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New insights into male gametogenesis: what about the spermatogonial stem cell niche?

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Abstract: The spermatogonial stem cells (SSCs) are at the foundation of spermatogenesis. They can self-renew and generate a large number of differentiated germ cells. A balance between SSC renewal and differentiation in the adult testis is essential to maintain normal spermatogenesis and fertility. These two processes are tightly regulated by intrinsic gene expression in the stem cells and extrinsic signals, including soluble factors and adhesion molecules from the surrounding microenvironment, known as the SSC niche. Few factors which are only found in germ cells are recognized as indispensable to SSC maintenance or differentiation. *Plzf*, a transcriptional repressor protein, and TAF-4b, a germ cell specific component of the RNA polymerase complex, are considered to be essential for SSC renewal, whereas the transcription factors *Sohlh1* and *2* appear to be crucial for spermatogonial differentiation. By providing glial cell-line-derived neurotrophic factor (GDNF) and stem cell factor (SCF) for SSCs and segregating them, the Sertoli cell is one of the major contributors to stem cell niche regulation Another Sertoli cell product, *ERM*, a transcription factor which is exclusively expressed in mature Sertoli cells, has been shown to be required for SSC self-renewal and maintenance of spermatogenesis in adult mice. It has been hypothesized that SSC niche regulation changes with age. SSC self-renewal could be controlled by GDNF during the perinatal period of development while it is dependent on *ERM*, during the pubertal period.

Key words: Spermatogenesis - Spermatogonial stem cells - Niche - Testis - Developmental biology

Introduction

Spermatogenesis is a cyclic and continuous process which starts at puberty and carries on throughout the majority of a male's life span. It takes place within the seminiferous tubules whose wall is composed of an innermost basal lamina surrounded by a layer of peritubular cells. The tubules contain the seminiferous epithelium populated by a mixture of germ cells and Sertoli cells, the only somatic cells in the seminiferous tubules. The Sertoli cells play a crucial nursing role for germ cells and are believed to coordinate important events of spermatogenesis [1].

The development of the germ cells belonging to the same generation occurs in successive mitotic, meiotic and post-meiotic phases [2]. Spermatogonia proliferate through mitotic amplifying divisions, then some of them undergo meiosis to give rise to haploid spermatids which are subsequently transformed into spermatozoa. The spermatogonial stem cells (SSCs) are at the foundation of spermatogenesis. They can self-renew and generate a large number of differentiated germ cells [3]. A balance between SSC renewal and differentiation in the adult testis is essential to maintain normal spermatogenesis and fertility. These two processes are tightly regulated by intrinsic gene expression in the stem cells and extrinsic signals, including soluble factors and adhesion molecules from the surrounding microenvironment, known as the niche.

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Spermatogonial stem cells

In men, the Adark spermatogonia are recognized as the true spermatogonial stem cells with a low mitotic activity under normal conditions and function as regenerative reserve. The undifferentiated A_{pale} spermatogonia function as progenitor cells producing high numbers of differentiating daughter cells. They are followed by only one generation of B spermatogonia before the derivation of spermatocytes [4]. Much of our understanding of SSC biology comes from the data provided by transplantation techniques and long-term cultures of mouse SSCs [3,5,6] as well as by mutant or knock-out mice. In this respect, it must be underlined

