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The transcription factor Ets-Related Molecule (ERM) is essential for spermatogonial stem cell maintenance and self-renewal[1]

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Abstract

This review discusses how recent methodological developments that have facilitated study of spermatogonial stem cells (SSCs) in vitro and in vivo have been used in conjunction with knockout mice to gain important insights into the factors that regulate SSC maintenance, self-renewal and differentiation. We first consider the critical role of the GDNF/RET/GFRα1 signaling pathway in regulating SSCs. We then subsequently focus on what has been learned about SSC maintenance from a newly developed knockout mouse lacking the transcription factor ets-related molecule (ERM). ERM is necessary for maintenance of spermatogonial stem cells (SSC) in the testis; in the absence of ERM, the first wave of spermatogenesis occurs normally, but all SSCs differentiate and are lost during this time. This is the first time a transcription factor has been shown to be essential for stem cell self-renewal. Understanding how ERM, the GDNF/RET/GFRα1 signaling pathway and other factors act to maintain SSCs will advance our understanding of how these cells, and stem cells in general, are regulated. This work has potential clinical implications for human and animal infertility, and may also provide novel contraceptive targets and strategies.

Keywords: Sertoli cell, germ cell, testis, spermatogenesis.

Introduction

Stem cells have become a major focus of both the research community and the general public due to their great medical potential, as well as the rapid scientific progress in stem cell biology. In the seminiferous tubules of the testis, spermatogonial stem cells (SSCs) are responsible for the generation of the cells that subsequently undergo elaborate modifications during the spermatogenic process to give rise to functional male gametes. In this review, we initially discuss landmark methodological developments in SSC biology over approximately the past decade that have provided critical tools for progress in this area. These techniques have opened up unprecedented opportunities to address some of the most basic problems in this field. The remainder of the review will be devoted to a discussion of the rapid and exciting progress in our understanding of one of these areas, how SSC maintenance, self-renewal and differentiation are regulated.

The recent development of a variety of knockout mouse model systems (Meng *et al*., 2000; 2001; Buaas *et al*., 2004; Costoya *et al.,* 2004; Chen *et al.,* 2005; Falender *et al*., 2005; Naughton *et al*., 2006; Oatley *et al*., 2006) where SSC maintenance has been perturbed offers unique experimental systems for understanding SSC regulation. These models have great promise for providing mechanistic insights into how key processes in SSCs are controlled, an area where almost no information was available only a few years ago. This review will focus on what has been learned from these knockout systems, as well as crucial questions knockouts will help to answer in the future. These knockout systems in which the SSCs are perturbed will expand our understanding of testicular stem cells, but since there appears to be substantial conservation in terms of how various stem cells are regulated, the basic principles governing SSC development revealed from these model systems will have broader implications in all facets of stem cell biology.

Recent developments in SSC biology offer unprecedented opportunities

Stem cells have been a focus of testicular biologists well before the current wave of interest in stem cells and their medical applications. Elegant work by Clermont, Huckins and others almost a half-century ago (Clermont, 1962; Huckins and Clermont, 1968) documented the biology of what would eventually be called the spermatogonial stem cells in the seminiferous epithelium of the testis. These studies were mostly descriptive, as only a few tools such as tritiated thymidine autoradiography to assess cell proliferation were available to supplement their meticulous histological observations. However, these classical studies clearly indicate that the basic biology of stem cells was appreciated in the testis long before stem cells were widely studied in many other organs.

In the intervening years, increasing attention has been focused on stem cells in hematopoietic,

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gastrointestinal, hair follicle, skin and other organs (Fuchs *et al*., 2004), as well as embryonic stem (ES) cells. The development of mouse ES cells provided an early experimental system in which stem cells could be studied both in vitro and in vivo (Martin and Evans, 1974), and these cells have been used extensively over the years to understand the factors governing both decisions to remain a stem cell and to differentiate into various lineages. Landmark discoveries, such as the definitive identification of hematopoietic and other stem cells and development of methodologies to purify and study the differentiation of these cells in vitro, catapulted certain areas of stem cell biology forward (Fuchs *et al*., 2004). The recent development and culture of human ES cells (Thomson *et al*., 1998) opened possibilities for using these cells therapeutically to treat human disease. The immense medical potential of human ES cells has led to broad interest in these cells and rapid progress in almost all aspects of their biology, despite the extensive moral, ethical and legal complications that come with potential medical uses of these cells.

