sca-1(pos) cells in the mouse mammary gland represent an enriched progenitor cell population. Dev Biol 2002 245:42-56

8 Murayama A, Matsuzaki Y, Kawaguchi A, Shimazaki T, Okano H. Flow cytometric analysis of neural stem cells in the developing and adult mouse brain. J Neurosci Res 2002 69:837-847

9 Shimano K, Satake M, Okaya A, Kitanaka J, Kitanaka N, Takemura M, Sakagami M, Terada N, Tsujimura T. Hepatic oval cells have the side population phenotype defined by expression of ATP-binding cassette transporter ABCG2/BCRP1. Am J Pathol 2003 163:3-9

10 Kubota H, Avarbock MR, Brinster RL. Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells. Proc Natl Acad Sci U S A 2003 100:6487-6492

11 Lassalle B, Bastos H, Louis JP, Riou L, Testart J, Dutrillaux B, Fouchet P, Allemand I. 'Side population' cells in adult mouse testis express Bcrp1 gene and are enriched in spermatogonia and germinal stem cells. Development 2004 131:479-487

12 Falciatori I, Borsellino G, Haliassos N, Boitani C, Corallini S, Battistini L, Bernardi G, Stefanini M, Vicini E. Identification and enrichment of spermatogonial stem cells displaying side-population phenotype in immature mouse testis. FASEB J 2004 18:376-378

13 Lo KC, Lei Z, Rao CV, Beck J, Lamb DJ. De novo testosterone production in luteinizing hormone receptor knockout mice after transplantation of Leydig stem cells. Endocrinology 2004 145:4011-4015

14 Oulad-Abdelghani M, Bouillet P, Decimo D, Gansmuller A, Heyberger S, Dolle P, Bronner S, Lutz Y, Chambon P. Characterization of a premeiotic germ cell-specific cytoplasmic protein encoded by Stra8, a novel retinoic acid-responsive gene. J Cell Biol 1996 135:469-477

15 Lei ZM, Mishra S, Zou W, Xu B, Foltz M, Li X, Rao CV. Targeted disruption of luteinizing hormone/human chorionic gonadotropin receptor gene. Mol Endocrinol 2001 15:184-200

16 Spangrude GJ, Heimfeld S, Weissman IL. Purification and characterization of mouse hematopoietic stem cells. Science 1988 241:58-62

17 van de Rijn M, Heimfeld S, Spangrude GJ, Weissman IL. Mouse hematopoietic stem-cell antigen Sca-1 is a member of the Ly-6 antigen family. Proc Natl Acad Sci U S A 1989 86:4634-4638

18 Van Vlasselaer P, Falla N, Snoeck H, Mathieu E. Characterization and purification of osteogenic cells from murine bone marrow by two-color cell sorting using anti-Sca-1 monoclonal antibody and wheat germ agglutinin. Blood 1994 84:753-763

19 Reiser H, Coligan J, Palmer E, Benacerraf B, Rock KL. Cloning and expression of a cDNA for the T-cell-activating protein TAP. Proc Natl Acad Sci U S A 1988 85:2255-2259

20 LeClair KP, Palfree RG, Flood PM, Hammerling U, Bothwell A. Isolation of a murine Ly-6 cDNA reveals a new multigene family. EMBO J 1986 5:3227-3234
21 Spangrude GJ, Brooks DM. Mouse strain variability in the expression of the hematopoietic stem cell antigen Ly-6A/E by bone marrow cells. Blood 1993 82:3327-3332
22 Codias EK, Cray C, Baler RD, Levy RB, Malek TR. Expression of Ly-6A/E alloantigens in thymocyte and T-lymphocyte subsets: variability related to the Ly-6a and Ly-6b haplotypes. Immunogenetics 1989 29:98-107
23 Ito CY, Li CY, Bernstein A, Dick JE, Stanford WL. Hematopoietic stem cell and progenitor defects in Sca-1/Ly-6A-null mice. Blood 2003 101:517-523
24 Stanford WL, Haque S, Alexander R, Liu X, Latour AM, Snodgrass HR, Koller BH, Flood PM. Altered proliferative response by T lymphocytes of Ly-6A (Sca-1) null mice. J Exp Med 1997 186:705-717
25 Bonyadi M, Waldman SD, Liu D, Aubin JE, Grynpas MD, Stanford WL. Mesenchymal progenitor self-renewal deficiency leads to age-dependent osteoporosis in Sca-1/Ly-6A null mice. Proc Natl Acad Sci U S A 2003 100:5840-5845
26 Bellows CG, Wang YH, Heersche JN, Aubin JE. 1,25-Dihydroxyvitamin D3 stimulates adipocyte differentiation in cultures of fetal rat calvaria cells: comparison with the effects of dexamethasone. Endocrinology 1994 134:2221-2229
27 Aubin JE. Bone stem cells. J Cell Biochem Suppl 1998 30: –31 73-82
28 Kotton DN, Summer RS, Sun X, Ma BY, Fine A. Stem cell antigen-1 expression in the pulmonary vascular endothelium. Am J Physiol Lung Cell Mol Physiol 2003 284:L990-996
29 Heller CG, Lalli MF, Pearson JE, Leach DR. A method for the quantification of Leydig cells in man. J Reprod Fertil 1971 25:177-184
30 Teerds KJ, Closset J, Rommerts FF, de Rooij DG, Stocco DM, Colenbrander B, Wensing CJ, Hennen G. Effects of pure FSH and LH preparations on the number and function of Leydig cells in immature hypophysectomized rats. J Endocrinol 1989 120:97-106
31 Buehr M, Gu S, McLaren A. Mesonephric contribution to testis differentiation in the fetal mouse. Development 1993 117:273-281
32 Martineau J, Nordqvist K, Tilmann C, Lovell-Badge R, Capel B. Male-specific cell migration into the developing gonad. Curr Biol 1997 7:958-968
33 de Bruijn MF, Ma X, Robin C, Ottersbach K, Sanchez MJ, Dzierzak E. Hematopoietic stem cells localize to the endothelial cell layer in the midgestation mouse aorta. Immunity 2002 16:673-683
34 Medvinsky A, Dzierzak E. Definitive hematopoiesis is autonomously initiated by the AGM region. Cell 1996 86:897-906
35 Sainio K, Raatikainen-Ahokas A. Mesonephric kidney—a stem cell factory?. Int J Dev Biol 1999 43:435-439
36 Ginsburg M, Snow MH, McLaren A. Primordial germ cells in the mouse embryo during gastrulation. Development 1990 110:521-528
37 Asakura A, Seale P, Girgis-Gabardo A, Rudnicki MA. Myogenic specification of side population cells in skeletal muscle. J Cell Biol 2002 159:123-134
38 Montanaro F, Liadaki K, Schienda J, Flint A, Gussoni E, Kunkel LM. Demystifying SP cell purification: viability, yield, and phenotype are defined by isolation parameters. Exp Cell Res 2004 298:144-154
39 De Rooij DG, Van Bragt MP. Leydig cells: testicular side population harbors transplantable Leydig stem cells. Endocrinology 2004 145:4009-4010
40 Mendis-Handagama SM, Ariyaratne HB. Differentiation of the adult Leydig cell population in the postnatal testis. Biol Reprod 2001 65:660-671
41 Teerds KJ, De Rooij DG, Rommerts FF, van der Tweel I, Wensing CJ. Turnover time of Leydig cells and other interstitial cells in testes of adult rats. Arch Androl 1989 23:105-111

Chapter 3: Expression of pluripotency marker, UTF1, is restricted to a subpopulation of early A spermatogonia in rat testis

Maaike P.A. van Bragt[1], Hermien L. Roepers-Gajadien[1], Cindy M. Korver[2], Jan Bogerd[1], Akihiko Okuda[3], Bart J.L. Eggen[4], Dirk G. de Rooij[1,2] and Ans M.M. van Pelt[2]

Department of Endocrinology and Metabolism, Faculty of Science, Utrecht University, Utrecht, Netherlands

2 Center for Reproductive Medicine, Academic Medical Center, 1105 AZ Amsterdam, the Netherlands

3 Division of Developmental Biology, Research Center for Genomic Medicine, Saitama Medical University, 1397-1 Yamane, Hidaka, Saitama 350-1241, Japan

4 Department of Developmental Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, 9751 NN Haren, the Netherlands

REPRODUCTION. 2008 APRIL

Abstract

The population of early A spermatogonia includes stem cells that possess spermatogonial stem cell properties. Recent reports suggest that these cells have the ability to regain pluripotent properties. Here we show that expression of pluripotency marker undifferentiated embryonic cell transcription factor 1 (UTF1) is restricted to distinct germ cells within the testis. In embryonic and neonatal testes, all gonocytes were found to strongly express UTF1. During further testicular development, expression of UTF1 was restricted to a subset of A spermatogonia and with the increase in age the number of cells expressing UTF1 decreased even more. Ultimately, in adult rat testes, only a small subset of the A spermatogonia expressed UTF1. Remarkably, even in testes of vitamin A deficient rats, in which the early A spermatogonia are the only type of spermatogonia, only a subset of the spermatogonia expressed UTF1. In adult rat testis expression of utf1 is restricted to a subpopulation of the PLZF positive early A spermatogonia. Furthermore, the observed distribution pattern of UTF1 expressing cells over the different stages of the cycle of the seminiferous epithelium suggests that expression of UTF1 is restricted to those A_s, A_{pr} and short chains of A_{al} spermatogonia that are in the undifferentiated state and therefore maintain the ability to differentiate into A1 spermatogonia in a next round of the epithelial cycle or possibly even in other directions when taken out of their testicular niche.

Introduction

Spermatogenesis is a continues process starting with spermatogonial stem cells [1, 2], which were until recently thought of as unipotent stem cells only being able to form cells of the spermatogenic lineage [3, 4]. However, recent reports suggest that these cells have a greater potential. Testes of both neonatal and 6 weeks old mice were reported to contain pluripotent cells or cells able to obtain these properties *in vitro* [5-7]. Previously, it was described that primordial germ cells (PGCs), of both human and mouse origin, could give rise to pluripotent stem cells [8, 9]. Finally, PGCs, gonocytes and spermatogonial stem cells express several genes believed to be essential for pluripotency, e.g. octamer binding transcription factor 3/4 (Oct3/4) and Sry box 2 (Sox2) [10, 11].

Another pluripotency-associated gene is UTF1 [12]. This gene was found to be expressed in embryonic stem (ES) cells, embryonal carcinoma (EC) cells and PGCs [12-14]. In adult mice, UTF1 mRNA was only detected in ovary and testis [12]. In EC and ES cells, protein expression of UTF1 was found to be localized to the nucleus and subnuclear fractionation and mobility assays revealed that UTF1 was a chromatin-

associated protein with histone-like properties [15]. Differentiation of EC cells is accompanied by a rapid reduction in UTF1 levels, indicating a stem cell specific function [12, 14, 15]. Furthermore, it was suggested that activation of UTF1 is an important mechanism by which OCT3/4 maintains the stem cell state of ES cells [16]. Experiments indicated that UTF1 plays a role in the proliferation rate and teratoma-forming capacity of ES cells [16]. However, recently it was proposed that UTF1 might be involved in the maintenance of a specific epigenetic profile that allows lineage-specific differentiation of ES and EC cells while it is not involved in stem cell renewal [15].

In order to learn more about a possible role of UTF1 in spermatogenesis, we now studied the localization of UTF1 in the testis at different developmental stages during pre- and postnatal life. During testicular development, expression of UTF1 was restricted to gonocytes and A spermatogonia. Cell counts revealed a decrease in the number of UTF1 positive cells during testicular development and expression in adult rat testes was found to be restricted to a subpopulation of early A spermatogonia.

Material and methods

Animals

Testes from Wistar rats of different ages (16 and 18dpc, 4, 9, 13, 17, 24, 42, 56, and 70 dpp) were snap frozen or fixed for 6 hours in 4% formaldehyde, followed by overnight fixation in diluted Bouin's fluid (71% (v/v) 0.9% picric acid, 24% (v/v) 37% formaldehyde, 5% (v/v) acetic acid). Diluted Bouin's fixed testes, total testis RNA and RNA isolated from A spermatogonia (93% pure) of (VAD) rats were obtained during previous studies [17, 18]. The A spermatogonia were isolated by enzymatic digestion and percoll gradient centrifugation. Purity was determined by Nomarski microscopy [18]. All procedures were performed according to regulations provided by the ethical committee of the University of Utrecht that also approved of the experiments.

DNA Sequencing and RT-PCR

T to determine the rat UTF1 sequence, primers were designed by comparing human and mouse mRNA sequence (accession numbers AB011076 and NM009482, respectively). RT-PCR was performed on cDNA from total testis of adult rat. DNA fragments of approximately 1000 bp were isolated from gel and subcloned into pcDNA3.1/V5-His TOPO vector (Invitrogen, Carlsbad, CA, USA). Plasmid DNA of 2 clones was prepared for DNA sequence analysis. DNA sequence analyses were performed on automated ABI PRISM 310 and 377 DNA sequencers, using Dye

Terminator cycle sequencing chemistry (all from Applied Biosystems, Foster city, CA, USA). Sequence alignment was performed using ClustalW and Genedoc [19, 20]. Amino acid identity and similarity to mouse and human UTF1 sequences were determined with MatGAT 2.0 [21]. RT-PCR on cDNA of total rat testes was performed with the following primers;
Utf1 fw: 5'-cgctcagattcagctcttcc-3', rv: 5'-cgaaggaacctcgtagatgc-3' (333bp),
Pou5f1 fw: 5'-cgaggagtcccaggatatga-3', rv: 5'-gttccacctcacacggttct-3' (334bp),
Sox2 fw: 5'-accagctcgcagacctacat-3', rv: 5'-ccctcccaattcccttgtat-3' (388bp),
Zfp145 fw: 5'-tctgtctgctgtgtgggaag-3', rv: 5'-gtggcagagtttgcactcaa-3' (353bp).

Cell counts and staging

Cells expressing UTF1 and/or PLZF were counted in testes of rats at various ages. Cell numbers were expressed per tubule cross-section. Cell counts were performed until at least 100 tubule cross-sections per testis were scored. Numbers were expressed as means ± SEM. Statistical analysis (paired t-test) was performed with Graphpad Prism 4 (Graphpad Software, San Diego, USA). Adjacent sections were stained with periodic acid Schiff's (PAS) reagent and Mayer's hematoxylin in order to determine the epithelial stage of the tubule cross-sections.

Immunohistochemistry

For immunolocalization of UTF1 and PLZF, adjacent 5μm sections of diluted Bouin's fluid fixed testes were mounted on TESPA coated glass slides. For antigen retrieval, sections were boiled in a microwave (H2500, Bio-rad, Veenendaal, The Netherlands) at 98°C in 0.01M sodium citrate pH6.0. Sections for localization of PLZF were boiled 3 times 10 min. For localization of UTF1, boiling time depended on the antibody used, 1 time 10 min. (AB3383, Chemicon, Temecula, CA, USA; ab24273, Abcam, Cambridge, UK and antibody generated by Okuda *et al.* [12]) or 1 time 20 min. (antibody kindly provided by Dr. Eggen [13, 15]). Endogenous peroxidase was blocked with 0.35% H_2O_2 in PBS for 15 min. After blocking in 5% normal goat serum (NGS) or normal horse serum (NHS) (Vector laboratories, Burlingame, CA, USA), slides were incubated overnight at 4°C with 1:100 UTF1 (AB3383, ab24273, antibody Dr. Okuda), 1:200 (UTF1 antibody Dr. Eggen) or 1:50 PLZF (OP128, Calbiochem, San Diego, CA, USA) in 0.1% BSAc in PBS (Aurion, Wageningen, The Netherlands). Secondary biotinylated goat anti rabbit (BA-1000, Vector laboratories, Burlingame, CA, USA) or horse anti mouse (PK6102, Vector Laboratories) was used 1:200 in 0.1% BSAc in PBS for one hour at RT. Horseradish peroxidase avidin-biotin complex reaction was performed according to the manufacturers protocol (PK6102). Bound antibody

was finally detected by diaminobenzidine (DAB, Sigma, St. Louis, USA). Sections were counterstained with Mayer's hematoxylin. For negative control first antibody was replaced with normal rabbit or normal mouse IgG (sc-2025, sc-2027, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for UTF1 and PLZF, respectively. Figure 3 shows UTF1 immunolocalization with AB3383 antibody.

Immunofluorescence

Co-immunolocalization of UTF1 and PLZF was studied in diluted Bouins's fluid fixed testes of rats at 42, 56 and 70 dpp. Five micrometer paraffin sections were mounted on TESPA-coated glass. For antigen retrieval, sections were boiled at 98°C in 0.01M sodium citrate for 10 min. Sections were blocked in 5% NHS in 0.1% BSAc and incubated overnight at 4°C with 1:100 UTF1 (AB3383) and 1:50 PLZF (OP128) antibody in 0.1% BSAc. To fluorescently label the UTF1 positive cells, slides were incubated with 1:100 goat anti rabbit alexa 488 (A11008, Molecular Probes, Leiden, The Netherlands) in 0.1% BSAc for 1 hour. PLZF antibody was labeled with 1:200 biotinylated horse anti mouse (PK6102) for 1 hour, followed by incubation with 1:200 streptavidin alexa Fluor 495 conjugated (S11227, Molecular Probes) in 0.1% BSAc. Sections were mounted with VECTAshield (Vector Laboratories).

Results

UTF1 mRNA is expressed in testis of neonatal, pubertal and adult rats

To determine *Utf1* mRNA expression in the rat testis of different ages we first obtained the rat *Utf1* sequence. Adult rat total testis RNA was subjected to RT-PCR using primers derived from the human and mouse *Utf1* cDNA sequences.

The resulting rat *Utf1* cDNA sequence (GenBank Acc. No. EU176857) encodes a predicted protein of 338 amino acids and comparison between rat and mouse or human UTF1 showed an overall amino acid identity of 93% and 65% and similarity of 96% and 73%, respectively (Fig.1). Previously, based on the human and mouse UTF1 protein sequences, two conserved domains, CD1 and CD2, were identified [22]. Analysis of the corresponding domains in rat UTF1 revealed identities of 96% and 87% for CD1 and 100% and 86% for CD2 compared to mouse and human UTF1, respectively (Fig.1).

To determine *Utf1* expression during rat testicular development, RT-PCRs were performed on total testis RNA of rats of various ages (Fig.2). *Utf1* mRNA was detected in neonatal, pubertal and adult rat testes and in testes of vitamin A deficient (VAD) rats (Fig.2).

```
                *         20         *         40         *
rat    : MLLRPRRLPAFAP---PSPGSPDAELRAAGDVPVTTSDAFATSGAMADPGSPK :  50
mouse  : MLLRPRRLPAFSP---PSPASPDAELRSAGDVPVTTSDAFATSGGMAEPGSPK :  50
human  : MLLRPRRPPPLAPPAPPSPASPDPEPRTPGDAPGTPPRRPASPSALGELG--- :  50

             60         *         80         *        100
rat    : APVSPGSAQRTPWSARETELLLGTLLQPAVWRSLLLDRRQALPTYRRVSAALA : 103
mouse  : APVSPDSAQRTPWSARETELLLGTLLQPAMWRSLLLDRRQTLPTYRRVSAALA : 103
human  : LPVSPGSAQRTPWSARETELLLGTLLQPAVWRALLLDRRQALPTYRRVSAALA : 103

           *       120         *       140         *       16
rat    : RQQVRRTPAQCRRRYKFLKDKLRDSQGQPSGPFDDQIRQLMGLLGDDGPPRVR : 156
mouse  : RQQVRRTPAQCRRRYKFLKDKLRDSQGQPSGPFDNQIRQLMGLLGDDGPPRVR : 156
human  : QQQVRRTPAQCRRRYKFLKDKFREAHGQPPGPFDEQIRKLMGLLGDNGRKRPR : 156

         0         *       180         *       200         *
rat    : RRSAGPGRPQRRGRSAFSALAPAPAVVEQEAELPLAAENAEPAPALRFSSSTT : 209
mouse  : RRSTGPGRPQRRGRSSLSALAPAPAPVEQEAELPLAAENDEPAPALRFSSSTT : 209
human  : RRSPGSGRPQRARRPVPNAHAPAPSEPDATPLPTARDRDADPTWTLRFSPSPP : 209

             220         *       240         *       260
rat    : KSAGVRRITSSPPP---TAIDTLPPEPGHTLESSPTPTPDHDTENPNEPPGLS : 259
mouse  : KSAGAHRITSSPPL---TSTDTLPPEPGHTFESSPTPTPDHDVETPNEPPGLS : 259
human  : KSADASPAPGSPPAPAPTALATCIPEDRAPVRGPGSPPPPPAREDPDSPPGRP : 262

            *       280         *       300         *       3
rat    : QGRAS-PQVAPQSLNTALLQTLTHLGDISTVLGPLRDQLSTLNQHVEHLRGSF : 311
mouse  : QGRASSPQVAPQSLNTALLQTLTHLGDISTVLGPLRDQLSTLNQHVEHLRGSF : 312
human  : EDCAP-PPAAPPSLNTALLQTLGHLGDIANILGPLRDQLLTLNQHVEQLRGAF : 314

         20         *       340
rat    : DQTVSLAVGFILGSAASERGILGDLRQ : 338
mouse  : DQTVSLAVGFILGSAASERGILGDLRQ : 339
human  : DQTVSLAVGFILGSAAAERGVLRDPCQ : 341
```

Figure 1. Amino acid sequence of rat UTF1 protein compared to the mouse and human UTF1 protein sequences. Identities are marked by the gray boxes. The two conserved domains are underlined [22].

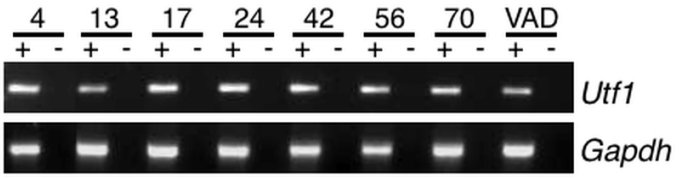


Figure 2. RT-PCR for Utf1 on total testis of rat of several ages and VAD total testis. Numbers express age in days post partum. + : cDNA, - : -RT control.

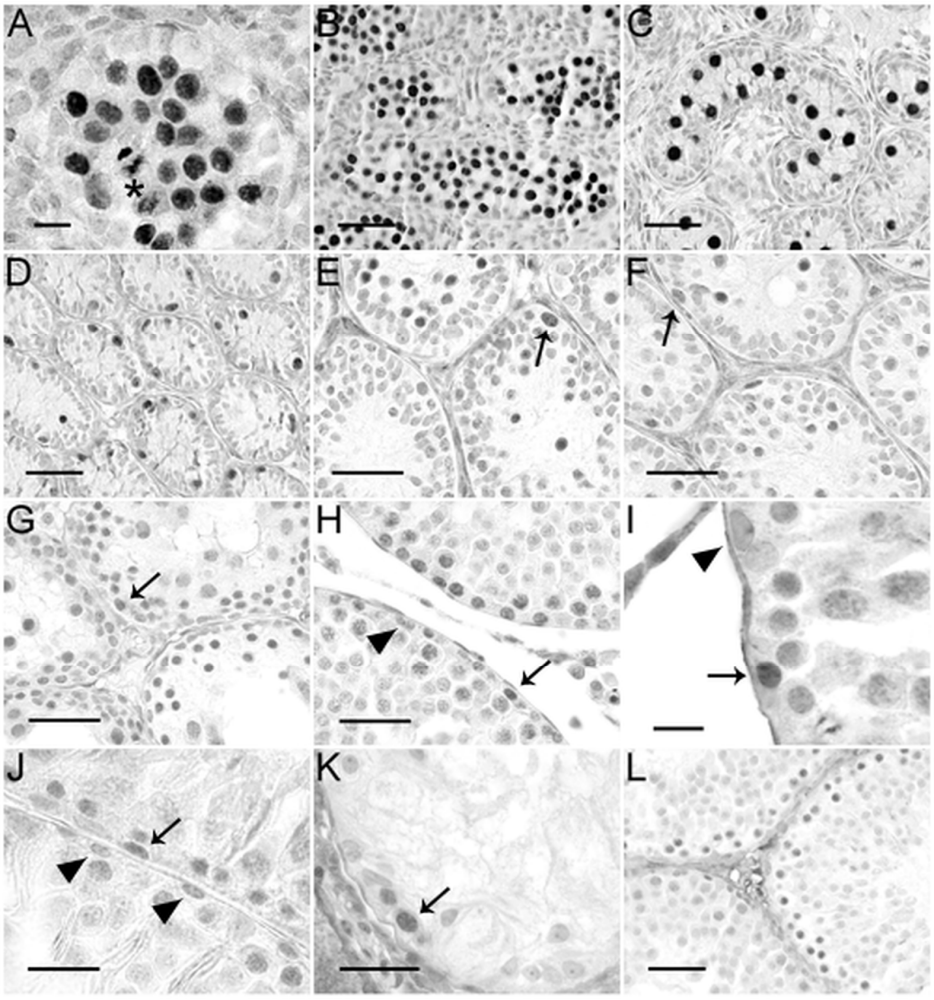


Figure 3. UTF1 expression in rat testes of different ages. A: 16dpc, B: 18dpc, C: 4dpp, D: 9dpp, E: 13dpp, F: 17dpp, G: 24dpp, H: 42dpp, I: 56dpp, J: 70dpp, K: adult VAD rat, L: negative control 70dpp. Asterisk: mitotic gonocytes, arrow: positive spermatogonia, arrowheads: negative spermatogonia. Bars: A, I: 100µm, B, C, D, L: 40µm, E, F, G, H, J, K: 50µm. Figure shows the immunolocalisation of UTF1 performed with the AB3383 antibody.