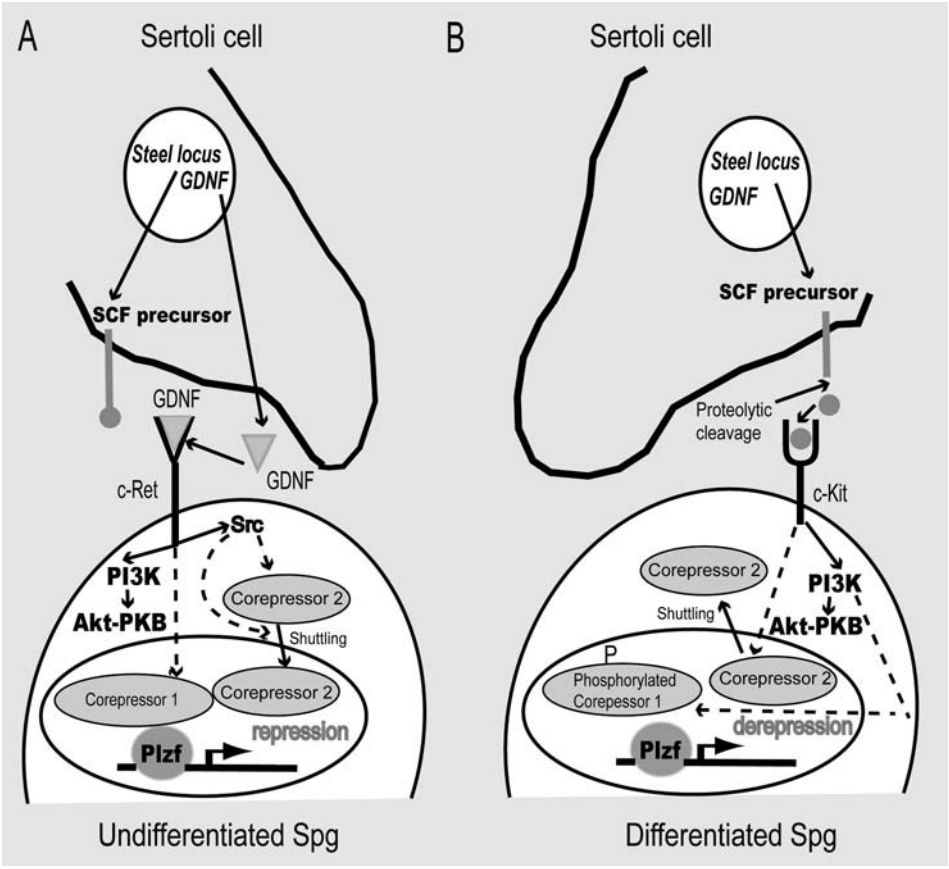

Fig. 1. A model for *Plzf* transcriptional repressor activity regulation in undifferentiated and differentiated spermatogonia. (modified figure of G.Berutti [43])