Lack of methodologies for definitive identification and culture of SSCs initially caused progress to lag in this area relative to other stem cell lineages such as the hematopoietic system. However, the past few years have seen a series of seminal developments in the biology of testicular stem cells that have increased our abilities to identify and work with these cells, as well as our understanding of their biology. One of the earliest advances, and perhaps the most critical, was an in vivo method for unequivocally determining whether or not a certain cell population contains SSCs. Brinster and colleagues (Brinster and Zimmermann, 1994) developed a transplantation system for assessing "stemness" in which a cell population obtained from the testis in vivo or cultured in vitro was injected into a testis where endogenous spermatogenesis was not present due to a genetic deficiency or chemical treatment. The ability of the injected cells to re-establish spermatogenesis in mice lacking germ cells (Brinster, 2002) provided a direct measure of whether or not a population of cells contains SSCs, and this technique has become the standard methodology for addressing this question. This technique can even be used to give estimates of the concentration of SSCs in the injected cells based on the number of germ cell colonies that result in the injected hosts. Fundamental progress also has been made in development of molecular markers for SSCs that can be used to identify and purify these cells in vivo and in vitro (Shinohara *et al*., 1999; Kubota *et al*., 2003; Ryu *et al.,* 2004; Hamra *et al*., 2004; 2005; Hofmann *et al*., 2005). In addition, culture systems that allow maintenance and even expansion of SSCs have been reported (Kubota *et al*., 2004; Hamra *et al*., 2005; Hofmann and Dym, 2005; Hofmann *et al*., 2005; Kanatsu-Shinohara *et al*., 2005; Aponte *et al*., 2006).

These advances have opened new avenues of investigation in this area that could not have been foreseen only a few years previously. In addition, other recent work has indicated that understanding SSCs might have extensive implications that go far beyond testicular biology. For example, the recent demonstrations that ES-like cells can be obtained from SSCs and can give rise to derivatives of all germ cell layers suggest that SSCs (Kanatsu-Shinohara *et al*., 2004; Guan *et al*., 2006) might be used to generate ES-like cells that could be used therapeutically without the legal and ethical complications that hamper use of ES cells derived from blastocysts. Similarly, Shinohara and co-workers (Kanatsu-Shinohara *et al*., 2006) recently demonstrated that knockout mice can be made using SSCs, rather than the ES cells typically used to generate knockout mice. This provocative finding suggests that this methodology could potentially be used to make knockouts in species where ES cells are unavailable, such as rats, sheep and cattle. Both the ability of SSCs to give rise to ES-like cells and the potential use of SSCs to generate knockouts in a wide variety of animals have significant medical and research implications far beyond testicular biology and emphasize the necessity and potential benefits of understanding how SSCs are regulated.

The significant methodological advances in identifying, purifying and culturing SSCs described above have facilitated similarly rapid progress in discovering and understanding the role of individual proteins that are important regulators of SSCs. These findings allow us to begin to elucidate the key players in SSC biology, and develop a mechanistic understanding of how stem cell decisions regarding maintenance, differentiation and self-renewal are made. Some proteins that appear to be essential for stem cell maintenance are SSC proteins, while others are Sertoli proteins that appear to be involved in the stem cell niche.

The GDNF signaling pathway regulates SSCs

Stem cells exist in specialized microenvironments called niches that are conducive to supporting these cells in their undifferentiated state. These niches have been shown to be essential for the maintenance of viable stem cell populations in the gut, skin, bone marrow and other organs (Fuchs *et al*., 2004; Li and Xie, 2005; Yamashita *et al*., 2005). The supporting cells not only physically establish the niche but also communicate with the stem cells to regulate maintenance, differentiation and proliferation. The spermatogonial stem cell niche lies along the basement membrane of the seminiferous epithelium and the Sertoli cell physically establishes and maintains the stem cell niche, although Sertoli, Leydig and myoid cells may all influence the niche through soluble factors.

Although the critical role of the niche in stem cell development in many organs has been appreciated for years, relatively little mechanistic information has been available describing the presumably reciprocal

communication between the stem cells and the cells providing the niche. This was especially true in the seminiferous epithelium, where despite awareness for many years that Sertoli cells controlled development of SSCs, no molecules known to be involved in this process had been identified.

A critical advance in our mechanistic understanding of the stem cell niche in the seminiferous epithelium came in 2000 with the demonstration that the TGFβ family member glial cell-derived neurotrophic factor (GDNF) was involved in SSC maintenance (Meng *et al*., 2000]. These authors reported that mice containing one null allele for GDNF gradually lost their SSCs during development. GNDF is produced by Sertoli cells, which establish the niche in which the SSCs reside. Loss of Sertoli cell GDNF interferes with self-renewal and maintenance of SSCs, and eventually the seminiferous epithelium in many testicular tubules consists of only Sertoli cells. Confirming the critical role of GDNF in SSCs, mice overexpressing GDNF had increased numbers of undifferentiated spermatogonia (Meng *et al*., 2001). These results clearly established GDNF as one Sertoli cell factor that was necessary for SSC maintenance and provided the first mechanistic insight into the precise nature of the communication from Sertoli cells that maintains SSCs in the stem cell niche.