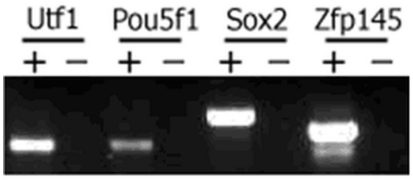


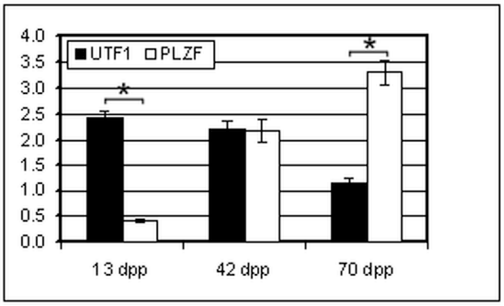
Figure 4. RT-PCR for Utf1, Pou5f1 (OCT3/4), Sox2 and Zfp145 (PLZF) on spermatogonia isolated from VAD rat testis. +: cDNA, - : -RT control.

Gonocytes and spermatogonia express UTF1

To determine which cell types in the testis express UTF1, we performed immunohistochemistry at different developmental stages during pre- and postnatal life. At 16 days post coïtum (dpc), a strong staining for UTF1 was present in the nuclei of all gonocytes (Fig.3a). In gonocytes undergoing mitosis, the condensed chromosomes were stained for UTF1 (Fig.3a). At 18 dpc and 4 days post partum (dpp) the expression was also strong and localized to the nuclei of all gonocytes (Fig.3b, c). At 9 dpp, when gonocytes have migrated to the basal membrane and have become spermatogonia, UTF1 expression was detected in almost all A spermatogonia (Fig.3d). At 13 dpp, staining was present in A spermatogonia, but not in B spermatogonia or preleptotene spermatocytes (Fig.3e). At later stages during development and in adult rat testes, when spermatogenesis was complete, UTF1 expression was restricted to A spermatogonia (Fig.3f –j). However, in testes of older rats, A spermatogonia that did not express detectable UTF1 levels were also observed. In VAD animals, seminiferous tubules only contain early A spermatogonia Sertoli cells and sporadically preleptotene spermatocytes [23, 24]. Immunohistochemistry showed that in VAD rat testes, UTF1 is expressed in spermatogonia (Fig.3k). Interestingly, also in VAD testes spermatogonia were observed that did not show expression of UTF1. Immunolocalization experiments were repeated with three other antibodies directed against UTF1, all confirming the localization of UTF1 to the nuclei of gonocytes and A spermatogonia. In accordance with the immunolocalization of UTF1 to the spermatogonia in VAD rat testes, RT-PCR results confirmed the expression of *Utf1* in spermatogonia isolated from these testes (Fig.4). We furthermore found that the early A spermatogonia marker *Zfp145* (PLZF) and the activators of *Utf1* transcription *Pou5f1* (OCT3/4) and *Sox2* were also expressed by the early A spermatogonia isolated from the VAD rat testes (Fig.4).

The number of UTF1 positive cells decreases with age

The expression pattern of UTF1 during testicular development indicated that the number of UTF1 positive cells in the testis decreased with age. To quantify this finding, the numbers of cells expressing UTF1 and promyelocytic leukemia zinc finger (PLZF) were determined. PLZF is a transcriptional repressor required for the self-renewal of spermatogonial stem cells and its expression is restricted to early A spermatogonia [25, 26]. The numbers of UTF1 and PLZF positive cells per tubule cross-section were scored in adjacent sections of rat testis of different ages (Fig.5). While the numbers of PLZF positive cells increased during testicular development, the number of UTF1 positive cells clearly decreased. Indicating that in adult rat, there is a clear reduction in UTF1 positive A spermatogonia whereas the number of PLZF positive A spermatogonia increased during testicular development.



Age	# Animals	UTF1	PLZF
9dpp	2	2.96	ND
13dpp	4	2.40	0.43
17dpp	2	2.25	0.57
42dpp	4	2.21	2.15
56dpp	2	1.36	1.81
70dpp	4	1.13	3.29

Figure 5. Number ± SEM of UTF1 and PLZF positive cells per tubule cross-section in testes of rats of various ages. ND: not determined. Asterisk indicates that a significant difference ($p \leq 0.05$) between the number of UTF1 and PLZF positive cells.

UTF1 expression is restricted to a subpopulation of early A spermatogonia

To study which type of A spermatogonia expressed UTF1 we performed double immunofluorescent labeling for UTF1 and PLZF. At 42 dpp almost all PLZF positive spermatogonia also expressed UTF1. However, at 56 dpp and 70 dpp, not all PLZF positive spermatogonia also expressed UTF1 (Fig.6). Cells positive for UTF1, but negative for PLZF were not observed. These findings indicate that in adult rat testis, UTF1 expression was restricted to a subpopulation of early A spermatogonia.

Pattern of the UTF1 positive cells during the epithelial cycle

Determining which early A spermatogonia express UTF1 is extremely difficult as early A spermatogonia are morphologically indistinguishable [27]. However, the numbers of the different types of early A spermatogonia follow a specific pattern during the epithelial stages. Studying the numbers of UTF1 expressing cells during the epithelial stage could thus possibly reveal the identity of UTF1 expressing cells.
The numbers of UTF1 positive spermatogonia per tubule cross-section were scored for the different epithelial stages (Fig.7). During epithelial stage VII-X, the highest number of UTF1 positive spermatogonia was observed. The number decreased slowly from stage X until stage II-III, after which an increase in the numbers of UTF1 positive cells was found during stage IV-VI. However, the numbers in stages IV-VI did not yet reach the level observed in stage VII. In comparison, the average number of PLZF positive spermatogonia per tubule cross-section remained relatively constant with exception of the decrease observed in stage XIV (Fig.7). During all stages the number of PLZF positive spermatogonia was higher then the number of spermatogonia that expressed UTF1. At stage VII the biggest overlap between UTF1 and PLZF expression was observed.

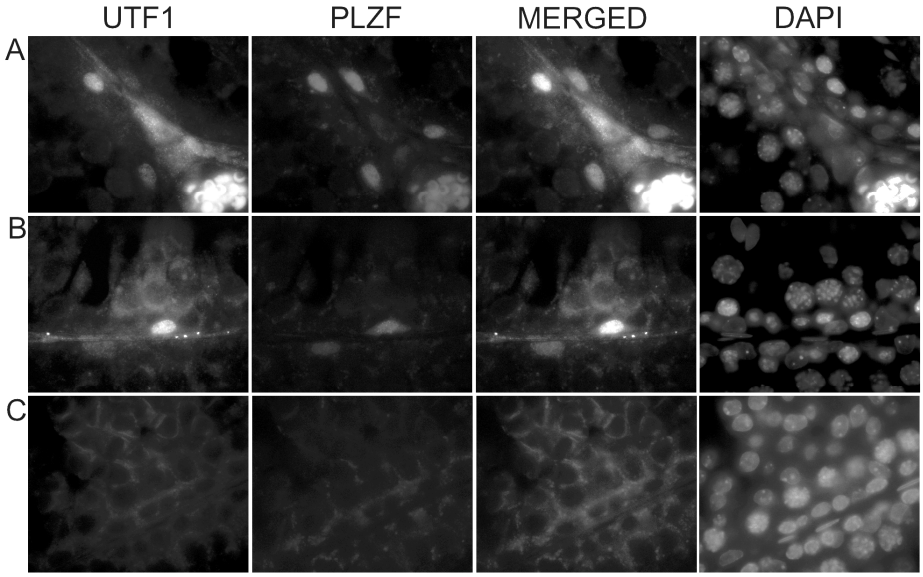


Figure 6. Co-localization of UTF1 and PLZF in 56dpp rat testes. A and B: partial co-localization of UTF1 and PLZF can be observed in the testis of two different 56dpp rats. C: negative control.

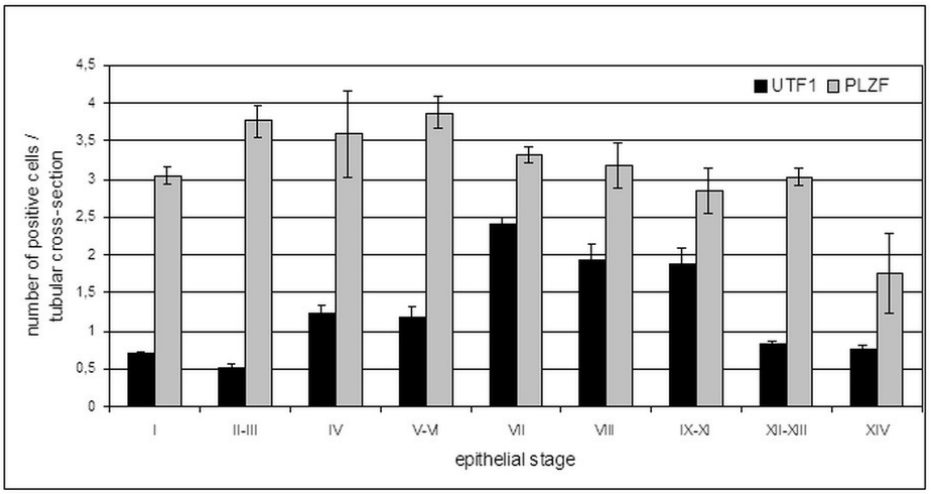


Figure 7. Average number ± SEM of UTF1 and PLZF positive cells per tubule cross-section for the different epithelial stages in 10 weeks old rat testes.

Discussion

The mechanisms whereby spermatogonial stem cells self-renew, differentiate and possibly maintain their pluripotent potential are not yet fully known. In order to unravel the underlying mechanisms, the identification of proteins involved in one or more of these processes is crucial. The pluripotency associated protein UTF1 is a prime candidate for being involved in the process of self-renewal and/or differentiation of spermatogonial stem cells and possibly in maintaining the pluripotent potential of these cells.

The rat sequence of the *Utf1* gene was found to be highly identical to that of mouse, but considerably different from the human sequence [22]. The identities of the conserved domains are higher compared to the overall identity and therefore the rat sequence supports the existence of the two conserved domains [22]. *Utf1* mRNA expression was found in testes of both developing as well as adult rats, indicating that *Utf1* expression is not restricted to a certain developmental stage.

In embryonic and postnatal (4dpp) rat testis, expression of UTF1 protein was found to be restricted to the gonocytes. Following further testicular development, expression of UTF1 was only observed in A spermatogonia. However, the number of cells per tubule cross-section that expressed UTF1 decreased with age and non-expressing type A spermatogonia were observed in the adult rat testes, suggesting that not all types of A spermatogonia expressed UTF1.

Even in testes of VAD rats, in which the early A spermatogonia are the only type of spermatogonia, expression of UTF1 was restricted to the spermatogonia. This finding was supported by RT-PCR which showed expression of Utf1 and its upstream factors Oct3/4 and Sox2 in A spermatogonia isolated from VAD rat testes. Surprisingly however, also in VAD rat testis spermatogonia were observed that did not express UTF1, indicating that UTF1 expression was restricted to a subpopulation of the early A spermatogonia.

As the different types of A spermatogonia are morphologically very difficult to distinguish, co-localization studies of UTF1 with PLZF were performed in order to reveal the identity of the UTF1 positive cells. In mice, expression of PLZF in the testis is known to be restricted to early A spermatogonia [25, 26]. To our knowledge localization of PLZF in rat testes was not described before. The expression pattern of PLZF we observed in both cross-sections as well as in whole mount seminiferous tubules of rat testes was similar to the expression seen in mice (data not shown). In addition, we determined the number of PLZF positive spermatogonia during the cycle of the seminiferous epithelium, which was to our knowledge not shown before.

The co-localization studies of UTF1 with PLZF showed an almost complete overlap in A spermatogonia of 42dpp old rats. However, in adult (56 and 70 days old) rats

only partial co-localization was observed, cells that expressed UTF1 always expressed PLZF, but cells expressing PLZF did not always express UTF1. These observations are in accordance with our cell counts of the numbers of UTF1 and PLZF positive cells per tubule cross-section in testes of rats of various ages (Fig.5). At 42 dpp, when in almost all cells UTF1 and PLZF are co-expressed, counting revealed equal numbers of UTF1 and PLZF expressing cells. At 10 weeks, approximately 3 times more PLZF expressing cells were counted compared to the number of cells expressing UTF1, thereby confirming the co-localization experiments that not all PLZF positive cells expressed UTF1.

Thus, expression of UTF1 in adult rat testis is restricted to a subpopulation of early A spermatogonia as shown by the lower number of UTF1 positive cells compared to the number of PLZF positive cells, the partial co-localization of UTF1 with PLZF and the observation that in testis of VAD rats not all early A spermatogonia expressed UTF1.

The important question then is which types of early A spermatogonia in adult rat testis expressed UTF1. Unfortunately, our attempts to immunohistochemically stain for UTF1 on whole mounts of seminiferous tubules were not successful, not allowing us to identify the UTF1-expressing spermatogonia. In order to shed more light on this issue we determined the number of UTF1 positive spermatogonia during the cycle of the seminiferous epithelium. A pattern was found in which the number of UTF1 positive spermatogonia peaked in stages VII through X, gradually decreasing thereafter until stage III and then increasing again. This pattern is quite different from that of the total numbers of early A spermatogonia throughout the epithelial cycle which shows an increase from about stage X to stage III, almost similar numbers from stage III till VII and sharply decreasing thereafter as the A_{al} spermatogonia differentiate into A1 spermatogonia [28]. As A_{al} spermatogonia form the largest group within the population of early A spermatogonia, their pattern is similar to this latter pattern and thus does not reflect the pattern of the UTF1 expressing cell numbers we observed in this study. Surprisingly, the observed expression pattern of UTF1 also does not reflect the pattern of the numbers of A_s or/and A_{pr} spermatogonia which remains about the same during the epithelial cycle [28]. Furthermore, very few divisions of early A spermatogonia occur from epithelial stage IV until IX [27]. Thus, as hardly any new early A spermatogonia are formed, the increase in UTF1 positive spermatogonia observed in epithelial stage VII, cannot be explained by the generation of new UTF1 positive cells by mitotic activity of existing UTF1 positive cells. It rather suggests that the observed pattern is related to the function of UTF1 and that its expression is stage specifically induced or upregulated to detectable levels in existing early A spermatogonia during epithelial stage IV to VII.

Recently, it was reported that in EC and ES cells, UTF1 has histone-like properties and it was postulated to be involved in maintaining stem cells in a specific epigenetic

profile, either by attracting chromatin-modifying proteins or by chromatin compaction [15]. In the testis, UTF1 could have a similar function as in EC and ES cells. It is therefore most likely that the increase in the number of UTF1 positive cells during stage II-III until VII represent the A_s, A_{pr} and A_{al} spermatogonia that do not differentiate into A1 spermatogonia but rather go through another round of the epithelial cycle. The observed distribution of UTF1 positive cells over the different epithelial stages supports this idea, as the number of UTF1 positive spermatogonia in stage II-III reflects the number of A_s and A_{pr} spermatogonia as determined by Huckins for this stage [28]. Furthermore, the early A spermatogonia that during stage VII did not differentiate, do not undergo mitotic divisions until stage X [27]. The decrease in the number of UTF1 positive cells per tubule cross section thus coincides with the mitotic divisions of the early A spermatogonia in stage X/XI, suggesting that UTF1 expression is gradually lost from the population of early A spermatogonia as new or larger syncitia of A_{al} spermatogonia are generated.

Therefore, we propose that those A_s, A_{pr} and short chains of A_{al} spermatogonia that do not differentiate into A1 spermatogonia express UTF1 and that these spermatogonia thus maintain the ability to differentiate in a next round of the epithelial cycle into A1 spermatogonia or possibly even in other directions when they are taken out of their testicular niche.

Recent reports, described the presence of pluripotent stem cells in both the neonatal and adult mouse testis [5, 6]. A point of debate was whether these stem cells possessed pluripotency capacity in vivo, or whether these cells acquired their pluripotency in vitro. However, non cultured cells, isolated from adult mouse testes were shown to be able to contribute to multiple tissues upon injection into blastocysts, indicating the presence of pluripotent stem cells in the adult mouse testis [6]. Since UTF1 is thought to play a role in maintaining the pluripotent capacity and our immunohistochemistry experiments localize the expression of UTF1 to the population of cells that are close to, if not identical to the population of germ cells with stem cell properties, our results suggest that cells with the capacity for pluripotency are present in the testis during all stages of development, including the adult stage. However, our results furthermore suggest that with increasing age the number of stem cells with the capacity for pluripotency decreases and that the isolation and culture of sufficient numbers of these cells will be more difficult from testes of adult animals than of young animals, an observation supported by findings previously reported by Kanatsu-Shinohara *et al.* [5].

In conclusion, we have shown that during all stages of testicular development expression of UTF1 is restricted to the population of germ cells that contains the stem cells. The distribution of UTF1 positive cells over the different epithelial stages suggests that UTF1 in the testis plays a role in maintaining the cells in an

undifferentiated state that allows differentiation into one or more directions. We therefore believe that further studies on the function of UTF1 in the testis will reveal more insight into the mechanism(s) of spermatogonial stem cell self-renewal and the potential pluripotent capacity.

References

1. Aponte PM, van Bragt MP, de Rooij DG, van Pelt AM. Spermatogonial stem cells: characteristics and experimental possibilities. Apmis 2005; 113: 727-742.
2. de Rooij DG, Russell LD. All you wanted to know about spermatogonia but were afraid to ask. J Androl 2000; 21: 776-798.
3. Smith A. A glossary for stem-cell biology. nature 2006; 441: 1060.
4. Hofmann MC, Braydich-Stolle L, Dym M. Isolation of male germ-line stem cells; influence of GDNF. Dev Biol 2005; 279: 114-124.
5. Kanatsu-Shinohara M, Inoue K, Lee J, Yoshimoto M, Ogonuki N, Miki H, Baba S, Kato T, Kazuki Y, Toyokuni S, Toyoshima M, Niwa O, Oshimura M, Heike T, Nakahata T, Ishino F, Ogura A, Shinohara T. Generation of pluripotent stem cells from neonatal mouse testis. Cell 2004; 119: 1001-1012.
6. Guan K, Nayernia K, Maier LS, Wagner S, Dressel R, Lee JH, Nolte J, Wolf F, Li M, Engel W, Hasenfuss G. Pluripotency of spermatogonial stem cells from adult mouse testis. Nature 2006; 440: 1199-1203.
7. Seandel M, James D, Shmelkov SV, Falciatori I, Kim J, Chavala S, Scherr DS, Zhang F, Torres R, Gale NW, Yancopoulos GD, Murphy A, Valenzuela DM, Hobbs RM, Pandolfi PP, Rafii S. Generation of functional multipotent adult stem cells from GPR125+ germline progenitors. Nature 2007; 449: 346-350.
8. Shamblott MJ, Axelman J, Wang S, Bugg EM, Littlefield JW, Donovan PJ, Blumenthal PD, Huggins GR, Gearhart JD. Derivation of pluripotent stem cells from cultured human primordial germ cells. Proc Natl Acad Sci U S A 1998; 95: 13726-13731.
9. Matsui Y, Zsebo K, Hogan BL. Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. Cell 1992; 70: 841-847.
10. Shi YQ, Wang QZ, Liao SY, Zhang Y, Liu YX, Han CS. In vitro propagation of spermatogonial stem cells from KM mice. Front Biosci 2006; 11: 2614-2622.
11. Pesce M, Wang X, Wolgemuth DJ, Scholer H. Differential expression of the Oct-4 transcription factor during mouse germ cell differentiation. Mech Dev 1998; 71: 89-98.
12. Okuda A, Fukushima A, Nishimoto M, Orimo A, Yamagishi T, Nabeshima Y, Kuro-o M, Boon K, Keaveney M, Stunnenberg HG, Muramatsu M. UTF1, a novel transcriptional coactivator expressed in pluripotent embryonic stem cells and extra-embryonic cells. EMBO J 1998; 17: 2019-2032.
13. Chuva de Sousa Lopes SM, van den Driesche S, Carvalho RL, Larsson J, Eggen B, Surani MA, Mummery CL. Altered primordial germ cell migration in the absence of transforming growth factor beta signaling via ALK5. Dev Biol 2005; 284: 194-203.
14. Nishimoto M, Fukushima A, Okuda A, Muramatsu M, Seki N, Hori TA, Orimo A, Yamagishi T, Nabeshima Y, Kuro-o M, Boon K, Keaveney M, Stunnenberg HG. The gene for the embryonic stem cell coactivator UTF1 carries a regulatory element which selectively interacts with a complex composed of Oct-3/4 and Sox-2. Mol Cell Biol 1999; 19: 5453-5465.
15. van den Boom V, Kooistra SM, Boesjes M, Geverts B, Houtsmuller AB, Monzen K, Komuro I, Essers J, Drenth-Diephuis LJ, Eggen BJL. UTF1 is a chromatin-associated protein involved in ES cell differentiation. 2007; 178: 913-924.
16. Nishimoto M, Miyagi S, Yamagishi T, Sakaguchi T, Niwa H, Muramatsu M, Okuda A. Oct-3/4 maintains the proliferative embryonic stem cell state via specific binding to a variant octamer sequence in the regulatory region of the UTF1 locus. Mol Cell Biol 2005; 25: 5084-5094.
17. van Pelt AM, Roepers-Gajadien HL, Gademan IS, Creemers LB, de Rooij DG, van Dissel-Emiliani FM. Establishment of cell lines with rat spermatogonial stem cell characteristics. Endocrinology 2002; 143: 1845-1850.
18. van Pelt AM, Morena AR, van Dissel-Emiliani FM, Boitani C, Gaemers IC, de Rooij DG, Stefanini M. Isolation of the synchronized A spermatogonia from adult vitamin A-deficient rat

testes. Biol Reprod 1996; 55: 439-444.
19. Nicholas KB, Nicholas H.B. Jr., and Deerfield, D.W. GeneDoc: Analysis and Visualization of Genetic Variation. http://www.psc.edu/biomed/genedoc. 1997.
20. Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG, Thompson JD. Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res 2003; 31: 3497-3500.
21. Campanella JJ, Bitincka L, Smalley J. MatGAT: an application that generates similarity/identity matrices using protein or DNA sequences. BMC Bioinformatics 2003; 4: 29.
22. Fukushima A, Okuda A, Nishimoto M, Seki N, Hori TA, Muramatsu M. Characterization of functional domains of an embryonic stem cell coactivator UTF1 which are conserved and essential for potentiation of ATF-2 activity. J Biol Chem 1998; 273: 25840-25849.
23. Mitranond V, Sobhon P, Tosukhowong P, Chindaduangrat W. Cytological changes in the testes of vitamin-A-deficient rats. I. Quantitation of germinal cells in the seminiferous tubules. Acta Anat (Basel) 1979; 103: 159-168.
24. Van Pelt AM, De Rooij DG. The origin of the synchronization of the seminiferous epithelium in vitamin A-deficient rats after vitamin A replacement. Biol Reprod 1990; 42: 677-682.
25. Buaas FW, Kirsh AL, Sharma M, McLean DJ, Morris JL, Griswold MD, de Rooij DG, Braun RE. Plzf is required in adult male germ cells for stem cell self-renewal. Nat Genet 2004; 36: 647-652
26. Costoya JA, Hobbs RM, Barna M, Cattoretti G, Manova K, Sukhwani M, Orwig KE, Wolgemuth DJ, Pandolfi PP. Essential role of Plzf in maintenance of spermatogonial stem cells. Nat Genet 2004; 36: 653-659.
27. de Rooij DG. Stem cells in the testis. Int J Exp Pathol 1998; 79: 67-80.
28. Huckins C. The spermatogonial stem cell population in adult rats. I. Their morphology, proliferation and maturation. Anat Rec 1971; 169: 533-557.