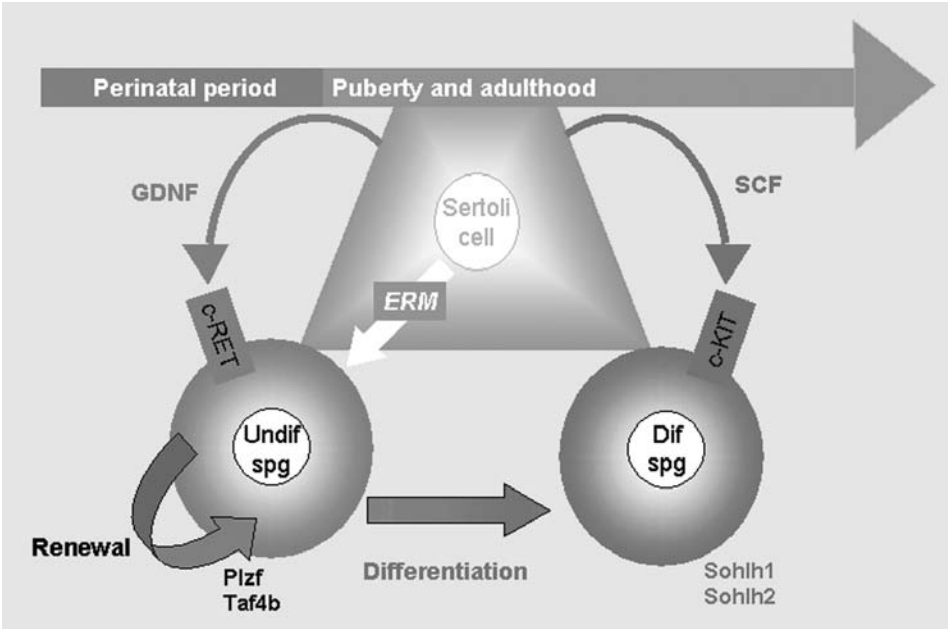

Fig. 2. Key players involved in SSC maintenance and differentiation. *Plzf* and *Taf4b*, which are expressed in undifferentiated spermatogonia (Undif spg), are essential for SSC self-renewal. *Sohlh1* and 2, which are expressed in differentiating spermatogonia (Dif spg) are critical in spermatogonial differentiation. The Sertoli cell is one of the major contributors to stem cell niche regulation by providing GDNF and *ERM*, indispensable for SSC self-renewal and SCF, necessary for SSC differentiation. SSC self-renewal could be controlled by GDNF during the perinatal period and by *ERM* during puberty and adulthood.

that, in mice, the A_{single} spermatogonia are considered to be the spermatogonial stem cells, corresponding to the human A_{dark} spermatogonia. The mouse A_{single} spermatogonia give rise successively to the undifferentiated Apaired and Aaligned spermatogonia, corresponding to the human undifferentiated A_{pale} spermatogonia. $A_{aligned}$ spermatogonia produce differentiating spermatogonia, termed A1 to A4 spermatogonia that are capable of further maturation into intermediate and type B spermatogonia [4]. Undifferentiated spermatogonia, including SSCs, are distinguishable from differentiating spermatogonia. They are defined by the surface phenotype MHC class I (MHC-I)- THY-1^+ c-KIT$^-$. The surface antigen THY-1 (CD90) is a glycosyl

phosphatidylinositol anchored glycoprotein of the Ig superfamily, which is also expressed on hematopoietic stem cells and pluripotent stem cells in culture [7]. As spermatogonia differentiate, the c-KIT tyrosine kinase receptor, which is found specifically on the surface of the differentiating A spermatogonia [8], is acquired concomitantly with a decrease in THY-1 expression [7].To date, few factors which are only found in germ cells are recognized as indispensable to SSC maintenance or differentiation.

Intrinsic proteins required for SSC maintenance

By studying mutant or knock-out mice, two groups have independently established that *Plzf* (Promyelocytic Leukaemia Zinc-Finger), a transcriptional repressor protein originally identified in haematopoietic cells [9], is essential for spermatogonial stem cell renewal [10, 11]. The study of the classic mutant luxoid has shown that young homozygous mutant mice produce limited number of normal spermatozoa and then progressively lose their germ line with age. After transplantation, germ cells of the testes of the luxoid mutants are not able to colonize the testes of recipient W/W^v mice, suggesting that the defect is intrinsic to the stem cells. The luxoid mutant contains a nonsense mutation in the gene *Zfp145* encoding *Plzf*, which regulates the epigenetic state of undifferentiated spermatogonia [10]. As also mentioned in this work, *Plzf* and the transcription factor *Oct4*, which is required to maintain the totipotency of embryonic stem cells, are coexpressed in undifferentiated spermatogonia from mice carrying a *Pou5f1-GFP* transgene. [10]. Likewise, mice with targeted disruption of *Zfp145* undergo a progressive loss of spermatogonia with age, associated with increases in apoptosis and subsequent alteration of tubule structure [11]. Microarray analysis of isolated spermatogonia from *Zfp145*-null mice has revealed modifications in the expression profile of spermatogenesis-associated genes, including the genes for RNA-binding proteins and cyclins and the *testis-specific X-linked* and *microrchidia* genes which are involved in spermatogonia differentiation [11]. Thus, the loss of *Plzf* function shifts the balance between self-renewal and differentiation, toward differentiation at the cost of self-renew. It has been suggested that *Plzf* is a cell-autonomous germ cell factor required for spermatogonial stem cell maintenance. But it might also interact with known signaling pathways, particularly in response to Sertoli-derived signals such as glial cell-line-derived neurotrophic factor (GDNF) and stem cell factor (SCF) [12].