Recent in vitro work has further emphasized the importance of GDNF for normal SSC function. GDNF stimulated proliferation of cultured SSCs (Kanatsu-Shinohara *et al*., 2004; Guan *et al*., 2006) and these cells reestablished spermatogenesis when grafted in vivo, confirming that they were still SSCs (Kubota *et al*., 2004). Microarray analysis of SSCs grown in vitro with and without GDNF has been used to identify genes downstream of GDNF (Hofmann *et al*., 2005; Oatley *et al*., 2006). Numb, a regulator of the Notch pathway, was identified as a GDNF target (Braydich-Stolle *et al*., 2006), but this was not confirmed in a subsequent microarray study that also attempted to identify GDNF targets in SSCs (Oatley *et al*., 2006), though the reason for this discrepancy is not clear. Another gene identified as a GDNF target in SSCs by microarray was B cell CLL/lymphoma 6, member B (Bcl6b) (Oatley *et al*., 2006). Bcl6b is a member of the POZ (poxvirus and zinc finger) family of transcriptional repressors that also includes another protein discussed subsequently in this review, Plzf, which also plays a critical role in SSC maintenance. Inhibition of Bcl6b in SSCs in vitro by siRNA indicated that this protein had a critical role in SSC maintenance. Furthermore, knockout mice lacking Bcl6b had an increased incidence of Sertoli cell-only tubules (Oatley *et al*., 2006), which confirmed the in vitro results and was consistent with a role for this protein as a downstream target of GDNF important for SSC maintenance.

Recent in vivo work has also revealed critical roles for GDNF receptors in SSC maintenance. As discussed above, work with mice containing one null allele for GDNF (Meng *et al*., 2000) revealed that GDNF was critical for SSC maintenance. However, mice homozygous for the *GDNF* knockout died neonatally, so the effects of loss of both alleles initially could not be easily determined. GDNF produced by Sertoli cells acts through the Ret tyrosine kinase and the GDNF family receptor α1 (GFRα1) receptor complex, which are located at the surface of SSCs. The critical role of GDNF clearly suggests that RET and/or GFRα1 might also be important or even essential in promoting self-renewal and maintenance of SSCs. However, like the *GDNF* knockout, knockout of either *RET* or *GFRα1* was also neonatally lethal, initially precluding analysis of the effects of these two molecules on SSC maintenance in the testis.

This obstacle has recently been elegantly overcome by Naughton *et al.* (2006) and the obligatory roles of each of these molecules in SSC maintenance has been demonstrated. To circumvent the neonatal lethality of the *GDNF*, *RET* and *GFRα1* knockouts, testes from these knockout animals were transplanted at birth to a subcutaneous site in a host animal, and development of the grafted testis was subsequently monitored. Total SSC loss occurred in testis lacking GDNF, RET or GFRα1, although normal spermatogenesis and maintenance of SSC populations was observed in WT testes that had been grafted neonatally. Thus, GDNF, RET and GFRα1 are all obligatory for SSC maintenance, emphasizing the essential role of the GDNF/RET/GFRα1 signaling pathway in SSCs.

The transcription factor ets-related molecule (ERM) is essential for maintenance of SSCs

The recent unexpected discovery that mice in which ets-related molecule (ERM; also know as ets variant 5) has been knocked out by gene targeting lose their SSCs (Chen *et al.,* 2005) has provided another powerful tool for understanding the factors controlling SSC maintenance. Following a brief introduction to ERM, we will provide a description of the newly developed ERM knockout (*ERM*$^{-/-}$) mice and the experiments that led to the conclusion that ERM is essential for maintenance of SSCs. Finally, we discuss some recent unpublished experiments and their contribution to our understanding of the mechanistic basis by which ERM promotes SSC maintenance.

ERM was discovered in 1994 by Monte *et al.* (1994), based on its similarities to another molecule in the Ets protein family, PEA3. ERM, PEA3 and ER81 comprise the PEA3 group of Ets transcription factors. The defining feature of this protein family is the ETS domain, a region of about 85 amino acids that comprises a sequence-specific DNA binding domain. The ETS domain is 95% identical in ERM compared to other Pea3 family members (PEA3, ER81) (Monte *et al*., 1994), although overall sequence identity is lower

(approximately 50%) for the entire molecule.