Chapter4: Controlled differentiation of spermatogonial stem cell line reveals genes involved in early steps of spermatogonial differentiation

Maaike P.A. van Bragt[1,2] Cindy M. Korver[2], Dirk G. de Rooij[1,2] and Ans M.M. van Pelt[2]

1 Center for Reproductive Medicine, Academic Medical Center, 1105 AZ Amsterdam, the Netherlands
2 Department of Endocrinology and Metabolism, Faculty of Science, Utrecht University, 3584 CH Utrecht, the Netherlands

Manuscript in preparation

Abstract

An *in vitro* model for spermatogonial stem cell differentiation was established using two spermatogonial stem cell lines (GC-5spg and Gc-6spg). Spermatogonial stem cell characteristics of these cell lines were studied in further detail and one of the cell lines was induced to differentiate *in vitro*. Upon addition of bone morphogenetic protein 4 (BMP4) to the culture medium, Smads (1/5/8) were phosphorylated and up-regulation of c-Kit was observed on both the mRNA and protein level. In addition, expression of the different BMP receptors was observed in both cell lines. Together these results indicate that BMP4 induced differentiation of the GC-6spg spermatogonial stem cells in a specific manner to at least late A_{al} / A1 spermatogonia.

To identify both direct and indirect targets of BMP4 induced differentiation, a time course DNA micro-array experiment was performed. Several members and downstream targets of the BMP/TGF pathway were found to be differentially regulated after both 4 and 10 hours, confirming that the observed differentiation was specifically induced by BMP4. The number of differentially regulated genes increased with time suggesting that BMP4 triggered a cascade of signaling events resulting in the initial steps of spermatogonial stem cell differentiation. In total 529 transcripts were found to be differentially regulated during at least one time point. Several of the differentially regulated genes were already known to play a role in differentiation and/or development.

In conclusion we have established an *in vitro* model for the initial steps of spermatogonial stem cell differentiation allowing the identification of genes involved in this differentiation process.

Introduction

In the testis spermatozoa are constantly produced by the process of spermatogenesis. At the base of this process is a small pool of spermatogonial stem cells that gives rise to either new spermatogonial stem cells (self-renewal) or to daughter cells committed to differentiate into spermatozoa, eventually [1, 2].

A balance should exist between self-renewal and differentiation of the spermatogonial stem cells in order to maintain spermatogenesis throughout live. Disruption of this balance will either result in the depletion of the seminiferous tubules or in the formation of spermatogonial stem cell tumors. These respective phenotypes were observed in testes of glial cell line derived neurothrophic factor (GDNF) transgenic mice [3]. Targeted knock down of GDNF resulted in a shift towards differentiation and eventually to the depletion of the seminiferous tubules of heterozygous knock out mice. In contrast, overexpression of GDNF resulted in the

accumulation of spermatogonial stem cells and finally in the formation of spermatogonial stem cell tumors. These phenotypes revealed the importance of GDNF as a regulator for spermatogonial stem cell fate decision [3].

Like GDNF, bone morphogenetic protein 4 (BMP4) was reported to influence spermatogonial stem cell fate decision. Spermatogonia isolated from adult mice that were cultured for 7 days in the presence of BMP4 gave rise to a lower number of colonies upon transplantation compared to cells that were cultured in the absence of BMP4, suggesting that BMP4 induced spermatogonial stem cell differentiation [4]. Furthermore, BMP4 was reported to induce the upregulation of the transmembrane tyrosine kinase receptor c-KIT in spermatogonia isolated from 4 day old mice [5]. C-KIT is expressed by differentiating spermatogonia, but not or at a low level by spermatogonial stem cells [6, 7]. Upregulation of c-KIT can thus be used as a marker for spermatogonial stem cell differentiation.

The establishment of GDNF as an inducer of spermatogonial stem cell self-renewal has led to a number of reports describing downstream targets and regulatory mechanisms of spermatogonial stem cell self-renewal [8-11]. However, the knowledge about the regulatory mechanisms involved in spermatogonial stem cell differentiation is still poor. Establishment of a model for the *in vitro* differentiation of a pure population of spermatogonial stem cells would allow the identification of genes involved in the process of spermatogonial stem cell differentiation.

Previously, two cell lines with spermatogonial stem cell characteristics were established in our lab [12]. These cells were derived from vitamin A deficient (VAD) rat testis and were shown to colonize depleted testes of recipient mice. Furthermore, these cell lines could be cultured without feeder cells and thus provide excellent models to study spermatogonial stem cells characteristics and possibly the mechanisms involved in their differentiation.

We here report the establishment of an *in vitro* model for the initial steps of spermatogonial stem cell differentiation. Using the model in a time course DNA micro-array revealed 529 differentially regulated transcripts, suggesting that these transcripts are involved in the initial steps of spermatogonial stem cell differentiation.

Material and methods

Cell culture

Spermatogonial stem cell lines, GC-5spg and GC-6spg, were cultured as described previously with a few minor adjustments [12]. Briefly, cells were cultured in MEM supplemented with nonessential amino acids (100IU/ml), penicillin/streptomycin (100g/ml), gentamicin (40g/ml), HEPES (15mM) (all from Life Technologies, Inc.,

Paisley, UK), sodium bicarbonate (0.12%), L-glutamine (4mM), platelet-derived growth factor-BB (10ng/ml), recombinant human basic fibroblast growth factor (10ng/ml), recombinant human LIF (10ng/ml), forskolin (20μM),), 1nM 17β-estradiol (all from Sigma, St. Louis, USA) and 2,5% FCS (10099141, Invitrogen, Carlsbad, USA) at 32°C and 5% CO_2. For the induction of differentiation of the GC-6spg cells, 50ng/ml BMP4 (R&D systems, Minneapolis, USA) was added to the culture medium and cells were subsequently cultured for the indicated periods of time.

Analysis of Marker Gene Expression and BMP4 receptors

Isolated spermatogonia of VAD rat testis and adult total testis were obtained during earlier published and unpublished experiments [12, 13]. Total RNA from isolated spermatogonia, total rat testis and the cell lines GC-5spg and GC-6spg was extracted using the FastRNA Pro Green kit (Qbiogene, Illkirch Cedex, France) according to the manufacturers protocol. RNA was DNase treated (79254, Qiagen, Valencia, USA) and purified on RNeasy MinElute columns (74204, Qiagen). Thereafter first-strand cDNA was synthesized using random primers and M-MLV Reverse transcriptase (28025-021, Invitrogen). RT-PCR was performed using the primers as summarized in table 1.

Table 1. Overview of primers used to characterize the spermatogonial stem cell lines GC-5spg and GC-6spg. Last column indicates the expected product size in base pairs (bp).

Gene	forward sequence	reverse sequence	size (bp)
Bmpr1a	taggatgtcggcttggtttc	ggcaaatatgcctttctcca	326
Bmpr1b	agaagccagacctcggtaca	cagaatgttctcgtgcctca	319
Bmpr2	ttggtagacaggagggaacg	cactgccattgttgttgacc	358
Cd9	tgggattgttcttcggattc	tgtggaacttgctgtggaag	318
Cd24	gagactcaggccaagaaacg	ggtcgaaggaaccaatgaaa	390
Integrinα6	ctcaggagctgaccctgaac	ggatgcctttctgaattgga	339
Integrinβ1	ctctccagaaggtggctttg	tgtgcccactgctgacttag	340
Cd90 (Thy1)	agctattggcaccatgaacc	acatgtagtcgccctcatcc	323
Pou5f1 (Oct3/4)	cgaggagtcccaggatatga	gttccacctcacacggttct	334
Zfp145 (PLZF)	tctgtctgctgtgtgggaag	gtggcagagtttgcactcaa	353

Western analysis

Protein lysates of GC-6spg cells and total testis were prepared with RIPA buffer (1% NP40, 0,5% sodium deoxycholate, 0,1%SDS, 1mM protease inhibitors (1836153, Roche, Mannheim, Germany). Protein concentrations were measured using bicinchoninic acid analysis (Pierce Chemical Co., Rockford, USA). Of each sample,

50μg was loaded on a 4-12% gradient gel (NP0323box, Invitrogen). Proteins were blotted on a polyvinylidene fluoride (PVDF) membrane (Milipore, Bedford, USA). The membrane was blocked with 5% Protifar (Nutricia, Zoetermeer, The Netherlands) in tris-buffered saline (TBS, 10mM tris-HCl, pH 8.0, 150mM NaCl), including 0,05% Tween-20 (TBT) for 30 min. at room temperature (RT). Thereafter, the membrane was incubated overnight at 4˚C with 1:400 c-KIT antibody (Oncogene Research, San Diego, USA) or with 1:1000 phosphorylated smad1/5/8 (9511, Cell Signaling Technology, Danvers, USA) in 5% Protifar in TBT. After 1 hour of incubation with horseradish peroxidase conjugated goat anti rabbit antibody (SC-2004, Santa Cruz biotechnology Inc, Santa Cruz, USA) diluted 1:5000 in 5% Protifar in TBT, the antigens were visualized using chemiluminescence (ECL, Amersham Bioscience, Buckinghamshire, UK) and exposed to hyperfilm (Amersham Bioscience). For loading control, alpha tubulin (ms-581-pc, Neomarkers, Fremont, USA) was used.

Micro-array analysis

RNA of GC-6spg cells cultured in the presence of BMP4 for 0, 4, 10, 48 or 168 hours was extracted using FastRNA Pro Green kit (Qbiogene). RNA was DNase treated, purified (Qiagen) and amplified by in vitro transcription using T7 RNA polymerase (AM1334, Ambion, Foster City, USA) on 1μg of total RNA. During in vitro transcription, 5-(3-aminoallyl)-UTP (Ambion) was incorporated into the single-stranded cRNA. Of each sample, 3μg cRNA was labeled with Cy3 or Cy5 dye (PA23001 or PA 25001, Amersham, Buckinghamshire, UK). Labeled cRNA was fragmented and of each alternatively labeled cRNA, 2μg was hybridized (5188-5242, Gene Expression Hybridization Kit, Agilent, Santa Clara, USA) on whole genome rat arrays (G4131A, Agilent, Santa Clara, USA) for 17 hours at 65˚C, according to the two-color micro-array based gene expression analysis manual (Agilent). After hybridization, slides were washed manually according to the manufacturers protocol and scanned in the Agilent G2565AA DNA Microarray scanner (100% laser power, 30% photomultiplier tube). Scanned images were quantified (Imagene 6.0.1, biodiscovery, El Segundo, USA) and lowess print-tip normalization was performed [14]. Further data analysis was performed using Genespring GX (Agilent).

Real-time PCR

From 5 independent culture experiments, in which GC-6spg cells were cultured for 0, 4, 10, 48 or 168 hours in the presence of BMP4, RNA was isolated (FastRNA Pro Green kit, Qbiogene), DNAse treated (79254 and 74104, Qiagen) and cDNA was synthesized using random primers. Quantitative RT-PCR was performed using taqman

gene expression assays (*Id2*: Rn01495280_m1, *Gadd45g*: Rn01435432_g1, *Gata2*: Rn00583735_m1, *Nr4a1*: Rn00577766_m1, *Cxcl12*: Rn00573260_m1, *Emp1*: Rn00563345_m1, *Kit*: Rn01641579_m1, Applied biosystems, Foster City, USA) on a ABI prism 7700 (Applied Biosytems). The results were normalized to 18S (4352930E, Applied biosystems) and the average of the fold difference and its significance were calculated using one-way ANOVA (Graphpad 4, Graphpad Software, San Diego, USA).

Results

Characterization of the spermatogonial stem cell lines GC-5spg and GC-6spg

Previously, we reported that the spermatogonial stem cell lines GC-5spg and GC-6spg were able to colonize depleted seminiferous tubules upon transplantation and that both cell lines expressed the spermatogonial marker octamer 4 (OCT3/4 also known as POU5f1) and no or low levels of c-KIT [12]. Since the establishment of these cell lines, several new genes have been identified that are expressed by spermatogonial stem cells [15-19]. Therefore we now performed RT-PCR to characterize the spermatogonial stem cell lines in more detail. Both cell lines expressed the membrane markers *Cd9*, *Cd24*, integrin alpha 6 (*Itga6*) and integrin beta 1 (*Itgb1*) (Fig.1). The membrane receptor *Cd90*, better known as *Thy-1* was clearly expressed by cell line GC-5spg, however only low levels of expression were found in the GC-6spg cell line. Expression of zfp145, which encodes for the transcriptional repressor promyelocytic leukemia zinc finger protein (PLZF), was found to be expressed in GC-6spg, but no detectable levels were found in the GC-5spg cell line (Fig.1).

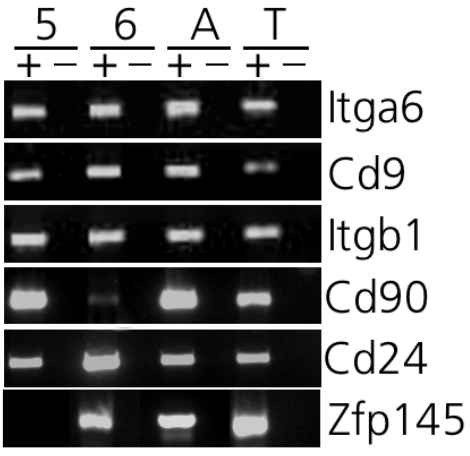


Figure 1. RT-PCR analysis of the spermatogonial stem cell lines GC-5spg and GC-6spg for genes known to be expressed by spermatogonial stem cells. 5: GC-5spg, 6: GC-6spg, VAD: spermatogonia isolated from vitamin A deficient rat , T: rat total testis.

BMP4 induces GC-6spg cells to differentiate

It was suggested that BMP4 could induce differentiation of adult spermatogonial stem cells [4]. Therefore, we investigated the ability of BMP4 to induce *in vitro* differentiation of GC-6spg spermatogonial stem cells. As to our knowledge, the first known molecular sign of spermatogonial stem cell differentiation is the up-regulation of c-KIT, the mRNA and protein expression levels of c-KIT were determined at several time points after addition of BMP4 to the culture medium (Fig.2). Quantitative RT-PCR revealed that the expression level of *c-Kit* mRNA was significantly up-regulated from 48 hours onwards (Fig.2a). Also at the protein level an increase of c-KIT expression was observed. Already after 8 hours there was a slight increase which became more apparent after 24 hours and remained high for at least 1 week (Fig.2b).

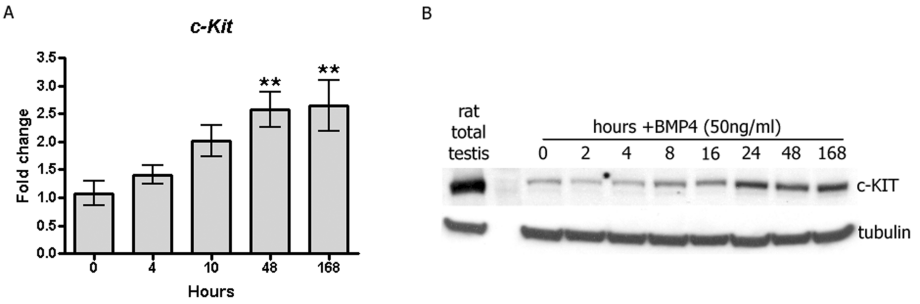


Figure 2. BMP4 induces in vitro differentiation of spermatogonial stem cells GC-6spg as demonstrated by the upregulation of c-KIT on the mRNA (A) and protein level (B). Significant difference from the control (t=0), P<0.01 is indicated by two asterisks.

Rat spermatogonial stem cells express BMP4 receptors

BMPs signal through heteromeric complexes of type I (BMPR-IA / ALK3, BMPR-IB / ALK6) and type II (BMPR-II) serine/threonine kinase receptors [20]. To study if these receptors were expressed by our spermatogonial stem cell lines, RT-PCR was performed. As positive controls, RNA from total testis and from spermatogonia isolated from vitamin A deficient (VAD) rats were taken along. Expression of all three receptors was observed in both positive controls as well as in both spermatogonial stem cell lines, suggesting that BMP4 induced differentiation via activation of its receptors (Fig.3). No signal was observed in the -RT controls.

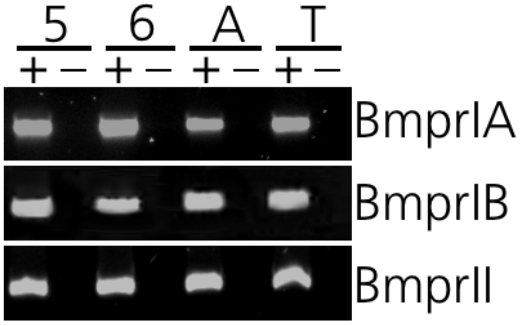


Figure 3. BMP4 receptors Bmprla, Bmprlb and BmprII are expressed by spermatogonial stem cell lines GC-5spg and GC-6spg. 5: GC-5spg, 6: GC-6spg, A: spermatogonia isolated from vitamin A deficient rat , T: rat total testis.

Smad's are phosphorylated upon addition of BMP4 to the culture medium

In order to confirm that the BMP4 induced differentiation of the GC-6spg cells was specifically induced via the BMP receptors we investigated if the downstream Smads 1, 5 and 8 were phosphorylated. Western blot analysis with an antibody specifically recognizing phosphorylated Smads 1, 5 and 8 showed that the Smads were phosphorylated within 15 minutes upon addition of BMP4 to the culture medium (Fig.4).

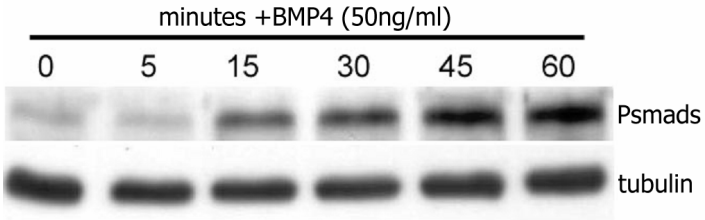


Figure 4. Smads 1, 5 and 8 were phosphorylated in GC-6spg spermatogonial stem cells upon addition of BMP4 to the culture medium.

Time course micro-array reveals 529 transcripts that might play a role in spermatogonial stem cell differentiation.

The now established model of spermatogonial stem cell differentiation was used for a time course DNA micro-array experiment to identify genes involved in the process of spermatogonial stem cell differentiation.

The GC-6spg cells were cultured in the presence of BMP4 for 0, 4, 10, 48 and 168 hours after which RNA was isolated, labeled and hybridized on Agilent whole rat genome oligo-DNA arrays. Multiple time points were chosen to enable the

identification of genes involved during different steps of BMP4 induced differentiation: before the up-regulation of *c-Kit* mRNA and protein (4 hours), after the first sign of up-regulation of *c-Kit* mRNA and protein (10 hours), after both mRNA and protein levels of *c-Kit* were clearly up-regulated (48 hours), and after 1 week when differentiation was established (168 hours). Expression levels at all time points were compared with those at 0 hours. In total 529 transcripts were found to be ≥ 2-fold regulated during at least 1 time point and as expected the number of differentially expressed genes increased over time, from 95 transcripts after 4 hours to 359 after 168 hours (table 2).

To verify the data obtained by the micro-array experiment, the expression levels for the different time points of 6 selected genes were determined by quantitative RT-PCR (Fig.5). All selected genes showed a similar expression profile compared to the micro-array data, verifying the data found by the micro-array (Fig.5). Expression of *Id2*, a known downstream target of BMP4, was sharply increased at 4 hours after which the expression decreased again. Expression of *Gata2*, another known downstream target of BMP4, was found to be increased at 4 hours but expression remained high. A similar expression pattern was observed for *Gadd45g*. Up-regulation of *Emp1* was observed at 48 and 168 hours after the addition of BMP4 to the culture medium. For *Nr4a1*, both a direct as well as an indirect effect was observed, at 4 and 168 hours a decrease in the expression of *Nr4a1* was observed. The expression of *Cxcl12* decreased from 10 hours onwards.

Time(hours)	up	down	total
4	84	11	95
10	75	29	104
48	119	89	208
168	170	189	359

Table 2. Number of transcripts found to be 2≥-fold regulated per time point in the GC-6spg cells upon the addition of BMP4 to the culture medium. In total 529 different transcripts were differentially regulated.

Table 3. Number of genes ≥2-fold up- or down-regulated for the indicated biological functions.

Cell differentiation	18 genes	Transport	30 genes
Cell proliferation	17 genes	Transcription	13 genes
Development	25 genes	Metabolism	27 genes
Apoptosis	11 genes	Cell cycle	6 genes
Signal transduction	24 genes	Cell adhesion	8 genes

Analysis of the micro-array data

Of the 529 transcripts identified by micro-array, 176 (33%) unique genes were assigned to at least one biological function according to the GO database. Most genes could be categorized to at least one of the categories listed in table 3. The genes known to play a role in differentiation, development and/or proliferation were summarized (Fig. 6).

Among the genes involved in differentiation are a number of genes that were reported to be involved in the differentiation of other stem cells, e.g. Pparγ and Emp1. Furthermore, analyzing the expression patterns of the different genes over time, it became clear that among the early (4 hours) and intermediate (10 hours) regulated genes several known downstream targets or members of TGF/BMP pathway were up-regulated, e.g.: *Id2*, *Gata2*, *Bhlhb2*, *Sno*, *Bmp3*, *Smad7*.

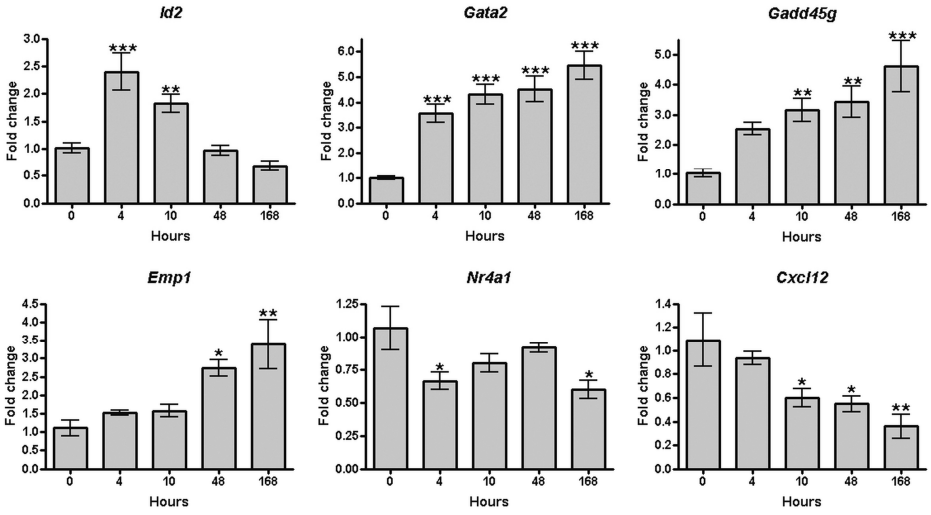


Figure 5. Verification of micro-array results by quantitative RT-PCR. Fold expression ± SEM is shown for the different genes. Significant difference from the control (t=0) is indicated by one asterisk (P<0.05), two asterisks (P<0.01) or three asterisk (P<0.001).

Discussion

The here described *in vitro* model of spermatogonial stem cell differentiation is to our knowledge the first in which the differentiation of a pure population of spermatogonial stem cells can be studied. Previously, we reported that the GC-5spg and GC-6spg cells were capable of colonizing a depleted testis upon transplantation

and that the cells expressed OCT3/4 [12]. We now also confirmed and extended the evidence for the spermatogonial stem cell character of both cell lines by performing

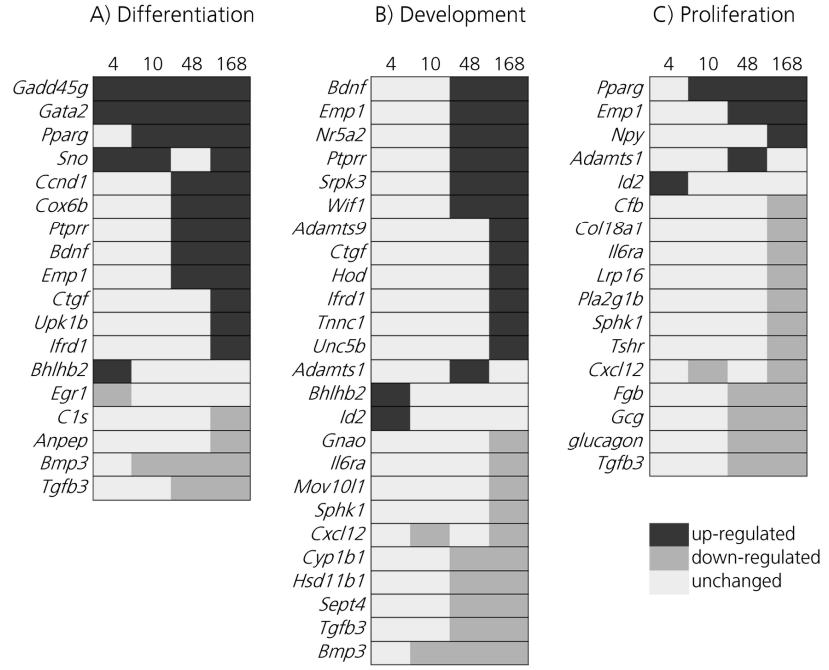


Figure 6. Genes known to play a role in A) differentiation, B) development, or C) proliferation that are ≥2-fold -regulated in GC-6spg cells upon induction of differentiation by BMP4.