TAF-4b is a germ cell specific component of the RNA polymerase complex (TFIID) and plays an important role in transcriptional regulation [13]. In the mouse, TAF4b is expressed in gonocytes in the post-natal testes and in spermatogonia and spermatids in the adult testes [14]. *TAF-4b*-null mice exhibit a unique testicular phenotype which includes normal fertility at early ages followed by a complete loss of fertility after 12 weeks, related to loss of germ cells and testicular degeneration. At birth, testes of *TAF-4b*-null males appear histologically normal. However, at post-natal day 3, gonocyte proliferation is impaired and expression of spermatogonial stem cell markers c-Ret (GDNF's coreceptor), *Plzf* and Stra8 (stimulated by retinoic acid gene 8) is reduced, suggesting that TAF-4b is essential for spermatogenesis maintenance, most specifically spermatogonial stem cell proliferation [14].

Intrinsic proteins required for SSC differentiation

As opposed to the two preceding germ cell-specific transcription factors, the spermatogenesis- and oogenesis-specific helix loop helix transciption factors *Sohlh1* and *2* appear to be crucial for spermatogonial differentiation. *Sohlh1* is preferentially expressed in mouse A1-A4 intermediate and B spermatogonia [15] and *Sohlh2* is present exclusively in differentiating A spermatogonia [16]. *Sohlh1* deficiency results in infertility by blocking spermatogonial differentiation into spermatocytes. Seven-day-old testes without *Sohlh1* lack LIM homeobox gene Lhx8 which may play an important function in spermatogenesis and show reduced expression of *Kit, Ngn3* and *Crabp1* which are involved in spermatogonia differentiation. Nevertheless, *Sohlh1*-null animals still express the testis-specific transcription factors *Etv5*, also known as *ERM, Taf4b* and *Plzf* , which are critical in self-renewal and proliferation of spermatogonia. The transcription factor *Zfp148*, critical in the formation of prespermatogonia from primordial germ cells is not affected either by the lack of *Sohlh1*. Conversely, *SOHLH2* protein is overexpressed in spermatogonia of $Sohlh1^{-/-}$ testes, suggesting that the leaky phenotype in $Sohlh1^{-/-}$ mice during gonadal development is partly due to transient compensatory effects of SOHLH2 [15].

The SSC niche

A niche is considered to be a subset of tissue cells and extracellular substrates which can indefinitely house one or more stem cells and control their self-renewal and progeny production. Stem cell activity in several organs, including gonads, skin and gut is regulated by niches [17]. Our understanding of germline niches essentially comes from the study of both male and female *Drosophila* gonads where the maintenance of the germ line stem cells depends on signals from a somatic cell which ensures the asymmetric division of stem cells, producing one cell for self-renewal and one

for differentiation [18]. The existence of niches in the mammalian testis has been demonstrated directly when early germ cells have been successfully transplanted into the seminiferous tubules of germ cell-depleted or -deficient recipient males [19].

The basal compartment of seminiferous tubule as stem cell niche

The Sertoli cell divides the seminiferous tubule into a basal compartment comprising mainly spermatogonia and an adluminal compartment where more advanced germ cells are sequestered behind a blood-testis barrier formed by tight junctions between adjacent Sertoli cells. Transplanted cells colonize sites on the wall of the tubule, each of them corresponding morphologically to the basal compartment. The latter is the only place in which SSCs can reside to maintain themselves and must therefore be regarded as the niche for SSCs. [20]

The successful restoration of spermatogenesis in infertile *Steel/Steel^{dickie}* mice by wild-type Sertoli cell transplantation [21] has confirmed that the Sertoli cell is one of the major contributors to stem cell niche regulation. However, other possible players may be involved in SSC niche formation. These players include the basement membrane of the seminiferous tubule, which is shared with the surrounding peritubular cells, and, possibly, some signals external to the seminiferous tubules.