RT-PCR for several markers known to be expressed by spermatogonial stem cells.

Furthermore, we demonstrated the expression of BMP4 receptors by early A spermatogonia and by both our spermatogonial stem cell lines. Expression of Bmprla and BmprII was previously reported in spermatogonia isolated from pubertal mice [5]. Our data now extends these findings by demonstrating that all three BMP4 receptors, i.e. Bmprla, Bmprlb and BmprII are expressed in early A spermatogonia of adult VAD rats, in which the A_s, A_{pr} and A_{al} are the only type of spermatogonia present.
Addition of BMP4 to the GC-6spg cells resulted in the phosphorylation of the BMP4 responsive Smads (1/5/8) and the up-regulation of c-KIT. Together these results indicate that BMP4 induced differentiation of the GC-6spg cells in a specific manner.

Unfortunately, it is at this point impossible to state how far differentiation of the GC-6spg cells is induced by addition of BMP4 to the culture medium. However, during spermatogenesis c-KIT is upregulated at the border of the differentiation of Aal to A1 spermatogonia [6]. The upregulation of *c-kit* in GC-6spg therefore suggests that the GC-6spg cells differentiated until at least late Aal. Up-regulation of SCP3, a

meiosis-specific gene, was not observed within one week upon the addition of BMP4 to the culture medium (data not shown) suggesting that differentiation does not reach until the spermatocyte stage [21]. A recent report describes that PLZF is a direct repressor of c-Kit transcription [22]. Previously, PLZF was reported to be essential for spermatogonial stem cell maintenance and expression was found to be restricted to the early A spermatogonia [18, 19]. Expression of PLZF should thus be down-regulated in the differentiated GC-6spg cells and would be interesting to study as an additional sign of differentiation. It will also be interesting to carry out a transplantation assay with the differentiated GC-6spg cells to check whether they lost there ability to colonize a recipient mouse testis [23]. Nevertheless, the increase in c-KIT expression suggests that the GC-6spg cells have differentiated until to at least the late Aa_l stage [6].

Many genes were found to be differentially expressed during the differentiation process. Our time course micro-array experiment revealed in total 529 transcripts to be differentially regulated during at least one of the time points studied. Of these transcripts about one-third (176) had a known biological function. Several of these genes were reported to play a role in differentiation, some even in the differentiation of stem cells.

For example, *Gata2*, a transcription factor expressed by brown adipocyte precursors and hematopoietic stem cells, was reported to suppress the differentiation of both these cell types [24, 25]. Reduction of GATA2 expression in these cells allowed brown adipogenesis and hematopoiesis, respectively [24, 26]. *Gata2* has also been reported to be up-regulated during the differentiation of embryonic stem cells in embryoid bodies and during hemangioblast development and differentiation [27, 28]. Hemangioblasts are the precursors of both hematopoietic and endothelial cells [29]. Furthermore, GATA2 has been reported to be a known downstream target of BMP4 [28, 30, 31]. Although expression of GATA2 in the testis to our knowledge has not been reported before, the expression levels we observed in the GC-6spg cells by both micro-array and quantitative PCR were high upon the induction of differentiation of these cells by BMP4. Furthermore, PLZF, a transcriptional repressor was shown to interact with GATA2 and modify its transactivation capacity, suggesting that within the spermatogonial stem cells and early A spermatogonia, PLZF could block the function of GATA2 [32].

Another example of a gene previously reported to have a function in differentiation is Epithelial membrane protein 1 (*Emp1*). This membrane protein was reported to be highly up-regulated during squamous cell differentiation and suggested to play a role in neuronal differentiation and neurite outgrowth [33, 34]. During the BMP4 induced differentiation, upregulation of *Emp1* was observed at 48 and 168 hours. Although, expression of *Emp1* mRNA was reported to be expressed in

human total testis, the localization of EMP1 was to our knowledge not reported [35].

We are not aware of other studies that aimed at identifying genes involved in the differentiation process of spermatogonial stem cells. Several studies aimed to identify genes differentially regulated between different populations of tesicular cells [36-39]. For example, Small *et al.* studied the difference between A spermatogonia, B spermatogonia, pachytene spermatcytes, round spermatids, myoid cells and Sertoli cells [39].

Other micro-array studies on spermatogonial stem cells were focused on identifying genes involved in spermatogonial self-renewal [8, 10]. All studies used addition of GDNF to the culture medium to secure spermatogonial stem cell self-renewal and one of the genes that was identified to be up-regulated was the transcription factor Egr-1 [10, 40]. Remarkably, we now found that the expression of this gene, Egr-1, is down-regulated upon the induction of differentiation of the GC-6spg cells by BMP4. The early growth response gene *Egr-1* is a transcription factor known to serve as an intermediary in a broad range of signal transduction processes in many different tissues [41].

In conclusion, we believe that with the establishment of the *in vitro* model for spermatogonial stem cell differentiation and by generating of a list of genes differentially regulated during the differentiation process we have developed a very strong research tool that can now be used to unravel the regulatory mechanisms involved in spermatogonial stem cell differentiation. Potentially, the *in vitro* model can also be used to identify additional and possibly earlier makers for spermatogonial stem cell differentiation and for screening of other potential inducers of spermatogonial stem cell differentiation.

References

1. Aponte PM, van Bragt MP, de Rooij DG, van Pelt AM. Spermatogonial stem cells: characteristics and experimental possibilities. Apmis 2005; 113: 727-742.
2. de Rooij DG, Russell LD. All you wanted to know about spermatogonia but were afraid to ask. J Androl 2000; 21: 776-798.
3. Meng X, Lindahl M, Hyvonen ME, Parvinen M, de Rooij DG, Hess MW, Raatikainen-Ahokas A, Sainio K, Rauvala H, Lakso M, Pichel JG, Westphal H, Saarma M, Sariola H. Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. Science 2000; 287: 1489-1493.
4. Nagano M, Ryu BY, Brinster CJ, Avarbock MR, Brinster RL. Maintenance of mouse male germ line stem cells in vitro. Biol Reprod 2003; 68: 2207-2214.
5. Pellegrini M, Grimaldi P, Rossi P, Geremia R, Dolci S. Developmental expression of BMP4/ALK3/SMAD5 signaling pathway in the mouse testis: a potential role of BMP4 in spermatogonia differentiation. J Cell Sci 2003; 116: 3363-3372.
6. Schrans-Stassen BH, van de Kant HJ, de Rooij DG, van Pelt AM. Differential expression of c-kit in mouse undifferentiated and differentiating type A spermatogonia. Endocrinology 1999; 140: 5894-5900.
7. Ebata KT, Zhang X, Nagano MC. Expression patterns of cell-surface molecules on male germ line stem cells during postnatal mouse development. Mol Reprod Dev 2005; 72: 171-181.
8. Braydich-Stolle L, Nolan C, Dym M, Hofmann MC. Role of glial cell line-derived neurotrophic factor in germ-line stem cell fate. Ann N Y Acad Sci 2005; 1061: 94-99.
9. Oatley JM, Avarbock MR, Brinster RL. Glial cell line-derived neurotrophic factor regulation of genes essential for self-renewal of mouse spermatogonial stem cells is dependent on Src family kinase signaling. J Biol Chem 2007; 282: 25842-25851.
10. Oatley JM, Avarbock MR, Telaranta AI, Fearon DT, Brinster RL. Identifying genes important for spermatogonial stem cell self-renewal and survival. PNAS 2006; 103: 9524-9529.
11. He Z, Jiang J, Kokkinaki M, Golestaneh N, Hofmann MC, Dym M. GDNF Up-regulates c-fos Transcription via the Ras/ERK1/2 Pathway to Promote Mouse Spermatogonial Stem Cell Proliferation. Stem Cells 2007.
12. van Pelt AM, Roepers-Gajadien HL, Gademan IS, Creemers LB, de Rooij DG, van Dissel-Emiliani FM. Establishment of cell lines with rat spermatogonial stem cell characteristics. Endocrinology 2002; 143: 1845-1850.
13. van Pelt AM, Morena AR, van Dissel-Emiliani FM, Boitani C, Gaemers IC, de Rooij DG, Stefanini M. Isolation of the synchronized A spermatogonia from adult vitamin A-deficient rat testes. Biol Reprod 1996; 55: 439-444.
14. Yang YH, Dudoit S, Luu P, Lin DM, Peng V, Ngai J, Speed TP. Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucleic Acids Res 2002; 30: e15.
15. Kubota H, Avarbock MR, Brinster RL. Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells. PNAS 2003; 100: 6487-6492.
16. Kanatsu-Shinohara M, Toyokuni S, Shinohara T. CD9 Is a Surface Marker on Mouse and Rat Male Germline Stem Cells. Biol Reprod 2004; 70: 70-75.
17. Shinohara T, Avarbock MR, Brinster RL. beta1- and alpha6-integrin are surface markers on mouse spermatogonial stem cells. Proc Natl Acad Sci U S A 1999; 96: 5504-5509.
18. Buaas F, Kirsh A, Sharma M, McLean D, Morris J, Griswold M, de Rooij D, Braun RE. Plzf is required in adult male germ cells for stem cell self-renewal. Nat Genet 2004; 36: 647-652
19. Costoya JA, Hobbs RM, Barna M, Cattoretti G, Manova K, Sukhwani M, Orwig KE, Wolgemuth DJ, Pandolfi PP. Essential role of Plzf in maintenance of spermatogonial stem cells. Nat Genet 2004; 36: 653-659.
20. Chen D, Zhao M, Mundy G. Bone morphogenetic proteins. Growth Factors 2004; 22: 233-41.
21. Parra MT, Viera A, Gomez R, Page J, Benavente R, Santos JL, Rufas JS, Suja JA. Involvement of

the cohesin Rad21 and SCP3 in monopolar attachment of sister kinetochores during mouse meiosis I. J Cell Sci 2004; 117: 1221-1234.
22. Filipponi D, Hobbs RM, Ottolenghi S, Rossi P, Jannini EA, Pandolfi PP, Dolci S. Repression of kit expression by Plzf in germ cells. Mol Cell Biol 2007.
23. Brinster R. Germline stem cell transplantation and transgenesis. Science 2002; 296: 2174-76.
24. Tsai J, Tong Q, Tan G, Chang AN, Orkin SH, Hotamisligil GS. The transcription factor GATA2 regulates differentiation of brown adipocytes. EMBO Rep 2005; 6: 879-884.
25. Persons D, Allay J, Allay E, Ashmun R, Orlic D, Jane S, Cunningham J, Nienhuis A. Enforced expression of GATA-2 blocks normal hematopoiesis. Blood 1999; 93: 488-499.
26. Dao MA, Nolta JA. Cytokine and integrin stimulation synergize to promote higher levels of GATA-2, c-myb, and CD34 protein in primary human hematopoietic progenitors from bone marrow. Blood 2007; 109: 2373-2379.
27. Lu SJ, Hipp JA, Feng Q, Hipp JD, Lanza R, Atala A. GeneChip analysis of human embryonic stem cell differentiation into hemangioblasts: an in silico dissection of mixed phenotypes. Genome Biol 2007; 8: R240.
28. Lugus JJ, Chung YS, Mills JC, Kim SI, Grass J, Kyba M, Doherty JM, Bresnick EH, Choi K. GATA2 functions at multiple steps in hemangioblast development and differentiation. Development 2007; 134: 393-405.
29. Choi K. Hemangioblast development and regulation. Biochem Cell Biol 1998; 76: 947-956.
30. Oren T, Torregroza I, Evans T. An Oct-1 binding site mediates activation of the gata2 promoter by BMP signaling. Nucleic Acids Res 2005; 33: 4357-4367.
31. Maeno M, Mead PE, Kelley C, Xu RH, Kung HF, Suzuki A, Ueno N, Zon LI. The role of BMP-4 and GATA-2 in the induction and differentiation of hematopoietic mesoderm in Xenopus laevis. Blood 1996; 88: 1965-1972.
32. Tsuzuki S, Enver T. Interactions of GATA-2 with the promyelocytic leukemia zinc finger (PLZF) protein, its homologue FAZF, and the t(11;17)-generated PLZF-retinoic acid receptor alpha oncoprotein. Blood 2002; 99: 3404-3410.
33. Jetten AM, Suter U. The peripheral myelin protein 22 and epithelial membrane protein family. Prog Nucleic Acid Res Mol Biol 2000; 64: 97-129.
34. Wulf P, Suter U. Embryonic expression of epithelial membrane protein 1 in early neurons. Brain Res Dev Brain Res 1999; 116: 169-180.
35. Chen Y, Medvedev A, Ruzanov P, Marvin KW, Jetten AM. cDNA cloning, genomic structure, and chromosome mapping of the human epithelial membrane protein CL-20 gene (EMP1), a member of the PMP22 family. Genomics 1997; 41: 40-48.
36. Small CL, Shima JE, Uzumcu M, Skinner MK, Griswold MD. Profiling gene expression during the differentiation and development of the murine embryonic gonad. Biol Reprod 2005; 72: 492-501.
37. Chan WY, Lee TL, Wu SM, Ruszczyk L, Alba D, Baxendale V, Rennert OM. Transcriptome analyses of male germ cells with serial analysis of gene expression (SAGE). Mol Cell Endocrinol 2006; 250: 8-19.
38. Wu SM, Baxendale V, Chen Y, Pang AL, Stitely T, Munson PJ, Leung MY, Ravindranath N, Dym M, Rennert OM, Chan WY. Analysis of mouse germ-cell transcriptome at different stages of spermatogenesis by SAGE: biological significance. Genomics 2004; 84: 971-981.
39. Shima JE, McLean DJ, McCarrey JR, Griswold MD. The murine testicular transcriptome: characterizing gene expression in the testis during the progression of spermatogenesis. Biol Reprod 2004; 71: 319-330.
40. Hofmann MC, Braydich-Stolle L, Dym M. Isolation of male germ-line stem cells; influence of GDNF. Dev Biol 2005; 279: 114-124.
41. Baron V, Adamson E, Calogero A, Ragona G, Mercola D. The transcription factor Egr1 is a direct regulator of multiple tumor suppressors including TGFbeta1, PTEN, p53, and fibronectin. Cancer Gene Ther 2006; 13: 115-124.

Chapter 5: Activin A induced differentiation of a spermatogonial stem cell line indicates role for *Cxcl12* in regulating spermatogonial stem cell fate

Maaike P.A. van Bragt[1,2] Cindy M. Korver[2], Dirk G. de Rooij[1,2] and Ans M.M. van Pelt[2]

[1] Department of Endocrinology and Metabolism, Faculty of Science, Utrecht University, 3584 CH Utrecht, the Netherlands
[2] Center for Reproductive Medicine, Academic Medical Center, 1105 AZ Amsterdam, the Netherlands

In preparation

Abstract

The underlying regulatory mechanisms of spermatogonial stem cell differentiation are poorly understood. We now used our previously established GC-6spg spermatogonial stem cell line to study the role of activin A in spermatogonial stem cell differentiation. We report that upon addition of activin A to the GC-6spg cells expression of *c-Kit* was up-regulated on both the mRNA and protein level. Furthermore, we show that the various activin A receptors are expressed by GC-6spg cells. These results suggest that activin A can induce differentiation of spermatogonial stem cells in a direct and specific manner.

Furthermore, the expression pattern of a number of genes was followed during activin A induced differentiation. These selected genes were previously identified to be involved in BMP4 induced differentiation. Comparison of the expression patterns revealed only a partial overlap in the genes involved in activin A and BMP4 induced differentiation. Expression of *Id2*, *Gata2*, *Gadd45g, Emp1* and *Nr4a1* was not significantly affected during activin A induced differentiation, in contrast to the effects observed during BMP4 induced differentiation of the GC-6spg cells. Interestingly, expression of *Cxcl12* was down regulated during both activin A and BMP4 induced differentiation which together with its known function in hematopoietic stem cells maintenance and primordial germ cell migration suggests a role for CXCL12 in spermatogonial stem cell maintenance. The present results suggest that activin A and BMP4 induce differentiation of GC-6spg cells via different pathways that however, partly overlap.

Introduction

Spermatogonial stem cells, like other stem cells, have the ability to proliferate indefinitely and to produce daughter cells which can terminally differentiate into a particular cell type [1]. Spermatogonial stem cells are unique in that they are the only cells in the adult animal that undergo self-renewal and contribute genes to the next generation [2, 3]. Furthermore, spermatogonial stem cells have recently been reported to be pluripotent or capable of regaining pluripotent capacity *in vitro* [4-7]. Spermatogonial stem cell division results in either two new stem cells (self-renewal) or in two daughter cells committed to differentiate. The committed daughter cells stay connected by an intercellular bridge, due to incomplete cytokinesis, and undergo several more mitotic, meiotic and differentiation steps to eventually become spermatozoa [8, 9].

Little is known about the mechanisms that underlie the regulation of spermatogonial stem cell self-renewal and differentiation. This lack of knowledge is

mainly caused by the fact that spermatogonial stem cells are difficult to isolate, identify and maintain *in vitro* [2, 9]. Nevertheless, research on spermatogonial stem cell self-renewal has made progress during the last decade since the discovery of GDNF, BCL6b and PLZF as factors promoting self-renewal [10-12]. However, less is known about the mechanisms of spermatogonial stem cell differentiation.

Recently, we described the potential of BMP4 to induce differentiation of the spermatogonial stem cell line GC-6spg [13]. The GC-6spg cell line was previously generated in our lab by single cell cloning upon transfection of SV40 large T antigen into early A spermatogonia isolated from vitamin A deficient rats [14]. The GC-6spg spermatogonial stem cells were able to colonize depleted mice testes upon transplantation and expressed several spermatogonial stem cells markers, i.e. *Cd90*, *Itga6*, *Itgb1*, *Cd9*, *Cd24* and *Pou5f1* [13, 14].

Upon addition of BMP4 to the GC-6spg cells, BMP4 responsive Smads were phosphorylated and expression of c-KIT was up-regulated on both the mRNA and protein level, indicating that BMP4 induced differentiation of GC-6spg cells in a specific manner [13]. Micro-array analysis revealed numerous genes differentially regulated during BMP4 induced differentiation of these cells [13].

Like BMP4, activin A was reported to reduce stem cell maintenance in *vitro* [15]. Cell suspensions containing spermatogonial stem cells, isolated from cryptorchid mice, that were cultured for 7 days on STO feeder cells in the presence of activin A or BMP4 generated less colonies upon transplantation than spermatogonia that were cultured in the absence of activin A or BMP4 [15]. Activin was also suggested to play a role in spermatocyte differentiation and proliferation of spermatogonia [16, 17]. Inhibin, an inhibitor of activin was reported to suppress the numbers of intermediate and late spermatogonia after intratesticular injection *in vivo* [18].

Sertoli cells and peritubular cells were reported to produce activin [19, 20]. Activins act through binding of type II receptors and phosphorylation of type I receptors, which in turn phosphorylates the downstream Smads 2 and 3 [21]. For activin, two subtypes of each receptor have been described (i.e. ActRIIA, ActRIIB, ActRIA and ActRIB). Expression of activin receptors in rodents was reported for most testicular cell types, i.e. Leydig and Sertoli cells contained ActRIIA, the A1 and A2 spermatogonia expressed ActRIIB and the spermatocytes and spermatids expressed ActRIIA. Presence of actRIA was reported in spermatids (reviewed in [22]).

We now show that mRNA of all four activin receptors was expressed by both GC-5spg and GC-6spg spermatogonial stem cells and that activin A can induce differentiation of GC-6spg cells, as demonstrated by the up-regulation of *c-Kit*, both on mRNA and protein level. Furthermore, the expression levels of six genes, found to change in expression level during BMP4 induced differentiation, were followed during activin A induced differentiation of the GC-6spg cells.

Material and methods

Cell culture

Spermatogonial stem cell line GC-6spg was cultured as described previously [13]. Cells were cultured in MEM supplemented with nonessential amino acids (100 IU/ml), penicillin/streptomycin (100 g/ml), gentamicin (40 g/ml), HEPES (15 mM) (all from Life Technologies, Inc., Paisley, UK), sodium bicarbonate (0.12%), L-glutamine (4 mM), platelet-derived growth factor-BB (10 ng/ml), recombinant human basic fibroblast growth factor (10 ng/ml), recombinant human LIF (10 ng/ml), forskolin, 1 nM 17β-estradiol (20 βμM) (all from Sigma, St. Louis, USA) and 2,5% FCS (10099141, Invitrogen, Carlsbad, USA) at 32°C and 5% CO_2. Activin A (50 ng/ml, R&D systems, Minneapolis, USA) was added to the culture medium and the cells were subsequently cultured during the indicated times.

Analysis of activin receptors expression

Total RNA from cell lines GC-5spg and GC-6spg was extracted using the FastRNA Pro Green kit (Qbiogene, Illkirch Cedex, France) according to the manufacturers protocol. RNA isolated from adult rat total testis and the GC-5spg spermatogonial stem cells was obtained during earlier experiments [14, 23]. RNA was DNase treated and purified on Rneasy MinElute columns (79254 and 74104, Qiagen, Valencia, USA). Thereafter first-strand cDNA was synthesized using random primers and M-MLV Reverse transcriptase (28025-021, Invitrogen, Carlsbad, USA). RT-PCR for the activin A receptors was performed using the following primers. Rat activin receptor 1(*actRIA*) fw: 5'-ggtcaacccgaagctttaca-3', rv: 5'-aaaaggcataccgcaaacac-3' (333kb), rat activin receptor 1b (*actRIB*) fw: 5'-gggaaccaaacgatacatgg-3', rv: 5'-gcttagctgggacaaagtc-3' (371kb), Rat activin receptor 2 (*actRIIA*) fw: 5'-aagatggcctaccctcctgt-3', rv: 5'-tagccacaggtccacatcaa-3' (303kb) rat activin receptor 2b (*actRIIB*) fw: 5'-ggagaacccccaggtgtatt-3' rv: 5'-tcttcacagccacaaagtcg-3' (371kb).

Western Blot analysis for c-KIT

The expression level of c-KIT in GC-6spg cells cultured in the absence or presence of activin A was determined by western blot analysis as described before [13]. Briefly, whole protein extracts were obtained by lysing the GC-6spg cells in RIPA buffer (1% NP40, 0,5% sodium deoxycholate, 0,1%SDS, 1 mM protease inhibitors, Roche, Mannheim, Germany). Thereafter 50 ug protein lysate was loaded on a 4-12% gradient bis-tris gel (Invitrogen, Carlsbad, USA). Protein was blotted on PVDF

membrane (Milipore, Bedford, USA) and c-KIT protein was detected with an antibody specifically directed to c-KIT (Oncogene Research, San Diego, USA). For loading control, alpha tubulin (ms-581-pc, Neomarkers, Fremont, USA) was used.

Quantitative RT-PCR

Using the FastRNA Pro Green kit (Qbiogene) total RNA was isolated from 5 independent culture experiments in which the GC-6spg cells were cultured for 0, 4, 10, 48 or 168 hours in the presence of 50 ng/ml activin A. RNA was DNase treated and cDNA was synthesized using random primers. Quantitative RT-PCR was performed using taqman gene expression assays (*Id2*: Rn01495280_m1, *Gadd45g*: Rn01435432_g1, *Gata2*: Rn00583735_m1, *Nr4a1*: Rn00577766_m1, *Cxcl12*: Rn00573260_m1, *Emp1*: Rn00563345_m1, *Kit*: Rn01641579_m1, Applied biosystems, Foster City, USA). The results were normalized to 18S (4352930E, Applied biosystems) and the average of the fold change (±SEM) and its significance were calculated using one-way Anova (Graphpad 4, Graphpad Software, San Diego, USA).

Results

Spermatogonial stem cell cell-lines express activin A receptors

Activins signal through type I (actRIA or actRIB) and type II (actRIIA or actRIIB) activin receptors. To investigate if these receptors are expressed by GC-5spg and GC-6spg spermatogonial stem cells, we performed RT-PCR for all 4 receptors on both cell lines. All activin receptors were found to be expressed by both cell lines (Fig.1). The -RT controls were negative and total testis which was taken along as a positive control showed a clear band for all receptors (Fig.1).

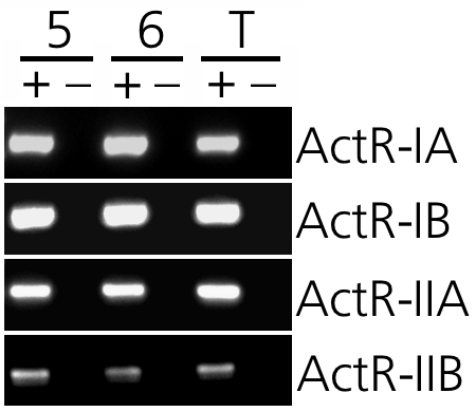


Figure 1. Expression analysis of the different activin receptors. 5: GC-5spg, 6: GC-6spg, T: total rat testis. All four receptors are expressed by the spermatogonial stem cell cell-lines.

Activin A induces spermatogonial stem cell differentiation

In order to study the potential of activin A to induce differentiation of spermatogonial stem cells, GC-6spg cells were cultured in the presence of 50 ng/ml activin A during several time intervals up to 1 week. As c-KIT was previously found to expressed in differentiating spermatogonia and spermatocytes and no or only low levels of expression were observed in spermatogonial stem cells, we used c-KIT as a marker for differentiation [24]. Expression of *c-Kit* mRNA was found to be significantly up-regulated within 4 hours after the addition of activin A to the medium. On the protein level, up-regulation of c-KIT was observed within 8 hours after the addition of activin A and expression of *c-Kit* remained elevated during at least one week (Fig.2b).

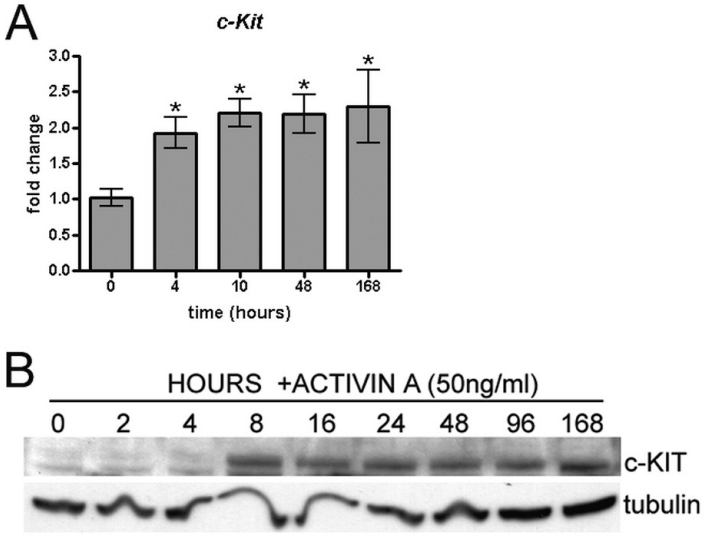


Figure 2. Activin A induced in vitro differentiation of spermatogonial stem cell line GC-6spg as demonstrated by the up-regulation of c-KIT on the mRNA (A) and protein level (B). Significant difference from the control (t=0), P<0.05 is indicated by an asterisk.

Cxcl12 Down-regulation marks spermatogonial stem cell differentiation

Previously, we identified 529 transcripts to be differentially regulated during BMP4 induced differentiation of GC-6spg spermatogonial stem cells [13]. To study if during activin A induced differentiation the same genes were differentially regulated, we performed quantitative RT-PCR on 6 selected genes and compared their expression patterns with those observed during BMP4 induced differentiation (Fig.3).

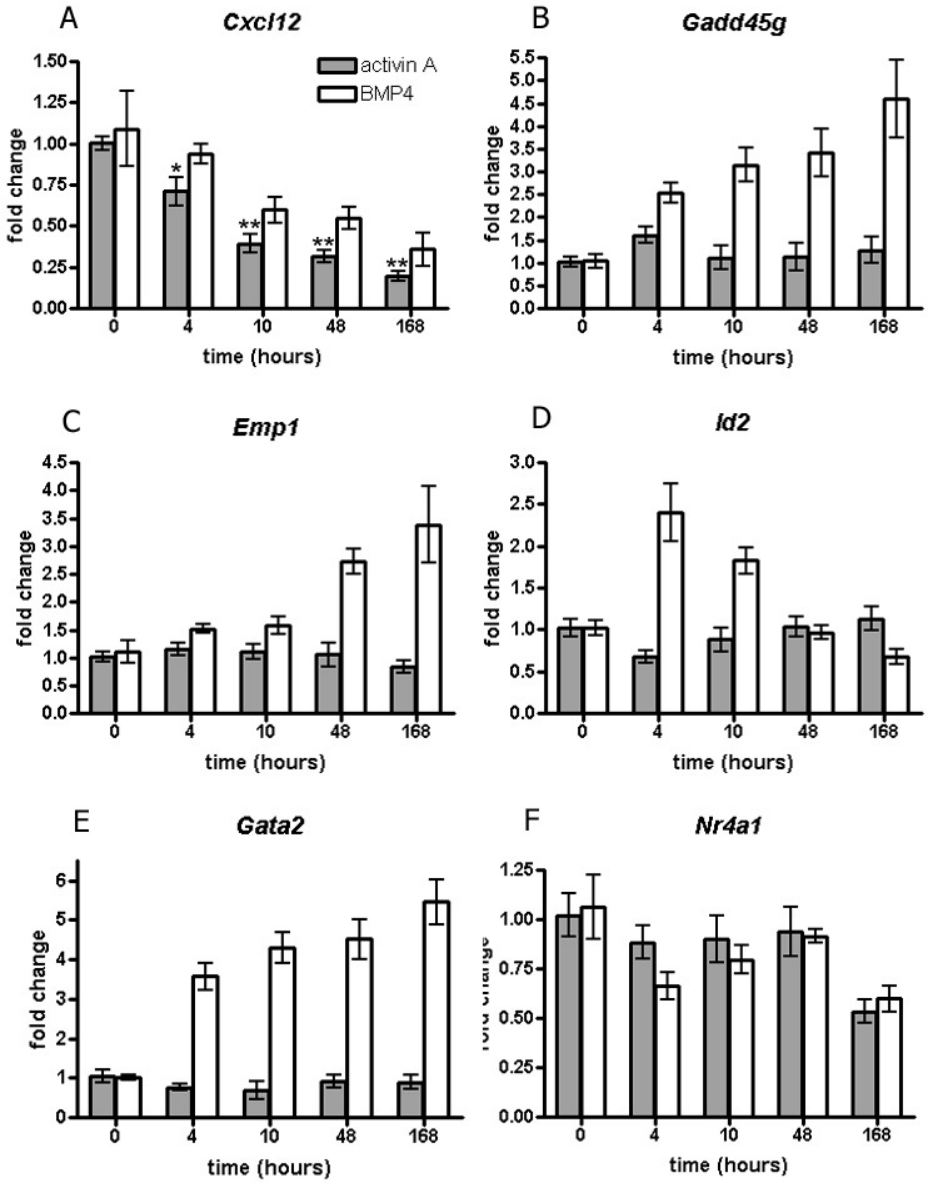


Figure 3. Comparison of the fold change (± SEM) of the indicated genes by GC-6spg cells upon the induction of differentiation by activin A (grey bars) or BMP4 (white bars). Significant difference from the control (t=0) is indicated by one asterisk (p<0,01) or two asterisks (p<0,001) for the activin A samples. Significance for the BMP4 experiment was reported elsewhere [13].

Expression levels of *Id2*, *Gadd45g*, *Emp1* and *Gata2* did not change during differentiation induced by activin A in contrast to the up-regulation of these genes previously observed during BMP4 induced differentiation. The expression levels of *Nr4a1* over time seemed to follow the same trend as was observed during BMP4 induced differentiation, however no significant effects were measured (Fig.3). Already after 4 hours of activin A induced differentiation, the expression of *Cxcl12* was significantly down-regulated and with time a further decrease was observed (Fig.3). Also during BMP4 induced differentiation of the GC-6spg spermatogonial stem cells, a significant down-regulation of *Cxcl12* was observed from 10 hours onwards [13].

Discussion

The present results clearly demonstrate that activin A can induce differentiation of GC-6spg spermatogonial stem cells *in vitro.* Interestingly, the activin A induced differentiation was accompanied by a different pattern of changes in gene expression than that previously observed during BMP4 induced differentiation of the GC-6spg cells. However, both activin A and BMP4 induced differentiation caused a clear down-regulation of *Cxcl12* suggesting that *Cxcl12* down-regulation can be used as a molecular marker for spermatogonial stem cell differentiation and that *Cxcl12* plays a role in spermatogonial stem cell maintenance.

Furthermore, we show here that all four activin receptors are expressed in the GC-5spg and GC-6spg cell lines. This suggests that spermatogonial stem cells express activin receptors and that spermatogonial stem cells therefore have the potential to be directly affected by activin A. This notion is strongly supported by the clear up-regulation of c-KIT on both the mRNA and protein level upon the addition of activin A to the culture medium of the GC-6spg cells. As the GC-6spg cells have been cultured in the absence of feeder cells, the observed effect indicates that activin A has a direct effect on GC-6spg spermatogonial stem cells.

As up-regulation of c-KIT is a marker of spermatogonial stem cell differentiation, the observed effect strongly suggests that activin A induced differentiation of GC-6spg cells *in vitro*. Previously, Nagano *et al*. reported a decrease in the number of spermatogonial stem cells upon culture of early A spermatogonia in the presence of activin A and feeder cells [15]. It was suggested that activin A induced *in vitro* differentiation of the spermatogonial stem cells as a result of the proliferation of committed spermatogonia by recruiting stem cells into the differentiation process to sustain the proliferation of non-stem spermatogonia or by a negative feedback mechanism from the expanding population of advanced spermatogonia to spermatogonial stem cells [15]. Our results, now suggest that activin A has a direct effect on the differentiation of spermatogonial stem cells.

Previously, we demonstrated the potential of GC-6spg cells to differentiate upon the addition of BMP4 to the medium. The now discovered potential of activin A underlines the usefulness of GC-6spg cells as a model system to study regulatory mechanisms of spermatogonial stem cell differentiation. Especially as the mechanisms by which BMP4 and activin A induce differentiation appear to be distinct. The expression of a number of genes, previously identified to be differentially regulated during BMP4 induced differentiation, were now studied during activin A induced differentiation. The expression of *Id2*, *Gata2*, *Gadd45g* and *Emp1*, was not significantly affected during activin A induced differentiation of the GC-6spg cells, in contrast to the upregulation observed for these genes during BMP4 induced differentiation. Although not significant, the expression pattern of *Nr4a1* during activin A induced differentiation appeared similar to the pattern observed during BMP4 induced differentiation. Notably, the expression of *Cxcl12* was downregulated during both activin A and BMP4 induced differentiation of GC-6spg cells. In addition, activin A is known to signal via activin receptors and the downstream Smads 2 and 3, while BMP4 signals via BMP receptors and the Smads 1, 5 and 8 [25]. Together, these findings indicate that activin A and BMP4 induce differentiation of GC-6spg cells, but the signaling pathways that are triggered by BMP4 and activin A have both distinct and common components. Additional experiments should be performed to identify more genes involved in activin A induced differentiation as this will allow unraveling of the specific signaling pathways of both BMP4 and activin A induced differentiation as well as common players in spermatogonial stem cell differentiation.

The expression of *Cxcl12* was down regulated during both activin A and BMP4 induced differentiation of GC-6spg cells. The CXC chemokine, CXCL12 is also known as stromal cell-derived factor 1 (SDF-1) or pre-B-cell-growth-stimulating factor (PBSF) and has been reported to be required for chemotaxis, homing and survival of hematopoietic stem and progenitor cells [26-30]. The primary physiologic receptor for CXCL12 is CXCR4, but CXCR7 has also been reported to bind CXCl12 [31].

Immunohistochemical staining revealed that expression of CXCR4 was restricted to the macrophages in the testicular interstitium [26]. To our knowledge the expression pattern of CXCR7 in mammalian testis was not reported. However, it was recently shown that CXCR7 functions primarily in the somatic environment and that CXCR7 is crucial for a proper migration of primordial germ cells toward their targets [32]. Previously, it was reported that in chick and mouse CXCL12 was required for the migration of primordial germ cells towards the genital ridge [33]. This suggests that in the adult testis, signaling between CXCl12 and CXCR7 is essential for maintaining the stem cells in their niche and thus for the maintenance of the stem cell. Upon induction of differentiation, expression of CXCl12 may be downregulated in order to allow the stem cells to leave the stem cell niche and to differentiate. Further studies

on the expression pattern of CXCL12 and CXCR7 and their function in the adult testis are required to identify their precise role within the regulation of spermatogonial stem cell maintenance and/or differentiation.

In conclusion, we report here that the different activin receptors are expressed by the GC-6spg spermatogonial stem cells and that activin A can in a direct manner induce the differentiation of these cells. In addition, we demonstrate that activin A and BMP4 induce differentiation via both distinct and identical downstream targets. Expression of Cxcl12, became down-regulated during both activin A and BMP4 induced differentiation, suggesting an important function for CXCl12 in the maintenance of spermatogonial stem cells.

References

1. Alison, M.R., et al., An introduction to stem cells. J Pathol, 2002. 197(4): p. 419-23.
2. Aponte, P.M., et al., Spermatogonial stem cells: characteristics and experimental possibilities. Apmis, 2005. 113(11-12): p. 727-42.
3. Oatley, J.M. and R.L. Brinster, Spermatogonial stem cells. Methods Enzymol, 2006. 419: p. 259-82.
4. Guan, K., et al., Pluripotency of spermatogonial stem cells from adult mouse testis. Nature, 2006. 440(7088): p. 1199-203.
5. Kanatsu-Shinohara, M., et al., Generation of pluripotent stem cells from neonatal mouse testis. Cell, 2004. 119(7): p. 1001-12.
6. Seandel, M., et al., Generation of functional multipotent adult stem cells from GPR125+ germline progenitors. Nature, 2007. 449(7160): p. 346-50.
7. Kanatsu-Shinohara, M., et al., Pluripotency of a Single Spermatogonial Stem Cell in Mice. Biol Reprod, 2008.
8. Moens, P.B. and A.D. Hugenholtz, The arrangement of germ cells in the rat seminiferous tubule: an electron-microscope study. J Cell Sci, 1975. 19(3): p. 487-507.
9. de Rooij, D.G. and L.D. Russell, All you wanted to know about spermatogonia but were afraid to ask. J Androl, 2000. 21(6): p. 776-98.
10. Meng, X., et al., Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. Science, 2000. 287(5457): p. 1489-93.
11. Oatley, J.M., M.R. Avarbock, and R.L. Brinster, Glial cell line-derived neurotrophic factor regulation of genes essential for self-renewal of mouse spermatogonial stem cells is dependent on Src family kinase signaling. J Biol Chem, 2007. 282(35): p. 25842-51.
12. Hofmann, M.C., L. Braydich-Stolle, and M. Dym, Isolation of male germ-line stem cells; influence of GDNF. Dev Biol, 2005. 279(1): p. 114-24.
13. van Bragt, M., et al., Controlled differentiation of spermatogonial stem cell line reveals genes involved in early steps of spermatogonial differentiation. in preparation.
14. van Pelt, A.M., et al., Establishment of cell lines with rat spermatogonial stem cell characteristics. Endocrinology, 2002. 143(5): p. 1845-50.
15. Nagano, M., et al., Maintenance of mouse male germ line stem cells in vitro. Biol Reprod, 2003. 68(6): p. 2207-14.
16. Meinhardt, A., et al., Activin maintains the condensed type of mitochondria in germ cells. Mol Cell Endocrinol, 2000. 168(1-2): p. 111-7.
17. Mather, J.P., et al., Activin stimulates spermatogonial proliferation in germ-Sertoli cell cocultures from immature rat testis. Endocrinology, 1990. 127(6): p. 3206-14.
18. van Dissel-Emiliani, F.M., et al., Inhibin reduces spermatogonial numbers in testes of adult mice and Chinese hamsters. Endocrinology, 1989. 125(4): p. 1899-903.
19. de Winter, J.P., et al., Activin is produced by rat Sertoli cells in vitro and can act as an autocrine regulator of Sertoli cell function. Endocrinology, 1993. 132(3): p. 975-82.
20. de Winter, J.P., et al., Peritubular myoid cells from immature rat testes secrete activin-A and express activin receptor type II in vitro. Endocrinology, 1994. 135(2): p. 759-67.
21. Loveland, K.L. and G. Hime, TGFbeta superfamily members in spermatogenesis: setting the stage for fertility in mouse and Drosophila. Cell Tissue Res, 2005. 322(1): p. 141-6.
22. de Jong, F.H., Testicular activin--too hot to handle? Eur J Endocrinol, 1997. 137(5): p. 448-9.
23. van Pelt, A.M., et al., Isolation of the synchronized A spermatogonia from adult vitamin A-deficient rat testes. Biol Reprod, 1996. 55(2): p. 439-44.
24. Schrans-Stassen, B.H., et al., Differential expression of c-kit in mouse undifferentiated and differentiating type A spermatogonia. Endocrinology, 1999. 140(12): p. 5894-900.
25. Miyazawa, K., et al., Two major Smad pathways in TGF-beta superfamily signalling. Genes Cells, 2002. 7(12): p. 1191-204.

26. Habasque, C., et al., Study of the HIV-1 receptors CD4, CXCR4, CCR5 and CCR3 in the human and rat testis. Mol Hum Reprod, 2002. 8(5): p. 419-25.
27. Kim, C.H. and H.E. Broxmeyer, In vitro behavior of hematopoietic progenitor cells under the influence of chemoattractants: stromal cell-derived factor-1, steel factor, and the bone marrow environment. Blood, 1998. 91(1): p. 100-10.
28. Lapidot, T. and O. Kollet, The essential roles of the chemokine SDF-1 and its receptor CXCR4 in human stem cell homing and repopulation of transplanted immune-deficient NOD/SCID and NOD/SCID/B2m(null) mice. Leukemia, 2002. 16(10): p. 1992-2003.
29. Broxmeyer, H.E., et al., Transgenic expression of stromal cell-derived factor-1/CXC chemokine ligand 12 enhances myeloid progenitor cell survival/antiapoptosis in vitro in response to growth factor withdrawal and enhances myelopoiesis in vivo. J Immunol, 2003. 170(1): p. 421-9.
30. Broxmeyer, H.E., et al., Rapid mobilization of murine and human hematopoietic stem and progenitor cells with AMD3100, a CXCR4 antagonist. J Exp Med, 2005. 201(8): p. 1307-18.
31. Balabanian, K., et al., The chemokine SDF-1/CXCL12 binds to and signals through the orphan receptor RDC1 in T lymphocytes. J Biol Chem, 2005. 280(42): p. 35760-6.
32. Boldajipour, B., et al., Control of Chemokine-Guided Cell Migration by Ligand Sequestration. Cell, 2008. 132(3): p. 463-473.
33. Stebler, J., et al., Primordial germ cell migration in the chick and mouse embryo: the role of the chemokine SDF-1/CXCL12. Dev Biol, 2004. 272(2): p. 351-61.

Chapter 6: Summarizing discussion

Spermatogenesis is a fascinating process, capable of producing millions of highly differentiated cells per day [1]. The process is complex and requires the constant interaction between germ cells and somatic cells. Several known growth factors and signaling pathways are known, however likely more, yet unknown factors, pathways and other regulatory mechanisms play a role [2].

In this thesis, emphasis was placed on unraveling regulatory mechanisms involved in the initial process of spermatogenesis, i.e. the mechanisms that regulate spermatogonial stem cell self-renewal and differentiation. To be more precise, research was focused on identifying genes that play a role in spermatogonial stem cell self-renewal and differentiation.

Genes were identified using two approaches, i.e., a candidate approach (chapters 2 and 3) and a genomics approach (chapters 4 and 5). For the candidate approach, genes were selected based on their previously reported function in self-renewal and differentiation of other stem cells. The possible function of these candidate genes in spermatogonial stem cells was studied and the results of two of these candidate genes were described in this thesis. For the genomic approach a model for spermatogonial stem cell differentiation was established using the GC-6spg spermatogonial stem cell line. Upon establishment of this model, micro-array and quantitative RT-PCR studies were performed which allowed the identification of genes differentially regulated during spermatogonial stem cell differentiation.

Candidate approach: SCA-1 and the side population

Stem cell antigen 1 (SCA-1), also known as LY6A/E, was selected because SCA-1 is frequently used as a marker to isolate hematopoietic stem cells and was shown to play a role in the repopulating capacity of these cells and the development of committed progenitor cells, megakaryocytes, and platelets [3, 4]. Furthermore, two independent research groups showed that SCA-1 was expressed by the testicular side population [5, 6]. However, these results were confusing as one of the research groups showed that the testicular side population contains spermatogonial stem cells, while the other group reported that the testicular side population cells did not possess repopulation capacity [5, 6].

Our results (chapter 2) showed that expression of SCA-1 was restricted to the testicular interstitium. Immunohistochemistry only revealed SCA-1 staining of peritubular myoid cells, spherical-shaped peritubular mesenchymal cells and depending on the mouse strain of endothelial cells. No phenotypic defects were observed in Ly6a$^{-/-}$ mice testes suggesting that the function of SCA-1 in testis development and spermatogenesis is redundant. Nevertheless, the observed results were important as they revealed the identity of SCA-1 positive cells and demonstrated

that SCA-1 does not play a critical role during spermatogonial stem cell self-renewal and differentiation.

Furthermore, comparing the different reports on the testicular side population, it became clear that parameters like Hoechst 33342 concentration and incubation time define which cell types are isolated by the side population technique [5-10]. For example, the testicular side population isolated by Lo *et al.* contained stem cells of the Leydig cell lineage [7, 11]. Use of the side population technique as an isolation method for spermatogonial stem cells would require extensive optimization as the protocols described so far were not efficient. The protocol used by Lassalle *et al.* resulted in a 15-fold enrichment in stem cell activity as determined by transplantation assay [6]. In comparison, a 166-fold enrichment was obtained by FACS sorting testicular cells on light-scattering properties, positive staining for α6-integrin and negative or low αv-integrin expression [12]. A 700-fold enrichment was obtained using a 400bp fragment of the stra8 locus to direct gene expression of a surface protein tag to a population of testicular cells containing repopulation activity [13]. In addition to the lack of a good protocol to isolate spermatogonial stem cells with the side population technique, the Hoechst 33342 dye, used for this technique has been reported to be toxic for cells [14].

The side population technique is therefore, in my opinion, not the preferred method to isolate spermatogonial stem cells from testes. A much purer population could be obtained using a combination of surface markers (e.g. ITGα6^{+}, THY1^{+}, SCA1^{-}, GFRα1^{+}) to sort spermatogonial stem cells from cryptorchid or VAD animals with FACS or MACS. Alternatively, highly pure populations of spermatogonial stem cells could be isolated by FACS from testes of transgenic animals which express a reporter protein (e.g. green fluorescent protein, GFP) under the control of the PLZF or UTF1 promoter.

Candidate approach: UTF1

Undifferentiated embryonic cell transcription factor 1 (UTF1), known to be expressed by embryonic stem cells and embryonal carcinoma cells, was quickly down-regulated upon the induction of differentiation of these cells [15, 16]. In adult mice expression of *Utf1* mRNA was found to be restricted to the ovary and the testis [15].

Our results (chapter 3) showed that within the testis, expression of UTF1 was restricted to a small subset of germ cells. In embryonic and neonatal testes, all gonocytes were found to strongly express UTF1. During further testicular development, expression of UTF1 was restricted to a subset of A spermatogonia and with increasing age the number of cells expressing UTF1 decreased even more. Ultimately, in the adult rat testis, only a small subset of the type A spermatogonia expressed UTF1. Remarkably, even in testes of vitamin A deficient rats that exclusively

contain A_s, A_{pr} and A_{al} type spermatogonia, only a subset of the spermatogonia expressed UTF1. In the adult rat testis, expression of UTF1 was restricted to a subpopulation of the PLZF positive early A spermatogonia. Furthermore, the distribution pattern of UTF1 expressing cells over the different stages of the cycle of the seminiferous epithelium suggested that expression of UTF1 was restricted to those spermatogonia that were in an undifferentiated state and that these cells had the potential to differentiate into spermatozoa or possibly even in other directions when taken out of their testicular niche (chapter 3).

These results were exciting as UTF1 expression was restricted to a small population of germ cells likely including the spermatogonial stem cells. Unfortunately, two questions were not answered. First, what percentage of the early A spermatogonia expressed UTF1? Second, how were the UTF1 expressing cells divided over the different types of early A spermatogonia, i.e. A_s, A_{pr} and A_{al} spermatogonia? These questions might be answered by immunohistochemical staining of whole mounts of seminiferous tubules. The early A spermatogonia are located on the basal lamina of the seminiferous tubules and the topographical arrangement of the single, paired and short chains of aligned spermatogonia can easily be distinguished in whole mount preparations [17]. Immunohistochemical staining with an antibody directed to UTF1 on whole mount seminiferous tubules would thus allow calculation of the percentage of early A spermatogonia that express UTF1. In addition, the distribution of these positive cells over the A_s, A_{pr} and the A_{al} of various chain lengths could be determined. Unfortunately, several attempts and protocols failed, while an antibody directed to PLZF, taken as a control showed clear staining. This lack of staining is probably due to the fact that the antibodies directed to UTF1 worked best on diluted Bouin's fixed testes tissue. As this is a strong fixation method, the antibody is probably not capable of reaching the UTF1 protein within the nucleus of the early A spermatogonia in a whole mount assay.