The location of SSCs along the wall of the tubule suggests that they bind laminin, a major component of the basement membrane. In agreement with this assumption, spermatogonial transplantation assays have shown that the selection of mouse testis isolated cells with antibodies against beta 1- or alpha 6- integrin, known to bind to laminin, produces cell populations with a significantly enhanced ability to colonize recipient testes [22].

On the other hand, the non-random distribution of murine undifferentiated spermatogonia in niches located in those areas of the seminiferous tubule which border the interstitial tissue [23], suggests that specific factors produced by the vascular endothelial cells attract SSCs into these niches [5].

In mice, the beginning of SSC niche formation occurs during early postnatal development, while Sertoli cells are maturing. The number of available SSC niches dramatically increases from birth to sexual maturity [24] and then the population becomes stable. A normal functioning SSC-niche unit in the adult is required to ensure fertility. Thus, infertility in old males may result from the deterioration of the SSC niche [25] which may be secondary to the impaired endocrine support of Sertoli cell function. Nevertheless, it cannot be excluded that both SSCs and somatic environment in the testis are involved in the aging process [26].

Sertoli cell growth factors

A key contribution to the SSC niche is GDNF, a protein member of the TGF-β superfamily, produced and secreted by Sertoli cells from birth through adulthood [27,28]. SSCs express GDNF receptors, i.e., GFRα1 and c-RET tyrosine kinase receptor ([27,29] which acts downstream by activating phosphatidyl-inositol 3' (PI-3')- kinase and/or the Src family of tyrosine kinases [30]. The GDNF signalling pathway, which is regulated by FSH, has been shown to be critical for the decision between SSC renewal and spermatogonial differentiation. Mice heterozygous for the GDNF knockout are viable, however they show a progressive depletion of testicular stem cells [27]. Conversely, mice which overexpress GDNF exhibit an increased number of undifferentiated spermatogonia, resulting in germ cell seminoma [31]. Other research has shown that forced expression of human GDNF by mouse Sertoli cells induces the expansion of the undifferentiated spermatogonial population in the seminiferous tubules of transplanted testes, with a detrimental effect on SCC differentiation [29]. A recent study, also using testis transplantation technology, has indicated that any disruption of the GDNF-mediated c-RET signaling results in a failure of spermatogenesis, due to deficits in SSC renewal [32]. Numerous additional *in vitro* data have also established that GDNF stimulates SSC proliferation without causing differentiation [33-37]. Six GDNF-regulated genes involved in SSC self-renewal and survival have been recently identified. The most responsive of the six, *Bcl6b*, a transcriptional repressor, belonging to the same POK (POZ and Kruppel) family as *Plzf*, might play an important role in SSC renewal [38].

SCF is another Sertoli cell-product, encoded by the Steel (Sl) locus, which binds to the c-KIT tyrosine kinase receptor [8]. Wild-type germ cells transplanted into the testes of *Steel/Steel^{dickie}* mice can still form colonies, but they fail to differentiate [39]. The SCF/c-KIT signal transduction system is required for germ cells to develop beyond type A spermatogonial stages but not for SSC maintenance and proliferation [40]. Homozygous mutant mice carrying a selective point mutation of the *c-KIT* gene which impairs SCF/c-KIT-induced activation of PI-3' kinase are sterile, due to a block in the initial steps of spermatogenesis. The mutation reduces by 90% SCF-induced PI3'-kinase-dependant activation of PKB/Akt, a serine/threonine kinase which plays a crucial role in the control of cell survival [41]. *Akt1*-null mice display a reduced spermatogenesis and spontaneous male germ cell apoptosis, thus confirming that the SCF/c-KIT PI3'-kinase pathway can be extended downstream to PKB/Akt [42].