Generation of transgenic reporter mice that express a reporter protein under the control of the UTF1 promoter would be an alternative. UTF1 expressing cells isolated from these mice could also be used to determine what percentage of the UTF1 expressing cells posses repopulation capacity. As repopulation capacity is believed to be a unique characteristic of the spermatogonial stem cells (A_s), it would be interesting to see if the percentage of cells that forms a colony is equal to the percentage of A_s spermatogonia that express UTF1 as determined by the whole mount experiment. The function of UTF1 in primordial germ cells, gonocytes and spermatogonial stem cells is unknown. In addition, the role of UTF1 in embryonic stem cells is under debate as some reports suggested UTF1 to be a transcriptional co-activator while a recent report described UTF1 as a stable chromatin-associated transcriptional repressor protein with a dynamic behavior similar to core histones [15,

18, 19]. Knock down of UTF1 in embryonic stem cells resulted in a substantial delay or block in the differentiation but did not affect the self-renewal capacity of the embryonic stem cells [19]. It was suggested that the function of UTF1 in embryonic stem cells could be the maintenance of a specific epigenetic profile required for differentiation either by attracting chromatin-modifying proteins or by chromatin compaction [19]. It was furthermore reported that expression of UTF1 in embryonic stem cells is rapidly down-regulated upon differentiation and that UTF1 therefore can be used as a marker for pluripotency [15, 19, 20].

The literature about UTF1 in embryonic stem cells and our findings, as described in chapter 3, have let us to develop a working hypothesis for the function of UTF1 in the pluripotent stem cell (Fig.1). In our hypothesis, UTF1 functions in a complex that maintains the DNA in such an epigenetic state that transcription of lineage specific genes is inhibited. However, when differentiation in a specific lineage is induced, the UTF1 complex is removed and the epigenetic state of the DNA is brought to a state that allows transcription of the lineage specific genes. Furthermore, we speculate that the UTF1 complex is only partly removed from genes involved in differentiation of other lineages, thereby permanently blocking differentiation in other directions. Knock down of UTF1 in embryonic stem cells resulted in the delay or block of differentiation [19], suggesting that the UTF1 complex can no longer be efficiently removed and that differentiation therefore is permanently blocked. However, this model should be tested in further research to either confirm or adjust the hypothesis. Confirmation that also in testicular cells, UTF1 functions in a complex and identification of the different components of this complex, could support and expand the hypothesis. As the model indicates that the complex blocks the transcription of many different genes, it is not likely that the complex binds to one specific DNA binding site. Nevertheless, it would be interesting to perform a chromatin immunoprecipitation assay (CHIP), in order to determine if the complex binds to specific sites on the DNA [21]. Furthermore, it would be exciting to knock down the different components of the complex to determine their function within the complex.

As only a small percentage of the testicular cells express UTF1 and *in vitro* experiments allow easier manipulation, the use of cell-lines would be preferable to identify the function of UTF1 in the spermatogonia. Unfortunately, expression of UTF1 could not be detected in any of the immortalized germ cell lines generated in our lab, i.e. the gonocyte (G53-3-1, G53-3-2 and G53-3-4) and the spermatogonial stem cell (GC-5spg and GC-6spg) cell-lines (not shown). However, the GS and ES-like cells described by Kanatsu-Shinohara *et al.* expressed UTF1 and these cells could therefore be used to test the hypothesis and to study the function of UTF1 in the testis [22, 23].

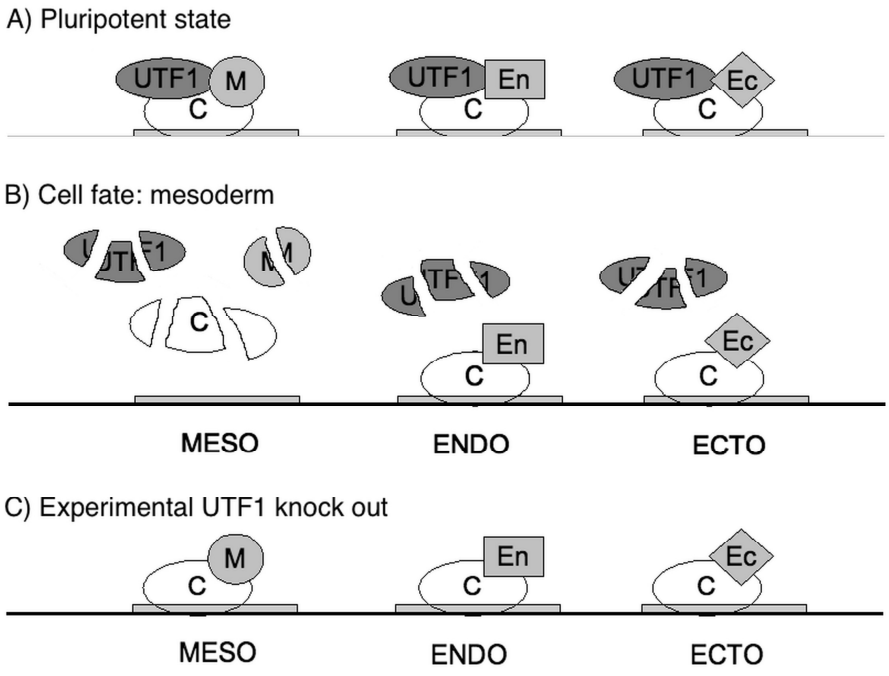


Figure 1. Hypothetical model for the function of UTF1.

In an undifferentiated pluripotent stem cell (A), UTF1 functions in a complex that maintains the DNA in such a state that the lineage specific genes can not be transcribed. Although symbolically drawn as three components, each component may actually consist of multiple proteins. The component indicated with C is symbolic for the structure that renders the DNA in a state that does not allow transcription. Histones and DNA compaction are likely to be part of component C. The components indicated with M, En or Ec are lineage specific components, which may function as specific recognition sites for differentiation inducing signals.

When differentiation is induced, in for example the mesodermal direction (B), the whole complex detaches from the mesodermal specific genes, allowing their transcription (B). The different components of the structure are broken down. UTF1 expression is down-regulated upon the differentiation of the cell. However, the complex is only partial removed from the genes involved in the differentiation in the other directions, thereby blocking the differentiation in these directions permanently, pluripotency is then lost.

When in an experimental model UTF1 is knocked out (C), all differentiation pathways are blocked as the complex can no longer be (efficiently) removed and no transcription of lineage specific genes can occur.

MESO: mesodermal specific promotor/gene, ENDO: endodermal specific promotor/gene, ECTO: ectodermal specific promotor/gene.

Candidate approach: BMI-1

Besides SCA-1 and UTF1, a number of other proteins were examined for their possible function in spermatogonial stem cell self-renewal and differentiation. For example, B lymphoma MO-MLV insertion region 1 (BMI-1), a transcriptional repressor that belongs to the polycomb group. This protein was reported to be required for the efficient self-renewal of neural and hematopoietic stem cells [24, 25]. Bmi-1$^{-/-}$ mice are viable, but exhibit defects in hematopoiesis, skeletal patterning, neurological functions and development of the cerebellum [26]. As a result of these phenotypic defects, homozygous knock out mice were difficult to breed (personnel communication with members of the group of Dr. M. van Lohuizen, NKI, Amsterdam, the Netherlands). Bmi-1 mRNA was reported to be expressed in the testis [27].

We performed immunohistochemical staining and observed expression of BMI-1 in Sertoli cells, spermatogonia and spermatocytes. However, as the results obtained by immunohistochemistry were variable, the identification of the type of spermatogonia and spermatocytes that expressed BMI-1 was unreliable. The morphology of Bmi-1$^{-/-}$ mice testes on both FVB and C57BL6/J backgrounds was studied in collaboration with van der Stoop and van Lohuizen. An enlarged rete testis was observed on the FVB background, however to our surprise we also observed this phenotype in the wild type animals. Taking this abnormality into account, there were no further visible effects on spermatogenesis. A recent report showed that BMI-1 was expressed in undifferentiated spermatogonia and that overexpression of BMI-1 could promote spermatogonia proliferation, while repression of endogenous Bmi-1 by RNAi resulted in inhibition of the proliferation [28].

Candidate approach: conclusion

Unraveling the mechanisms of spermatogonial stem cell self-renewal and differentiation by means of selecting genes on the base of literature studies is in my opinion not the best approach as the study of the possible role of each selected gene requires a large amount of time and resources. However, as at the start of the project no experimental model for spermatogonial stem cell self-renewal or differentiation was available we did use the direct gene approach. Our efforts did rule out SCA-1 as possible player in the regulation of spermatogonial stem cell self-renewal or differentiation and revealed the unique expression pattern of UTF1 in the testis. Further research on the expression pattern and the function of UTF1 will bring more insight in the mechanisms of spermatogonial stem cell self-renewal, differentiation and its potential role in the pluripotent capacity of spermatogonial stem cells.

Genomics approach

In theory, identification of genes involved in spermatogonial stem cell differentiation is simple. Obtain a pure population of spermatogonial stem cells, differentiate them in vitro and compare the undifferentiated cells with the differentiated cells in a micro-array experiment. The spermatogonial stem cells were present in the lab as immortalized cell lines and with the establishment of the protocol for their *in vitro* differentiation we generated an excellent and unique model to study the regulatory mechanisms of the initial steps of spermatogonial stem cell differentiation.

The GC-6spg cells form a pure population of cells with spermatogonial stem cell properties which can be cultured indefinitely without feeder cells or high concentrations of fetal calf serum. In chapter 4, we showed that the GC-6spg cells expressed several spermatogonial stem cell markers which together with the earlier reported colonization potential confirmed the spermatogonial stem cell character of the GC-6spg cells [29].

In vitro differentiation of the GC-6spg cells was first established by addition of BMP4 to the culture medium (chapter 4). BMP4 was previously suggested to play a role in differentiation of spermatogonial stem cells [30, 31]. c-KIT negative spermatogonia isolated from neonatal mice acquired sensitivity to stem cell factor (SCF), the ligand for the c-KIT receptor, upon the addition of BMP4 to the culture medium [30]. Transplantation of spermatogonia cultured on feeder cells in the presence of BMP4 resulted in a lower number of colonies compared to transplantation of spermatogonia cultured in the absence of BMP4 [31]. These results suggested that BMP4 induced differentiation of the early A spermatogonia. Our data (chapter 4) reinforced the idea that BMP4 has the potential to induce differentiation of a pure population of adult spermatogonial stem cells in vitro. We showed that the GC-6spg cells expressed the different BMP4 receptors and upon addition of BMP4 to the medium the Smads 1, 5 and 8 were phosphorylated and expression of *c-Kit* was up-regulated on both the mRNA and protein level. Hence, differentiation of the cells occurred in a direct and specific manner.

Activin A was also shown to be capable of inducing differentiation of GC-6spg cells as c-Kit mRNA and protein were upregulated within 4 and 8 hours, respectively (chapter 5). Previously, it was reported that culture of spermatogonia in the presence of activin A resulted in the loss of repopulation capacity of these cells compared to spermatogonial stem cells cultured in the absence of activin A [31]. Furthermore, Activin A was suggested to play a role in spermatocyte differentiation and the proliferation of spermatogonia [32, 33]. Although we showed that the different activin A receptors were expressed by GC-6spg cells, phosphorylation of the

downstream targets Smad 2 and 3, was not studied yet. The data suggested that activin A, like BMP4, could induce in vitro spermatogonial stem cell differentiation in a specific manner as demonstrated by the up-regulation of c-KIT.

With the establishment of activin A as an additional inducer of differentiation, the GC-6spg cells became an even more powerful tool to study spermatogonial stem cell differentiation. Especially, because BMP4 and activin A signal via different pathways, i.e. activin A signals via the activin receptors and Smad 2 and 3, while BMP4 signals via BMP receptors and Smad 1, 5 and 8 [34]. Now, not only the specific signaling pathways of both BMP4 and activin A can be studied, it also allows the identification of common downstream factors in the process of differentiation.

Up-regulation of c-KIT can be used as a marker for the differentiation of early A spermatogonia into differentiating spermatogonia as A_{al} spermatogonia were found to gradually change from c-KIT negative to c-KIT positive cells before their differentiation into A1 spermatogonia [35]. However, additional evidence should be provided to confirm that BMP4 and activin A induced differentiation of GC-6spg cells in the direction of differentiated spermatogonia. After all, c-KIT is also expressed by primordial germ cells and gonocytes, in which case BMP4 and activin A induced de-differentiation instead of differentiation. Even more, in view of recent reports describing the pluripotent potential of spermatogonial stem cells, addition of BMP4 or activin A may have resulted in the up-regulation of c-KIT as a result of the differentiation in the direction of a different lineage, e.g. hematopoietic stem cells and its immediate progenitors or Leydig cells [36, 37]

Unfortunately, no other or earlier molecular markers are known that could confirm the first in vitro differentiation step of GC-6spg cells. Other molecular markers used to determine the degree of differentiation of testicular germ cells are proteins involved in meiosis (e.g. SCP1, SCP3, DMC1, SPO11) or proteins expressed in spermatids (e.g. HILS1, H1T2, PRM1 and PRM2) [38-44]. Down-regulation of the early A spermatogonial markers GFRα1 and PLZF, could potentially be used as markers for spermatogonial stem cell differentiation. Expression of GFRα1 was reported to be restricted to the A_s and A_{pr} spermatogonia [45]. However, no expression of GFRα1 was observed in GC-6spg cells. As no GDNF was present in the culture medium of GC-6spg cells the expression of GFRα1 was possibly lost during earlier passages. The expression level of PLZF upon induction of differentiation by either BMP4 or activin A was not studied but should be down-regulated as expression is known to be restricted to the early A spermatogonia [46, 47]. In addition, it was recently reported that PLZF directly represses the transcription of *c-Kit* [48]. Alternatively, transplantation assays could be performed to investigate if the differentiated GC-6spg cells did loose their potential to home to the stem cell niche. After all, it is believed that only the true spermatogonial stem cell can colonize the depleted recipient testis [49].

The low level of expression and the relatively high molecular weight of c-KIT (140-150 kDA) make the detection of its up-regulation by western blot analysis difficult. Also, western blot analysis is a time consuming technique and therefore the generation of an alternative method to detect c-KIT up-regulation would be ideal. Stable transfection of a fluorescent reporter under the control of the c-KIT promoter would allow fast identification of differentiation and thereby provide a tool for screening multiple factors for their differentiation potential, e.g. LIF, SCF, IGF and combinations of these and other factors.

Two students in our lab (Gregor van Bochove and Marloes Bergevoet) demonstrated that omitting FGF2 from the culture medium resulted in the up-regulation of c-KIT [50, 51]. However, the signal was weak and only occurred after at least two weeks of culture in the absence of FGF2. The effect of FGF2 depletion on GC-6spg cells could possibly be enhanced by blocking the FGF2 pathway by inhibitors such as SU5402 or PD173074 to prevent any signaling from FGF2, likely present in the FCS in the culture medium. Previously, no effect was observed on spermatogonial stem cell self-renewal when spermatogonia were cultured in the presence of FGF2 alone [31, 52]. However, a synergistic effect on the maintenance and expansion of spermatogonial stem cells was reported when both FGF2 and GDNF were present [53, 54]. Hence, FGF2 appears to play a role in spermatogonial stem cell fate decision. Nevertheless, it would be interesting to study if c-KIT expression is enhanced or expression of meiosis markers is up-regulated when GC-6spg cells are cultured in the presence of BMP4 or activin A while FGF2 is absent.

In conclusion, we have demonstrated that GC-6spg cells can be induced to differentiate by at least two growth factors and that the cells therefore provide an excellent tool to study the intrinsic regulation mechanisms of the initial steps of spermatogonial stem cell differentiation.

Micro-array analysis using the GC-6spg differentiation model

In order to generate a candidate list of genes involved in the initial steps of spermatogonial stem cell differentiation, a micro-array experiment was performed using the BMP4 induced differentiation of GC-6spg cells as a model (chapter 4). Expression levels of the BMP4 treated cells were compared with the expression levels of the non treated cells at four different time points (4, 10, 48 and 168 hours) after addition of BMP4 to the culture medium. Studying multiple time points allowed us to follow the events during the differentiation process with time. It allowed the identification of direct targets of BMP4 within spermatogonial stem cells as well as the identification of possible new markers of spermatogonial stem cell differentiation.

In total, 529 transcripts were identified by micro-array to be ≥ 2-fold regulated during at least one of the time points measured. Of these 529 transcripts, 176 (33%) unique genes could be assigned to at least one biological function category according to the Gene Ontology (GO) database. Out of the 176 genes, 6 were selected to be confirmed by quantitative RT-PCR. All 6 genes showed a similar expression pattern compared to the micro-array data indicating that the micro-array experiment was reliable and that the data obtained could be used to generate a list of genes that might play a function during spermatogonial stem cell fate decision and the initial differentiation steps.

Remarkably, most differentially regulated genes known to play a role in proliferation were down-regulated during the later time points (Fig.6, chapter 4). Although the effect of BMP4 on proliferation of GC-6spg was not studied by for example a tritiated thymidine or Brdu incorporation assay, the number of cells present in the BMP4 culture appeared to be lower then the number of cells present in the standard culture after 1 week. This difference became even more apparent after 10 days of culture. Together these results strongly suggest that BMP4 not only induced differentiation but also had a negative effect on the proliferation of the GC-6spg cells.

Furthermore, several genes involved in the differentiation of other cell types were differentially regulated during the differentiation of GC-6spg cells. These genes might also play a role during spermatogonial stem cell differentiation and should be considered first when selecting genes for further studies.

Upon the induction of differentiation by BMP4, the expression level of the transcription factor GATA2, was directly up-regulated and continued to increase thereafter in GC-6spg cells (Fig.5, chapter 4). Interestingly, GATA2 was reported to be a key transcription factor in the control of cell fate in hematopoietic stem and progenitor cells [55, 56]. Furthermore, PLZF was shown to interact with GATA-2 and to inhibit the transcriptional activity of GATA2 [55]. As PLZF was reported to be a transcriptional repressor essential for spermatogonial stem cell self-renewal it suggests that GATA2 inhibits the repressor activity of PLZF and/or that PLZF inhibits the transcription activity of GATA2. In addition, as PLZF was recently reported to be a direct inhibitor of c-Kit transcription and GATA2 was reported to collaborate in activating c-Kit, it is tempting to speculate that PLZF and GATA2 compete for the regulation of the c-Kit promoter [48, 57].

Although it is not clear from the literature if GATA2 is expressed in the testis or spermatogonial stem cells of adult animals, expression levels as measured in both the micro-array and the quantitative RT-PCR experiments were high. Western blot analysis of GATA2 on GC-6spg cells and total testis will reveal if Gata2 mRNA is also translated into protein. Immunohistochemistry on testis sections and/or whole mount seminiferous tubules can reveal the localization of GATA2 in vivo. Thereafter

experiments can be performed to see if PLZF and GATA2 interact with each other and it will be interesting to see if GATA2 target genes are elevated when PLZF is knocked out or vice versa. It should however be noted that the expression of GATA2 was not up-regulated during activin A induced differentiation of GC-6spg cells (chapter 5).

Among the 6 genes differentially regulated by BMP4, only two showed similar expression profiles upon activin A induced differentiation, e.g. Nr4a1 and Cxcl12 (chapter 5). As BMP4 and activin A bind to different receptors, which in turn phosphorylate distinct Smads, it is to be expected that the downstream signals that lead to differentiation only partially overlap. To identify how big the overlap is, more of the 529 transcripts that were differentially regulated during BMP4 induced differentiation should be tested with respect to their expression level during activin A induced differentiation. Preferably, a micro-array experiment should be performed in which the mRNA expression levels of the activin A treated GC-6spg cells could be compared with the non treated cells at the same four different time points (4, 10, 48 and 168 hours). Overlap in the BMP4 and activin A candidate list will results in a list of genes which are likely to be involved in spermatogonial stem cell differentiation independent of the initiator of differentiation.

Although the activin A induced down-regulation of Nr4a1 was not significant, both Nr4a1 and Cxcl12 were down-regulated during BMP4 and activin A induced differentiation (chapter 5). An initial targeted knockdown experiment of Nr4a1 in GC-6spg cells did not reveal up-regulation of c-KIT protein. Nevertheless, further research on the function of NR4a1 in apoptosis and proliferation of spermatogonial stem cells could be interesting as NR4a1 was previously reported to have an effect on these processes [58].

As described in chapter 5, the expression of Cxcl12 was down regulated during both activin A and BMP4 induced differentiation of GC-6spg cells. Furthermore, CXCl12 was reported to play a role in homing and maintenance of hematopoietic stem cells and in the migration of primordial germ cells toward the genital ridge [59-61]. Together, these results suggest that in the adult testis, signaling between CXCl12 and its receptor CXCR7 could be essential for maintaining the stem cell in its niche and thus for the maintenance of the stem cell. Studies on the expression pattern of CXCL12 and CXCR7 and further studies on their function in the testis will allow the identification of their role in spermatogonial stem cell maintenance and/or differentiation.

In conclusion, our models of spermatogonial stem cell differentiation have enabled us to detect a number of genes involved in this process. If additional experiments can be performed that confirm the differentiation of the GC-6spg cells in the direction of spermatozoa, these cells form a unique and exciting model which allows studies on intrinsic regulatory mechanisms of spermatogonial stem cell

differentiation. Expression of CXCl12 was found to be significantly down-regulated during both activin A and BMP4 induced differentiation and further research on CXCl12 and its receptor CXCR7 will reveal their possible role in maintaining the spermatogonial stem cells in the niche and therefore a role in spermatogonial stem cell maintenance.

Overall conclusion

In order to obtain more knowledge about the mechanisms of spermatogonial stem cell self-renewal and differentiation we aimed to identify genes involved in these processes. Using both a candidate and a genomics approach we revealed numerous genes that are very likely to be involved in these processes.

The restricted expression pattern of UTF1, described in this thesis and the know function of UTF1 in embryonic stem cells strongly suggest that UTF1 plays a role in maintaining the stem cell state of the spermatogonial stem cells. Additional (functional) experiments should be performed to determine the exact function of UTF1 in these cells.

For the genomics approach two protocols for in vitro differentiation of the GC6spg cells were established. Using BMP4 induced differentiation of the GC-6spg cells as a model in a time course micro-array experiment has revealed numerous transcripts that are differentially regulated during the differentiation process. Among those transcripts were a number of genes known to be involved in differentiation of other types of stem cells, suggesting that these genes are indeed involved in spermatogonial stem cell differentiation and that these genes are prime candidates for further studies on spermatogonial stem cell differentiation.