The question arises as to how GDNF/c-RET and SCF/c-KIT signalling may influence the transcriptional repressor activity of *Plzf* in SSCs maintained in a

undifferentiated state and in differentiating spermatogonia (Fig.1). It has been speculated that the GDNF/c-RET signalling triggers two signalling cascades in undifferentiated spermatogonia. The first, through PI-3'/ PKB/Akt, regulates the survival-apoptosis decision whereas the second, through still unknown mediators, could result in the enhancement of the binding of *Plzf* associated corepressors so as to repress transcription. In differentiating/differentiated spermatogonia, the SCF/c-KIT signalling could also trigger two signalling cascades. The first, through PI-3'/ PKB/Akt, is involved in the control of cell survival. The second, through so far still unidentified mediators, could mediate the phosphorylated state of a *Plzf* associated corepressor, thus resulting in its dissociation from the transcriptional repressor complex and therefore in derepression [43].

Sertoli cell ERM

ERM (*Ets* related molecule) belongs to a subfamily of *Ets* transcription factors which also includes *PEA3* and *ER81*, important for normal neuronal development [44,45]. *ERM* is expressed in several tissues including testis, brain, colon and lung [46,47]. A recent investigation has demonstrated that *ERM* is required for SSC self-renewal and maintenance of spermatogenesis in the adult mouse [48]. As reported in this study, testes from *ERM*-null mice at 4 weeks of age exhibit normal spermatogenic differentiation, indicating that a normal wave of spermatogenesis has occurred. However, after six weeks, although more advanced germ cells look normal, the underlying new generation of spermatogonia, spermatocytes and eventually spermatids fail to appear, which leads to a Sertoli-cell-only phenotype. Microarray and RT-PCR analyses of *ERM*-null testes at 4 weeks of age has revealed a great reduction in expression of spermatogonia-specific genes, including *Stra8, Dazl* (deleted azoospermia homolog), *Crabp* (cellular retinoic-acid-binding protein), *Lsh* (lymphoid-specific helicase), *Rbm* (RNA-binding-motif) and *Plzf* [48]. The results have also demonstrated that ERM expression is found exclusively in Sertoli cells, within the testis. Furthermore it is first detectable between 3 and 4 weeks of age, approximately during the period when Sertoli cells cease to divide, and last it persists throughout adulthood. In addition, microarray analysis of purified ERM-/- primary Sertoli cells has shown alterations in secreted factors known to regulate the hematopoietic stem cell niche, such as chemokines including SDF-1 (stroma cell-derived factor) and MMP-12 (matrix metalloproteinase 12) [49-51], whereas GDNF expression remained unchanged throughout [48].

By comparing data from *Gdnf*$^{-/-}$ [32] and *ERM*$^{-/-}$ mice [48], Hess and his collaborators [52] have hypothesized that SSC regulation changes with age:

- SSC self-renewal is regulated by GDNF during the perinatal period of development, for the reason that, in transplanted testes from Gdnf-/- newborn mice under the back skin of nude mouse hosts, stem cell self-renewal is inhibited and differentiation is enhanced, without spermatogenesis becoming established [32].
- Targeted disruption of ^{ERM} in mice provides data supporting the assumption that SSC regulation is dependent on *ERM*, during the pubertal period [48]. In *ERM*$^{-/-}$ testes, during the perinatal period when GDNF expression is normal and *ERM* is not yet produced, spermatogenesis takes place, indicating that GDNF is sufficient for maintaining SSC self-renewal and permitting the first wave of spermatogenesis. But, during the pubertal period when GDNF continues to be expressed after *ERM* is no longer functional, SSC self renewal is inhibited, resulting in the depletion of successive layers of new germ cells [48].

Concluding remarks

To date, some germ cell-specific transcription factors are considered to be essential for SSC self-renewal and spermatogonial differentiation. However, interactions with the Sertoli cell, which is one of the major components of the SSC niche, are crucial for maintaining stem cell character. Several molecules produced by Sertoli cells can regulate the capacity of the niche to support SSC maintenance and differentiation (Fig. 2).

Further delineation of the signalling transduction pathways which control SSC function may provide new insights into the pathogenesis of male germ cell diseases, such as infertility and cancer.

Furthermore, experimental modulation of niche-derived factor expression may lead to discover new methods for the treatment of male infertility and possibly to develop a novel male contraceptive.

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