References

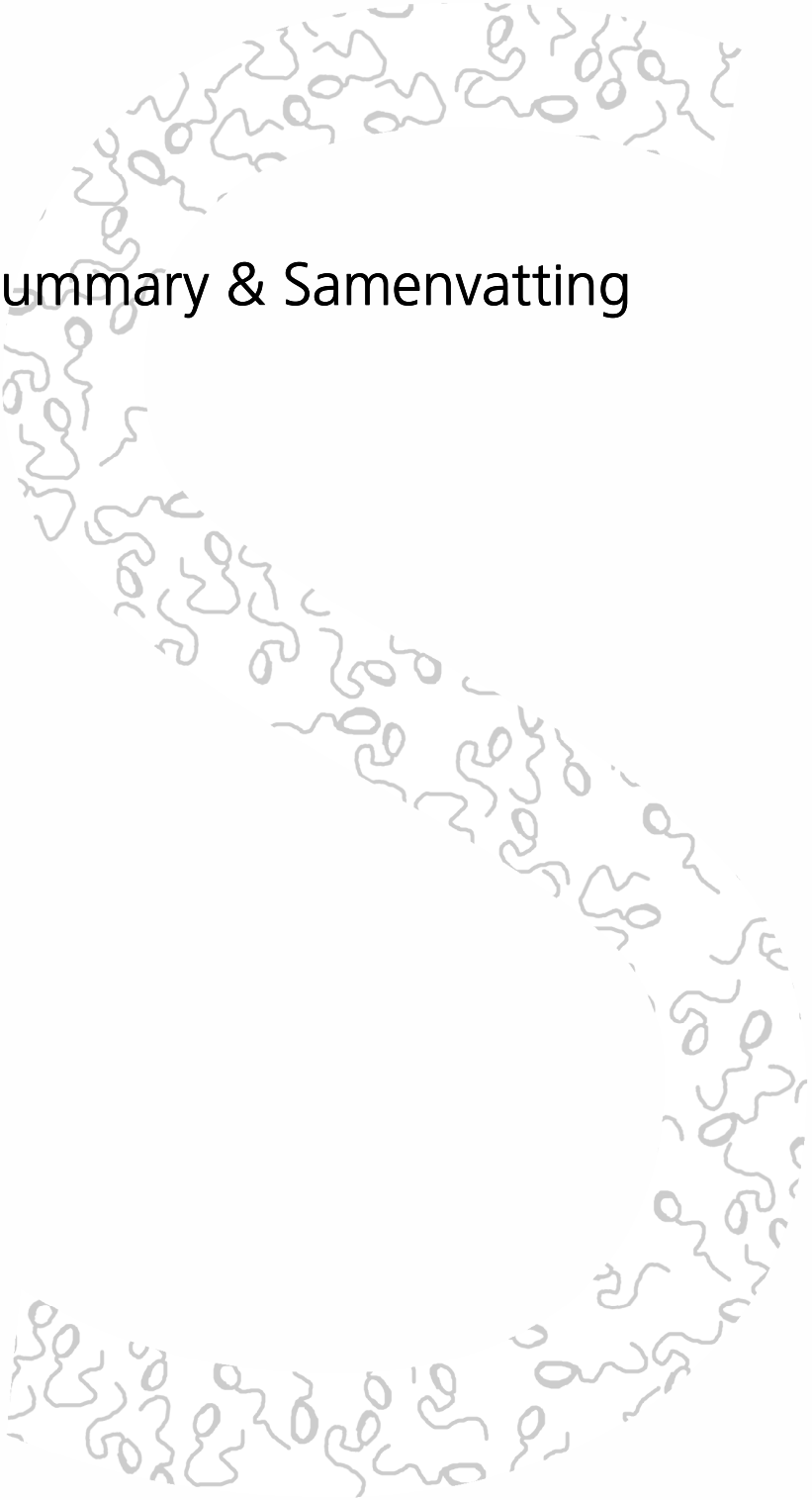
1. Johnson L, Varner DD, Roberts ME, Smith TL, Keillor GE, Scrutchfield WL. Efficiency of spermatogenesis: a comparative approach. Anim Reprod Sci 2000; 60-61: 471-480.
2. Sharpe RM. Local control of testicular function. Q J Exp Physiol 1983; 68: 265-287.
3. Spangrude GJ, Heimfeld S, Weissman IL. Purification and characterization of mouse hematopoietic stem cells. Science 1988; 241: 58-62.
4. Ito CY, Li CY, Bernstein A, Dick JE, Stanford WL. Hematopoietic stem cell and progenitor defects in Sca-1/Ly-6A-null mice. Blood 2003; 101: 517-523.
5. Kubota H, Avarbock MR, Brinster RL. Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells. PNAS 2003; 100: 6487-6492.
6. Lassalle B, Bastos H, Louis JP, Riou L, Testart J, Dutrillaux B, Fouchet P, Allemand I. 'Side Population' cells in adult mouse testis express Bcrp1 gene and are enriched in spermatogonia and germinal stem cells. Development 2004; 131: 479-487.
7. Lo KC, Lei Z, Rao CV, Beck J, Lamb DJ. De Novo Testosterone Production in Luteinizing Hormone Receptor Knockout Mice After Transplantation of Leydig Stem Cells. Endocrinology 2004; 145: 4011-4015.
8. Falciatori I, Borsellino G, Haliassos N, Boitani C, Corallini S, Battistini L, Bernardi G, Stefanini M, Vicini E. Identification and enrichment of spermatogonial stem cells displaying side-population phenotype in immature mouse testis. FASEB J 2004; 18: 376-378.
9. Lo KC, Brugh Iii VM, Parker M, Lamb DJ. Isolation and Enrichment of Murine Spermatogonial Stem Cells Using Rhodamine 123 Mitochondrial Dye. Biol Reprod 2005; 72: 767-771.
10. Montanaro F, Liadaki K, Schienda J, Flint A, Gussoni E, Kunkel LM. Demystifying SP cell purification: viability, yield, and phenotype are defined by isolation parameters. Exp Cell Res 2004; 298: 144-154.
11. De Rooij DG, Van Bragt MP. Leydig cells: testicular side population harbors transplantable leydig stem cells. Endocrinology 2004; 145: 4009-4010.
12. Shinohara T, Orwig KE, Avarbock MR, Brinster RL. Spermatogonial stem cell enrichment by multiparameter selection of mouse testis cells. Proc Natl Acad Sci USA 2000; 97: 8346-8351.
13. Giuili G, Tomljenovic A, Labrecque N, Oulad-Abdelghani M, Rassoulzadegan M, Cuzin F. Murine spermatogonial stem cells: targeted transgene expression and purification in an active state. EMBO Rep 2002; 3: 753-759.
14. Durand RE, Olive PL. Cytotoxicity, Mutagenicity and DNA damage by Hoechst 33342. J Histochem Cytochem 1982; 30: 111-116.
15. Okuda A, Fukushima A, Nishimoto M, Orimo A, Yamagishi T, Nabeshima Y, Kuro-o M, Boon K, Keaveney M, Stunnenberg HG, Muramatsu M. UTF1, a novel transcriptional coactivator expressed in pluripotent embryonic stem cells and extra-embryonic cells. EMBO J 1998; 17: 2019-2032.
16. Nishimoto M, Fukushima A, Okuda A, Muramatsu M, Seki N, Hori TA, Orimo A, Yamagishi T, Nabeshima Y, Kuro-o M, Boon K, Keaveney M, Stunnenberg HG. The gene for the embryonic stem cell coactivator UTF1 carries a regulatory element which selectively interacts with a complex composed of Oct-3/4 and Sox-2. Mol Cell Biol 1999; 19: 5453-5465.
17. de Rooij DG, Russell LD. All you wanted to know about spermatogonia but were afraid to ask. J Androl 2000; 21: 776-798.
18. Nishimoto M, Miyagi S, Yamagishi T, Sakaguchi T, Niwa H, Muramatsu M, Okuda A. Oct-3/4 maintains the proliferative embryonic stem cell state via specific binding to a variant octamer sequence in the regulatory region of the UTF1 locus. Mol Cell Biol 2005; 25: 5084-5094.
19. van den Boom V, Kooistra SM, Boesjes M, Geverts B, Houtsmuller AB, Monzen K, Komuro I, Essers J, Drenth-Diephuis LJ, Eggen BJL. UTF1 is a chromatin-associated protein involved in ES cell differentiation. 2007; 178: 913-924.

20. Tan S, Wang ST, Hentze H, Droge P. A UTF1-based selection system for stable homogeneously pluripotent human embryonic stem cell cultures. Nucleic Acids Res 2007; 35: e118.
21. Collas P, Dahl JA. Chop it, ChIP it, check it: the current status of chromatin immunoprecipitation. Front Biosci 2008; 13: 929-943.
22. Kanatsu-Shinohara M, Inoue K, Lee J, Yoshimoto M, Ogonuki N, Miki H, Baba S, Kato T, Kazuki Y, Toyokuni S, Toyoshima M, Niwa O, Oshimura M, Heike T, Nakahata T, Ishino F, Ogura A, Shinohara T. Generation of pluripotent stem cells from neonatal mouse testis. Cell 2004; 119: 1001-1012.
23. Kanatsu-Shinohara M, Miki H, Inoue K, Ogonuki N, Toyokuni S, Ogura A, Shinohara T. Long-term culture of mouse male germline stem cells under serum-or feeder-free conditions. Biol Reprod 2005; 72: 985-991.
24. Park IK, Qian D, Kiel M, Becker MW, Pihalja M, Weissman IL, Morrison SJ, Clarke MF. Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. Nature 2003; 423: 302-305.
25. Molofsky AV, Pardal R, Iwashita T, Park IK, Clarke MF, Morrison SJ. Bmi-1 dependence distinguishes neural stem cell self-renewal from progenitor proliferation. Nature 2003; 425: 962-967.
26. van der Lugt NM, Domen J, Linders K, van Roon M, Robanus-Maandag E, te Riele H, van der Valk M, Deschamps J, Sofroniew M, van Lohuizen M, et al. Posterior transformation, neurological abnormalities, and severe hematopoietic defects in mice with a targeted deletion of the bmi-1 proto-oncogene. Genes Dev 1994; 8: 757-769.
27. Gunster MJ, Raaphorst FM, Hamer KM, den Blaauwen JL, Fieret E, Meijer CJ, Otte AP. Differential expression of human Polycomb group proteins in various tissues and cell types. J Cell Biochem 2001; 81: 129-143.
28. Zhang S, Li D, Li E, Li L, Wang J, Wang C, Lu J, Zhang X. Expression localization of Bmi1 in mice testis. Mol Cell Endocrinol 2008.
29. van Pelt AM, Roepers-Gajadien HL, Gademan IS, Creemers LB, de Rooij DG, van Dissel-Emiliani FM. Establishment of cell lines with rat spermatogonial stem cell characteristics. Endocrinology 2002; 143: 1845-1850.
30. Pellegrini M, Grimaldi P, Rossi P, Geremia R, Dolci S. Developmental expression of BMP4/ALK3/SMAD5 signaling pathway in the mouse testis: a potential role of BMP4 in spermatogonia differentiation. J Cell Sci 2003; 116: 3363-3372.
31. Nagano M, Ryu BY, Brinster CJ, Avarbock MR, Brinster RL. Maintenance of mouse male germ line stem cells in vitro. Biol Reprod 2003; 68: 2207-2214.
32. Meinhardt A, McFarlane JR, Seitz J, de Kretser DM. Activin maintains the condensed type of mitochondria in germ cells. Mol Cell Endocrinol 2000; 168: 111-117.
33. Mather JP, Attie KM, Woodruff TK, Rice GC, Phillips DM. Activin stimulates spermatogonial proliferation in germ-Sertoli cell cocultures from immature rat testis. Endocrinology 1990; 127: 3206-3214.
34. Miyazawa K, Shinozaki M, Hara T, Furuya T, Miyazono K. Two major Smad pathways in TGF-beta superfamily signalling. Genes Cells 2002; 7: 1191-1204.
35. Schrans-Stassen BH, van de Kant HJ, de Rooij DG, van Pelt AM. Differential expression of c-kit in mouse undifferentiated and differentiating type A spermatogonia. Endocrinology 1999; 140: 5894-5900.
36. Okada S, Nakauchi H, Nagayoshi K, Nishikawa S, Miura Y, Suda T. In vivo and in vitro stem cell function of c-kit- and Sca-1-positive murine hematopoietic cells. Blood 1992; 80: 3044-3050.
37. Tsuchida J, Dohmae K, Kitamura Y, Nishimune Y. The role of the c-kit receptor in the regenerative differentiation of rat Leydig cells. Int J Androl 2003; 26: 121-125.
38. Sage J, Martin L, Meuwissen R, Heyting C, Cuzin F, Rassoulzadegan M. Temporal and spatial control of the Sycp1 gene transcription in the mouse meiosis: regulatory elements active in

the male are not sufficient for expression in the female gonad. Mech Dev 1999; 80: 29-39.
39. Parra MT, Viera A, Gomez R, Page J, Benavente R, Santos JL, Rufas JS, Suja JA. Involvement of the cohesin Rad21 and SCP3 in monopolar attachment of sister kinetochores during mouse meiosis I. J Cell Sci 2004; 117: 1221-1234.
40. Ahmed EA, van der Vaart A, Barten A, Kal HB, Chen J, Lou Z, Minter-Dykhouse K, Bartkova J, Bartek J, de Boer P, de Rooij DG. Differences in DNA double strand breaks repair in male germ cell types: lessons learned from a differential expression of Mdc1 and 53BP1. DNA Repair (Amst) 2007; 6: 1243-1254.
41. Smirnova NA, Romanienko PJ, Khil PP, Camerini-Otero RD. Gene expression profiles of Spo11-/- mouse testes with spermatocytes arrested in meiotic prophase I. Reprod 2006; 132: 67-77.
42. Iguchi N, Tanaka H, Yamada S, Nishimura H, Nishimune Y. Control of mouse hils1 gene expression during spermatogenesis: identification of regulatory element by transgenic mouse. Biol Reprod 2004; 70: 1239-1245.
43. Catena R, Ronfani L, Sassone-Corsi P, Davidson I. Changes in intranuclear chromatin architecture induce bipolar nuclear localization of histone variant H1T2 in male haploid spermatids. Dev Biol 2006; 296: 231-238.
44. Jedrzejczak P, Kempisty B, Bryja A, Mostowska M, Depa-Martynow M, Pawelczyk L, Jagodzinski PP. Quantitative assessment of transition proteins 1, 2 spermatid-specific linker histone H1-like protein transcripts in spermatozoa from normozoospermic and asthenozoospermic men. Arch Androl 2007; 53: 199-205.
45. von Schonfeldt V, Wistuba J, Schlatt S. Notch-1, c-kit and GFRalpha-1 are developmentally regulated markers for premeiotic germ cells. 2004: 235-239.
46. Buaas F, Kirsh AL, Sharma M, McLean DJ, Morris JL, Griswold MD, de Rooij DG, Braun RE. Plzf is required in adult male germ cells for stem cell self-renewal. Nat Genet 2004; 36: 647-652
47. Costoya JA, Hobbs RM, Barna M, Cattoretti G, Manova K, Sukhwani M, Orwig KE, Wolgemuth DJ, Pandolfi PP. Essential role of Plzf in maintenance of spermatogonial stem cells. Nat Genet 2004; 36: 653-659.
48. Filipponi D, Hobbs RM, Ottolenghi S, Rossi P, Jannini EA, Pandolfi PP, Dolci S. Repression of kit expression by Plzf in germ cells. Mol Cell Biol 2007.
49. Brinster RL. Germline stem cell transplantation and transgenesis. Science 2002; 296: 2174-76.
50. van Bochove G. Effecten van groei- en differentiatiefactoren op differentiatie, deling en eiwitexpressie in een spermatogoniale stamcellijn. 2002.
51. Bergevoet M. Search for a spermatogonial stem cell marker involved in differentiation or maintenance of spermatogonia using stem cell line, GC-6spg. 2004.
52. Kubota H, Avarbock MR, Brinster RL. Culture conditions and single growth factors affect fate determination of mouse spermatogonial stem cells. Biol Reprod 2004; 71: 722-731.
53. Shi YQ, Wang QZ, Liao SY, Zhang Y, Liu YX, Han CS. In vitro propagation of spermatogonial stem cells from KM mice. Front Biosci 2006; 11: 2614-2622.
54. Kubota H, Avarbock MR, Brinster RL. Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. Proc Natl Acad Sci U S A 2004; 101: 16489-16494.
55. Tsuzuki S, Enver T. Interactions of GATA-2 with the promyelocytic leukemia zinc finger (PLZF) protein, its homologue FAZF, and the t(11;17)-generated PLZF-retinoic acid receptor alpha oncoprotein. Blood 2002; 99: 3404-3410.
56. Tsai FY, Keller G, Kuo FC, Weiss M, Chen J, Rosenblatt M, Orkin SH. An early haematopoietic defect in mice lacking the transcription factor GATA-2. Nature 1994; 371: 221-226.
57. Lecuyer E, Herblot S, Saint-Denis M, Martin R, Begley CG, Porcher C, Orkin SH, Hoang T. The SCL complex regulates c-kit expression in hematopoietic cells through functional interaction with Sp1. Blood 2002; 100: 2430-2440.
58. Pols TW, Bonta PI, de Vries CJ. NR4A nuclear orphan receptors: protective in vascular disease? Curr Opin Lipidol 2007; 18: 515-520.

59. Lapidot T, Kollet O. The essential roles of the chemokine SDF-1 and its receptor CXCR4 in human stem cell homing and repopulation of transplanted immune-deficient NOD/SCID and NOD/SCID/B2m(null) mice. Leukemia 2002; 16: 1992-2003.
60. Boldajipour B, Mahabaleshwar H, Kardash E, Reichman-Fried M, Blaser H, Minina S, Wilson D, Xu Q, Raz E. Control of Chemokine-Guided Cell Migration by Ligand Sequestration. Cell 2008; 132: 463-473.
61. Stebler J, Spieler D, Slanchev K, Molyneaux KA, Richter U, Cojocaru V, Tarabykin V, Wylie C, Kessel M, Raz E. Primordial germ cell migration in the chick and mouse embryo: the role of the chemokine SDF-1/CXCL12. Dev Biol 2004; 272: 351-361.

Summary & Samenvatting



Summary

Within the testis, new spermatozoa are constantly produced by the process of spermatogenesis. At the base of this process is a small pool of spermatogonial stem cells that generate new stem cells as well as committed spermatogonia that will eventually differentiate in spermatozoa. A tight balance between self-renewal and differentiation must be maintained to prevent accumulation of stem cells or depletion of the seminiferous tubules.

The mechanisms by which self-renewal and differentiation are regulated are largely unknown. Therefore we have aimed at identifying genes that are involved in the self-renewal and/or differentiation of spermatogonial stem cells. Genes have been identified using both a candidate and a genomics approach. Candidate genes have been selected for their known function in the regulation of self-renewal and/or differentiation of other stem cells.

The gene *Ly6a/e*, also known as *Sca-1*, is frequently used for the isolation of hematopoietic stem cells and plays a role in the repopulating capacity of hematopoietic stem cells and the development of committed progenitor cells, megakaryocytes, and platelets. Furthermore, LY6a/ae is supposed to be expressed by the testicular side population, although the different reports disagree about the cell types that can be isolated from the testis with the side population technique. In this thesis (chapter 2), expression of SCA-1 has been localized to the mesenchymal and peritubular cells in the interstitial tissue and histological examination of *Ly6a/e* null mice testes has not revealed any abnormal phenotype. Together these observations indicate that LY6A/E is not expressed by spermatogonial stem cells and that LY6A/E does not play a role in the self-renewal or differentiation of the spermatogonial stem cells.

Expression of undifferentiated embryonic cell transcription factor 1 (*Utf1*) has previously been reported to be restricted to embryonic stem and embryonic carcinoma cells. In adult mice expression is restricted to the ovary and the testis. As expression of *Utf1* is rapidly down-regulated upon the induction of differentiation of embryonic stem cells, UTF1 has been referred to as a pluripotency marker. In chapter 3, expression of UTF1 in the rat testis is shown to be restricted to the population of cells that contains the stem cells, i.e. gonocytes in embryonic and neonatal rats and to a subpopulation of early A spermatogonia in adult rat testis. This result together with the recently proposed notion that UTF1 may be involved in the maintenance of a specific epigenetic profile suggests that UTF1 plays a role in maintaining the spermatogonial stem cells in an undifferentiated state.

In order to identify genes using a genomics approach, two protocols of spermatogonial stem cell differentiation have been established. Both, BMP4 and

activin A induce differentiation of the GC-6spg spermatogonial stem cell line (chapter 4 and 5 respectively). Expression of c-KIT, a marker of differentiated spermatogonia, is up-regulated and BMP4 and activin A receptors are expressed by GC-6spg cells. Using BMP4 induced differentiation of the GC-6spg cells as a model in a time course micro-array experiment has revealed numerous transcripts that are differentially regulated during the differentiation process (chapter 4). Among the differentially regulated genes are several TGFβ family members and downstream targets, e.g. *Id2*, *Smad7*, *Bambi* and *Sno*. Furthermore, a number of genes more generally known to play a role in stem cell maintenance or differentiation are identified, e.g. *Emp1*, *Gata2* and *Cxcl12*. Quantitative RT-PCR confirmed the expression pattern of six differentially regulated genes, indicating that the list of genes identified using this genomic approach can be used as candidate genes for further research.

Like BMP4, activin A induces differentiation of GC-6spg cells as demonstrated by the up-regulation of c-KIT. Our experiments indicate that activin A and BMP4 act via both distinct and identical downstream target genes (chapter 5).

Our results form a strong foundation for further research as multiple genes have been identified that likely play a role in spermatogonial stem cell self-renewal and/or differentiation. In addition, two distinct methods for *in vitro* differentiation of a spermatogonial stem cell line have been established, which can now be used as a unique model to study the process of spermatogonial stem cell fate and differentiation.

Samenvatting

Spermacellen worden in de testis gevormd tijdens een proces dat we spermatogenese noemen. Aan de basis van dit proces bevindt zich een kleine populatie spermatogoniale stamcellen die doormiddel van mitotische delingen zowel nieuwe stamcellen (zelfvernieuwing) vormt als ook dochtercellen die uiteindelijk zullen differentiëren in spermacellen. Het is cruciaal dat de verhouding tussen deze zelfvernieuwing en differentiatie in balans is. Verstoring van de balans kan resulteren in een opeenhoping van spermatogoniale stamcellen of in uitputting van de voorraad stamcellen.

Omdat het grotendeels onbekend is hoe zelfvernieuwing en differentiatie worden gereguleerd, hebben we geprobeerd om genen te identificeren die een rol spelen tijdens de zelfvernieuwing en differentiatie van de spermatogoniale stamcellen. Hiervoor is gebruik gemaakt van zowel een "kandidaat" als een "genomics" methode. Kandidaat genen zijn geselecteerd op basis van hun functie in de zelfvernieuwing of differentiatie van andere soorten stamcellen.

Het gen *Ly6a/e*, beter bekend als *Sca-1*, wordt vaak gebruikt voor de isolatie van hematopoietische stamcellen, is belangrijk voor de repopulatie capaciteit van deze stamcellen en speelt een rol in de ontwikkeling van bloedcel-voorlopers, megakaryocyten en bloedplaatjes. Bovendien, komt LY6a/e tot expressie in de zogenaamde 'testiculaire side population'. Er is echter onduidelijkheid over welke celtypen met deze techniek kunnen worden geïsoleerd uit de testis. In hoofdstuk 2, laten we zien dat LY6a/e tot expressie komt in de mesenchymale en peritubulaire cellen in het interstitium. Ook laten we zien dat er geen afwijkingen werden gevonden tijdens de histologische analyse van testes van *Ly6a/e* null-muizen. Deze resultaten tonen dus aan dat LY6a/e niet tot expressie komt in de spermatogoniale stamcellen en dat LY6a/e geen rol speelt in de zelfvernieuwing of differentiatie van de spermatogoniale stamcellen.

Eerder gepubliceerde onderzoeken laten zien dat expressie van *Utf1* is beperkt tot embryonale stamcellen en embryonale carcinoma cellen. In de volwassen muis, komt *Utf1* alleen tot expressie in het ovarium en de testis. Ook is beschreven dat expressie van *Utf1* zeer snel wordt uitgezet wanneer de embryonale stamcel wordt geïnduceerd om te differentiëren. UTF1 wordt daarom ook wel gebruikt als marker voor pluripotency. In hoofdstuk 3, laten we zien dat in de rat testis, UTF1 alleen tot expressie komt in die populatie van cellen die de stamcellen bevat. In het embryo en de pasgeboren rat zijn dat de gonocyten, in de volwassen rat komt UTF1 alleen tot expressie in een subpopulatie van de vroege A spermatogonia. Recentelijk is beschreven dat UTF1 mogelijk een rol speelt in het behouden van het DNA in een specifieke epigenetische staat. Dit samen met het door ons gevonden

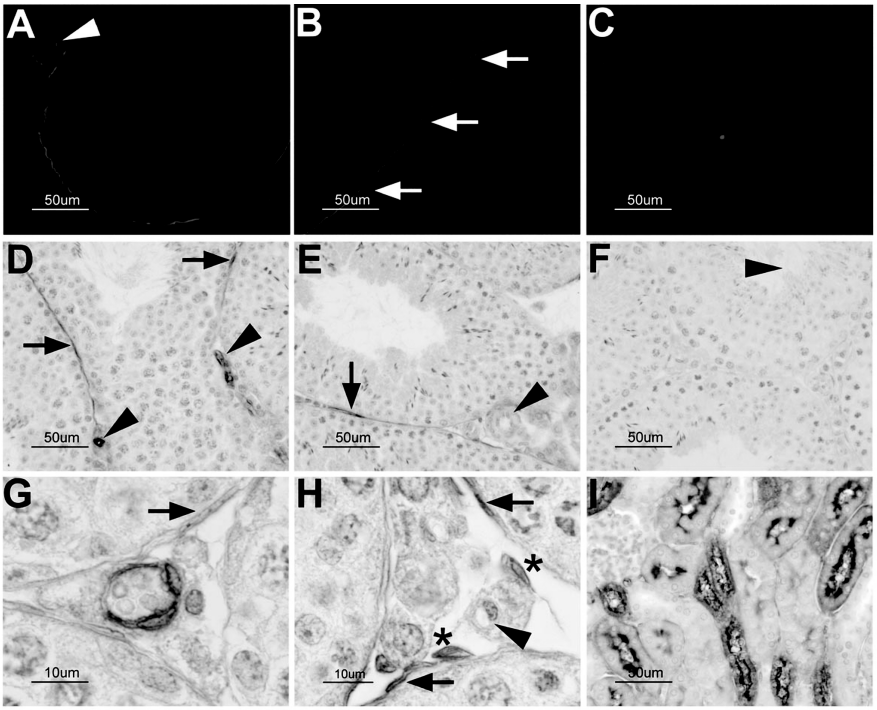
expressiepatroon in de testis suggereert dat UTF1 in de spermatogoniale stamcellen een rol kan spelen bij het behouden van de ongedifferentieerde staat.

Om genen te identificeren met behulp van de 'genomics' methode, zijn er twee modellen voor spermatogoniale stamcel differentiatie gegenereerd. Zowel BMP4 als activin A kan de GC-6spg cellen *in vitro* aanzetten tot differentiatie (hoofdstuk 4 en 5). Expressie van c-KIT, een marker voor gedifferentieerde spermatogonia, werd verhoogd en expressie van de verschillende receptoren voor BMP4 en activin A werd aangetoond in de GC-6spg cellen. Gebruik van het BMP4 geïnduceerde differentiatie model in een micro-array experiment leidde tot de identificatie van verscheidende genen waarvan de expressie veranderd tijdens het differentiatieproces (hoofdstuk 4). Onder de geïdentificeerde genen zijn leden van de TGFβ familie en bekende downstream targets van deze familie, bv. *Id2*, *Smad7*, *Bambi* and *Sno*. Ook genen waarvan bekend is dat ze een rol spelen in de zelfvernieuwing of differentiatie van andere stamcellen, werden geïdentificeerd als genen waarvan de expressie veranderd tijdens de differentiatie van spermatogoniale stamcellen, bv. *Emp1*, *Gata2* and *Cxcl12*. Kwantitatieve RT-PCR bevestigt het expressie patroon van 6 genen met een veranderde expressie. Hieruit kan worden geconcludeerd dat de lijst met genen die we hebben geïdentificeerd met deze 'genomics' methode gebruikt kunnen worden als kandidaat genen voor vervolgonderzoek.

Net als bij BMP4, zien we ook na toevoeging van activin A aan het medium van de GC-6spg dat de expressie van c-KIT wordt verhoogd, wat duidt op de differentiatie van deze cellen. Onze experimenten tonen verder aan dat bij de differentiatie die BMP4 en activin A induceren zowel verschillende als overlappende genen zijn betrokken (hoofdstuk 5).

De resultaten beschreven in dit proefschrift, vormen een stevig fundament voor vervolgonderzoek. Verscheidende genen die waarschijnlijk een rol spelen in spermatogoniale stamcel zelfvernieuwing en/of differentiatie zijn geïdentificeerd. Daarnaast, zijn er twee methodes ontwikkeld waarmee de spermatogoniale stamcel cellijn, GC-6spg, kan worden geïnduceerd om te differentiëren. Deze methoden maken van de cellijn een uniek model dat gebruikt kan worden voor vervolgonderzoek aan spermatogoniale stamcel zelfvernieuwing en differentiatie.

Color figures, acknowldgements, publications & curriculum vitae



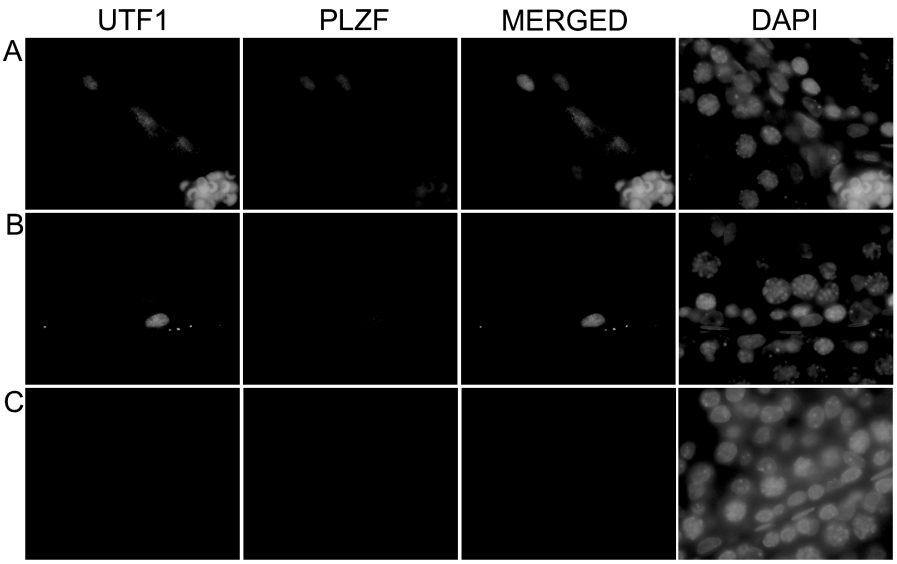


Figure 6, chapter 3. Co-localization of UTF1 and PLZF in 56dpp rat testes. A and B: partial co-localization of UTF1 and PLZF in the testis of two different 56dpp rats. C: negative control.

←--------. Figure1, chapter 2. *Immunolocalization of LY6A in testes of different mice strains. A–C) Immunofluorescence staining of LY6A in FVB (A), BALB/c (B), and Ly6a$^{-/-}$ testis (C). (D–I) Immunohistochemical staining of LY6A in FVB (D), BALB/c (E), and Ly6a$^{-/-}$ testis (F). (G–H) Higher magnification of FVB (G) and BALB/c testis (H). Indicated are endothelial cells (arrowheads), peritubular myoid cells (arrow), and spherical-shaped peritubular mesenchymal cells (asterisk).(I) Kidney; staining is observed in the renal vessels*

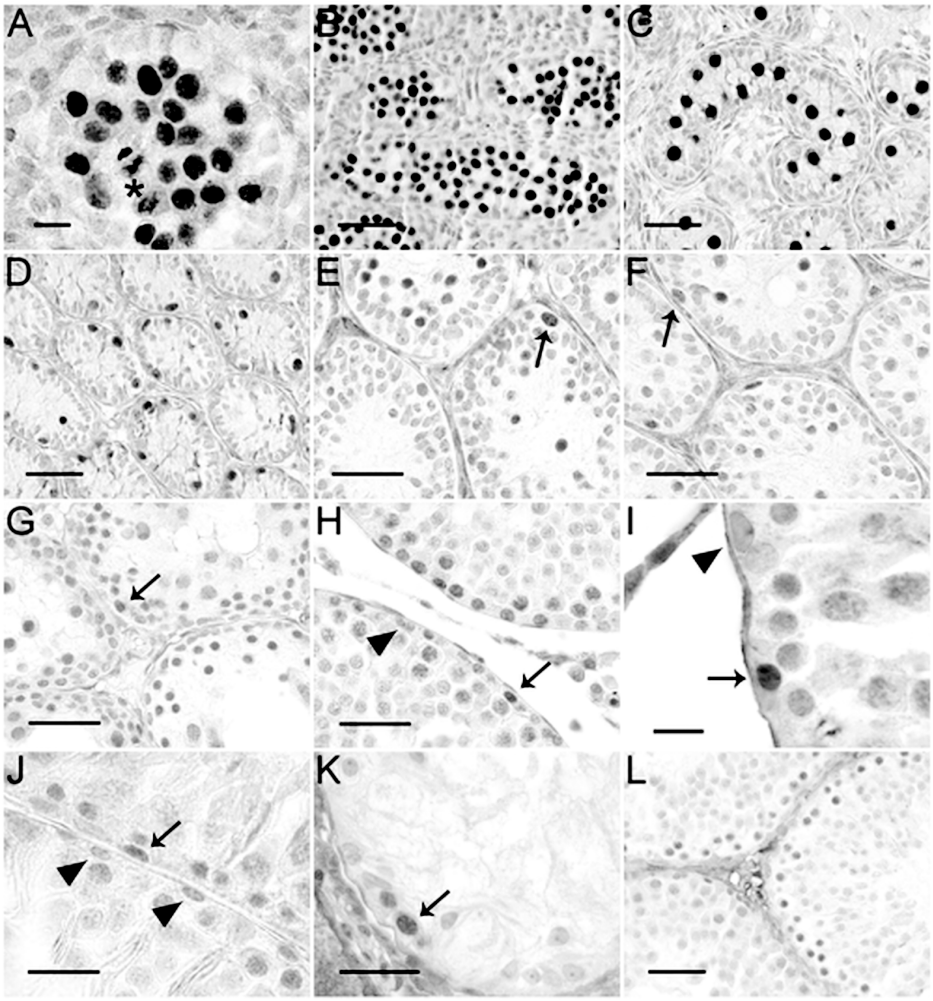


Figure 3, chapter 3. UTF1 expression in rat testis of different ages. A: 16dpc, B: 18dpc, C: 4dpp, D: 9dpp, E: 13dpp, F: 17dpp, G: 24dpp, H: 42dpp, I: 56dpp, J: 70dpp, K: adult VAD rat, L: negative control 70dpp. Asterisk: mitotic gonocytes, arrow: positive spermatogonia, arrowheads: negative spermatogonia. Bars: A, I: 100μm, B, C, D, L: 40μm, E, F, G, H, J, K: 50μm.

Dankwoord

De afgelopen (ruim) vier jaar heb ik veel mogen leren over spermatogenese en stamcellen en heb ik zelfs een beetje kunnen bijdragen aan de kennis over spermatogoniale stamcellen. Dat heb ik natuurlijk niet alleen gedaan en ik ben daarom ook iedereen die mij daarbij heeft geholpen zeer dankbaar.

Een aantal mensen wil ik in het bijzonder noemen en bedanken. Ten eerste natuurlijk mijn professor, Dick de Rooij, die mij een plekje in zijn lab gaf, Dick bedankt voor deze plek en voor het vele nakijkwerk van mijn schrijfsels. Jou talent en ervaring in het schrijven hebben mij zeer geholpen.

Ans, vanaf dag 1 stond je voor me klaar als co-promotor, wat betekende dat ik op ieder moment aan je bureau kon staan met een vraag, waarop jij meteen tijd maakte om die vraag te beantwoorden. Samen zijn we de oceaan overgestoken voor de American Testis Workshop en zijn we op zoek gegaan naar een boterham met kaas. De crackers met cheddar, waarmee we uiteindelijk op een bankje in het park neerstreken, waren een verademing na alle bagels en hamburgers. We vormden een goed team, of dat nou in Utrecht, Amsterdam, Tampa of Barcelona was en ik hoop dan ook dat we, ook op wetenschappelijk gebied, contact blijven houden.

Hermien, voor jou heeft de immuno al lang geen geheimen meer en ik denk dat ik er dankzij jou nu ook wel het een en ander vanaf weet. Dank daarvoor

Acknowledgements

During the last 4 years (and a bit) I learned a lot about spermatogenesis and stem cells and I could even contribute some knowledge about spermatogonial stem cells. Of course I didn't obtain the data described in this thesis alone and therefore I would like to thank everyone who helped me.

I especially would like to mention and thank the following persons. First, of course, my professor, Dirk de Rooij, who gave me a bench to work at. Dirk, thanks for having me in your lab and for the corrections and suggestions of the various drafts of my manuscripts. Your talent and experience in writing papers have been very meaningful for me.

Ans, from day 1 onwards you were there for me as my co-promotor. Whenever I appeared at your desk, you always made time to answer my questions. Together we crossed the ocean to visit the American Testis Workshop were we searched for a sandwich with cheese. Although we had to settle with crackers and cheddar on a bench in the park they were delicious after all the bagels and hamburgers. We were a good team, whether that was in Utrecht, Amsterdam, Tampa or Barcelona and I therefore hope that we will, also for scientific matters, keep in touch.

Hermien, for you immuno-histochemistry has no more secrets and I think that I now also know a few things about it. Thanks for that and for the

maar vooral ook bedankt voor de gezelligheid op het lab. De dagen die jij met Robin doorbracht waren duidelijk merkbaar op het lab.

De verhuizing van de muizengroep naar het AMC kwam in de laatste maanden van mijn aio-bestaan. Een drukke tijd waarin nog snel veel proeven gedaan moesten worden. Saskia, Cindy en Suzanne stonden te trappelen om aan het werk te gaan en ik wil daarom Cindy bedanken voor alle pcr's en kweken die ze heeft gedaan. Cindy, dat jou naam bij drie van mijn hoofdstukken staat bewijst wel dat je veel hebt gedaan. Bovendien was het ook gewoon heel leuk om met je te werken en kletsen.

Wytske, collega, carpoolmaatje, dorpsgenoot en paranimf, we hebben heel wat uurtjes doorgebracht in de Peugeots. Mijn gefrustreerde aio-hart kon bij jou altijd worden gelucht, maar gelukkig praten we meestal over andere dingen. Ik hoop dat we ook contact houden als Yuri en ik niet meer in Mijdrecht wonen. Je bent zeker reislustig genoeg dus bij deze ben je uitgenodigd om te komen logeren. (Ik kan je alleen nog geen adres geven).

Hugo, als ik op 16 juni over mijn schouder kijk zal jij daar naast Wytske staan. Jou komst op het lab heeft veel gezelligheid gebracht op het lab en daarbuiten. Zo was daar bijvoorbeeld het idee om naar Terschelling te gaan. We gingen in een busje, op professionele wijze bestuurd door Angel en we namen mee, Fernanda (i'm in the Netherlands), Sergio, Joana, Paula, Yuri en natuurlijk de "gezelligheid" (untranslatable ≅ good / happy atmosphere) you created in the lab. The days you spend with Robin were empty and quiet in the lab.

During the last months of my PhD project the mouse group moved to the AMC. A busy time in which still many experiments had to been done. Luckily, Saskia, Cindy and Suzanne were eager to get to work. I would therefore like to thank Cindy, for all the cultures and pcr's she performed. Cindy, the fact that your name is above three of my chapters, indicates how much work you have done. In addition, you are also a great person to work and chat with.

Wytske, paranimf, carpool partner, colleague and co-inhabitant of "de Ronde Venen", we spend many hours in the Peugeots. My frustrated phd-heart could always be emptied by you, but luckily we mostly talked about other things. I hope we will also keep in touch when Yuri and I move from "de Ronde Venen". You surely like to travel, so you are very welcome to stay with us for a holiday. (Although we don't have an address yet).

Hugo, when at june 16 I look over my shoulder I will see you standing besides Wytske. Your arrival in the lab has brought "gezelligheid" (untranslatable, ≅ joy and fun) in and outside the lab. For example, it was you who came up with the idea to go to Terschelling. We rented a van, professionally driven by Angel and we took along, Fernanda (I'm in the Netherlands), Sergio, Joana, Paula, Yuri

vliegers. Alhoewel ik denk dat we ons keurig hebben gedragen moest de brandweer er toch aan te pas komen. Het was een super weekend dat we snel over gaan doen. Hugo ik vind het heel fijn dat je mijn paranimf bent, maar nog liever ga ik komende zondag met je vliegeren.

Fernanda, jij bent een bijzonder dametje. Ik ga je missen als je teruggaat naar Fernando en Freud. Gelukkig duurt dat nog even en gaan we voor die tijd nog veel leuke dingen doen (bbq?). Dat doen we dan samen met Angel, king of the in situ, die met zijn enorme bos behoorlijk indruk maakte de eerste dag dat hij in het lab verscheen en later weer toen het eraf was, Paula, nu zelf aio op een gentherapieproject en die wel weet wat je met kip moet doen, bedankt voor het recept, Chen, bedankt voor de zeewier en de paddestoelen ze waren erg lekker en Paul, die mijn kamergenoot was totdat hij me liet zitten voor een werkplek met een eigen computer ☺.

Ook mijn andere kamergenoten, Pedro, Marcelo, Geert, Martijn en Katrien wil ik bedanken voor hun ideeën of gewoon gezellig praatjes.

Canan, met jou heb ik mijn eerste luchtmiles gemaakt. Ik was maar wat blij dat we samen waren toen op de terugreis vanuit Duitsland het vervangende vliegtuig propellers bleek te hebben. Samen hebben we Europa doorkuist: Rostock, Oslo, Milaan en Barcelona. Succes met je zeer spannende onderzoek.

and of course the kites. Although, I think, we behaved very well, the fire brigade of the island "Terschelling" might think different. It was a great weekend which we should redo this summer. Hugo, I think it is great that you want to be my paranimf, but I would like it even more if we went kiting next Sunday.

Fernanda, you are a special "misses" and I will definitely miss you when you go back to Fernando and Freud. Luckily, you are not leaving yet, so we still have time to do all kinds of fun stuff. (bbq?) Of course we will do this together with Angel, king of the in situ, who on his first day in the lab made quite an impression with his hair and again when it was gone). Chen, thanks for the seaweed and mushrooms, they were very tasty. Paula, who started her own PhD project on gene therapy and who knows what to do with chicken, thanks for the recipe and Paul, who was my roommate for a few weeks until he dumped me for a computer ☺.

I would also like to thank my other roommates, Pedro, Marcelo, Geert, Martijn and Katrien for their idea's and non-scientific chatting.

Canan, with you I made my first air miles. I was very happy that we were together when at the flight back from Germany the substitute plane appeared to have propellers. Together we "did" Europe; Rostock, Oslo, Milano and Barcelona. Good luck with your very exciting research.

Ook alle andere endo collega's (rudiger, Joke, Maijken, Sonia, Astrid, Takeshi, Prof. Hong, Wouter, Vincent, Emad, Jan en Henk) en studenten (Marloes, nicolien, Kim, Pedro, Sergio (2x), Hugo, Irene, Celi, Martha, Anniek, Jan-willem, Madhavi, Tresa, Michel (2x), Ana, Sophia, Rodrigo, Joana (3x), André, Danielle,/Daniella, Jao, Tim en Anita) wil ik graag bedanken voor de gezelligheid, inspiratie en practische tips. Ana, "mijn" student. Bedankt, het was leuk om samen aan UTF1 te werken

Naast de Endo's waren er in het Kruijtgebouw nog vele andere die mij hebben geholpen. Ik denk daarbij aan de buren van stofwisseling, de mannen van het aquarium en de fotografie. Mirjam, Maartje, Paulien, Thijs, Ton en Cor. (Allemaal bedankt voor de antilichamen, fedex verzendingen, microscopische plaatjes en taart tijdens de koffiepauzes, enz. Adri bedankt voor de mentor gesprekken.

De collega's uit het AMC (Cindy, Saskia, Suzanne, Sjoerd, Fulco, Hooman, Bita, Michiel, Liesbeth, Romana en Gianfranco) wil ik graag bedanken voor het enthousiasme waarmee we werden ontvangen in het AMC.

Hoofdstuk 2 was er niet gekomen zonder de hulp van Nadia Ciliberti en William Stanford, die testis van de SCA-1 null muizen verstuurde vanuit Canada..

Aki Okuda en Bart Eggen wil ik bedanken voor het versturen van UTF1 antilichamen en constructen en de hulp bij het schrijven van het UTF1 manuscript. Bart, ik vind het erg leuk dat

I also would like to thank all other Endo colleagues (rudiger, Joke, Maijken, Sonia, Astrid, Takeshi, Prof. Hong, Wouter, Vincent, Emad, Jan and Henk) and students (Marloes, Nicolien, Kim, Pedro, Sergio (2x), Hugo, Irene, Celi, Martha, Anniek, Jan-willem, Madhavi, Tresa, Michel (2x), Ana, Sophia, Rodrigo, Joana (3x), André, Danielle, Daniella, Jao, Tim en Anita) for the wonderful time I had in the lab in Utrecht, the inspiration and practical clues. Ana, "my" student thanks, I enjoyed working with you on UTF1.

Besides the Endo's there were many others within the Kruijt building who helped me. The neighbors of metabolic physiology, the aquarium- and photography- man, Maartje, Mirjam, Paulien, Thijs, Ton and Cor. Thanks for the antibodies, fedex shipments, microscopic pictures, scanned images and cake during the coffee breaks, etc.

The colleagues from the AMC (Cindy, Saskia, Suzanne, Sjoerd, Fulco, Hooman, Bita, Michiel, Liesbeth, Romana en Gianfranco), I would like to thank for the enthusiasm with which we were welcomed into the AMC.

I would like to thank Nadia Ciliberti and William Stanford for sending me the SCA-1 null mice from Canada. Chapter 2 would not have existed without your help, thanks.

I would like to thank Aki Okuda and Bart Eggen, for sending me UTF1 antibodies and constructs and for their help in writing the UTF1 manuscript. Bart I'm pleased that you are in my

je in mijn commissie zit en kijk er naar uit om met je te discussiëren over de functie van UTF1 in de testis.

Ik denk dat dit ook een mooie plek is om iedereen te bedanken die mij geleerd heeft hoe ik een pipet moet vasthouden en mij heeft laten zien hoe mooi de biologie is. Bedankt, juffrouw Jaspers en juffrouw Verhappen (Franciscus MAVO), alle leraren van het MLO en HLO, Dirk, Chris (Janssen Gilag), Ron, Harry (GDL), Agnes, Jos, Ed en Philip (LUMC), Carlie (jammer dat het niets is geworden met het uitknocken van TR3 in de spermatogoniale stamcellen), Karin, Viv en Astrid (AMC).

Tenslotte wil ik graag Yuri, mijn ouders, Breg, Joost, Karlijn, Femke, Marjan en Jean, Ome Jos, Tante Ria, Anna, Bart en Willemijn bedanken voor het zijn van mijn familie en thuis.

thesis committee and look forward to discuss the function of UTF1 in the testis with you.

I think this is also a nice place to thank the people who taught me how to hold a pipette and who showed me how beautiful biology is. Thanks to miss Jaspers and miss Verhappen (Franciscus MAVO), all teachers from MLO and HLO, Dirk, Chris (Janssen Gilag), Ron, Harry (GDL), Agnes, Jos, Ed and Philip (LUMC),Carlie (Pity that it didn't work out with knocking down TR3 in the spermatogonial stem cells), Hans Karin, Viv and Astrid (AMC).

Finally, I would like to thank Yuri, my parents, Breg, Joost, Karlijn, Femke, Marjan, Jean, uncle Jos, aunt Ria, Anna, Bart and Willemijn for being my family and home.

List of publications

van Bragt MP, Korver CM, de Rooij DG, van Pelt AM. Activin A induced differentiation of a spermatogonial stem cell line indicates role for *Cxcl12* in regulating spermatogonial stem cell fate. In preparation

van Bragt MP, Korver CM, de Rooij DG, van Pelt AM. Controlled differentiation of spermatogonial stem cell line reveals genes involved in early steps of spermatogonial differentiation. Manuscript in preparation.

van Bragt MP, Roepers-Gajadien HL, Korver CM, Bogerd J, Okuda A, Eggen BJ, de Rooij DG, van Pelt AM. Expression of the pluripotency marker, UTF1, is restricted to a subpopulation of early A spermatogonia in rat testis. Accepted Reprod. 2008.

van Rossum AG, **van Bragt MP**, Schuuring-Scholtes E, van der Ploeg JC, van Krieken JH, Kluin PM, Schuuring E. Transgenic mice with mammary gland targeted expression of human cortactin do not develop (pre-malignant) breast tumors: studies in MMTV-cortactin and MMTV-cortactin/-cyclin D1 bitransgenic mice. BMC Cancer. 2006 Mar; 6:58.

Aponte PM, **van Bragt MP**, de Rooij DG, van Pelt AM. Spermatogonial stem cells: characteristics and experimental possibilities. APMIS. 2005 Nov-Dec; 113(11-12):727-42.

van Bragt MP, Ciliberti N, Stanford WL, de Rooij DG, van Pelt AM. LY6A/E(SCA-1) expression in the mouse testis. Biol Reprod. 2005 Oct; 73(4):634-8.

De Rooij DG, **van Bragt MP**. Leydig cells: testicular side population harbors transplantable leydig stem cells. Endocrinology. 2004 Sep; 145(9):4009-10.

Arkenbout EK, **van Bragt M**, Eldering E, van Bree C, Grimbergen JM, Quax PH, Pannekoek H, de Vries CJ. TR3 orphan receptor is expressed in vascular endothelial cells and mediates cell cycle arrest. Arterioscler Thromb Vasc Biol. 2003 Sep; 23(9):1535-40.

Arkenbout EK, de Waard V, **van Bragt M**, van Achterberg TA, Grimbergen JM, Pichon B, Pannekoek H, de Vries CJ. Protective function of transcription factor TR3 orphan receptor in atherogenesis: decreased lesion formation in carotid artery ligation model in TR3 transgenic mice. Circulation. 2002 Sep; 106(12):1530-5.

Curriculum vitae

Maaike van Bragt werd geboren op 27 maart 1976 te Helmond, waar zij in 1992 haar MAVO diploma behaalde aan de Franciscus MAVO. In dat zelfde jaar startte zij haar MLO opleiding aan het technisch lyceum te Eindhoven. Na een stage onder leiding van Dirk Swerts bij Janssen Pharmaceutica te Beerse (België) en een stage onder leiding van Ron Timmermans en Dr. Harry van Herck in het GDL te Utrecht, behaalde zij haar MLO diploma in 1996. Hierna begon zij in 1996 aan haar HLO opleiding aan de Hogeschool van Utrecht. Deze opleiding ronde zij in 2001 af na een stage in de groep van Prof. Dr. Phillip Kluin en Dr. Ed Schuuring op de afdeling Pathologie van het LUMC te Leiden. Onder leiding van Dr. Agnes van Rossum deed zij hier onderzoek naar de rol van EMS1/cortactine in borstkliertumoren. Van juni 2001 tot juni 2003 was zij werkzaam als research analiste op de afdeling Biochemie van het AMC te Amsterdam. In de groep van Prof. dr. Hans Pannekoek werkte zij hier onder leiding van Dr. Carlie de Vries en Dr. Karin Arkenbout aan de functie van TR3 in artheriosclerosis. In juni 2003 begon zij als assistent in opleiding aan haar promotieonderzoek in de leerstoelgroep Endocrinologie van de Universiteit van Utrecht. Onder leiding van Prof. dr. Dirk de Rooij en Dr. Ans van Pelt verrichte zij het onderzoek waarvan de resultaten in dit proefschrift staan beschreven.