Mouse testis expresses high levels of ERM mRNA (Monte *et al.*, 1994; Chotteau-Lelievre *et al.*, 1997), and this organ, along with brain, colon and lung, has the greatest level of ERM mRNA. Although initial reports suggested a significant role for ERM in the testis, where ERM was produced and its function were unknown. Expression of ERM mRNA is also widespread in developing and/or adult lung, thymus, kidney, large intestine, mammary gland, lymphocytes, heart, salivary gland, skeletal muscle and other tissues (Chotteau-Lelievre *et al.*, 1997; 2001; Liu *et al.*, 2003; T'Sas *et al.*, 2005). Based on its pattern of expression in organs such as developing lung, ERM may play a role in epithelial-mesenchymal interactions (Liu *et al.*, 2003), but its function has not been established.

The recent fortuitous discovery that *ERM*$^{-/-}$ mice totally lose their SSCs after the first wave of spermatogenesis (Chen *et al.,* 2005) provided a new and unexpected tool for understanding factors controlling SSCs. The *ERM*$^{-/-}$ mice clearly indicated that this transcription factor, whose function was unknown in testis and other organs, is essential for SSC maintenance.

Testis development in ERM$^{-/-}$ mice

ERM$^{-/-}$ mice undergo a first wave of spermatogenesis (Chen *et al.,* 2005), but by 6 weeks of age there is widespread loss of germ cell layers and tubules show large variation in spermatogenesis (Fig. 1); some have only Sertoli cells, while others show maturation depletion or sequential loss of germ layers with aging. Recent work from our laboratories indicates that despite the persistence of spermatogenesis into early adulthood, SSC loss actually begins by the end of the first postnatal week and becomes complete by 5 weeks of age (Schlesser *et al.*, unpublished). Thus, while the first wave of spermatogenesis is ongoing there is a concomitant total loss of SSCs, which eventually leads to the Sertoli cell-only phenotype reported in adult *ERM*$^{-/-}$ mice.

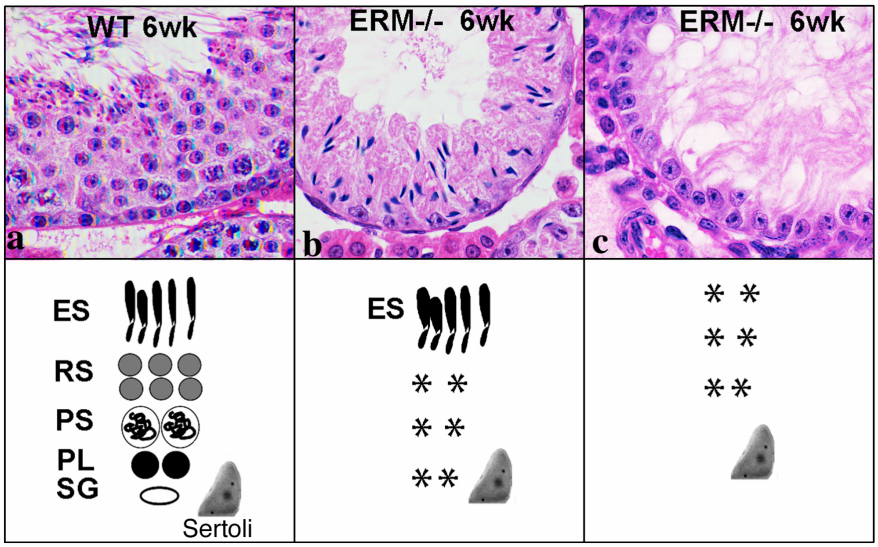

Figure 1. Testicular atrophy and Sertoli cell-only syndrome initiated by spermatogonial depletion in *ERM-/-* mice. Histology of wild-type (a) or *ERM-/-* (b, c) testes at 6 weeks of age. Loss of spermatogonia occurred during the first wave of spermatogenesis in *ERM-/-* testes. A schematic of seminiferous epithelium is shown below; elongated spermatid (ES); round spermatid (RS); pachytene spermatocyte (PS); preleptotene spermatocytes (PL); spermatogonium (Sg); Sertoli cell (Sertoli); and missing cell populations (*). From Chen et al, Nature 2005; 436: 1030-1034.

ERM is expressed in juvenile Sertoli cells

A variety of lines of evidence indicated that ERM was expressed in juvenile and adult Sertoli cells (Chen *et al.,* 2005), Immunohistochemical localization of ERM using a monoclonal antibody developed against an ERM-specific region, without sequence homology to Pea3 or ER81, showed specific nuclear staining only in Sertoli cells in the juvenile and adult animal (Fig. 2). This immunostaining was neutralized by pre-incubating ERM antibody with purified ERM protein. ERM mRNA was expressed in the *W/W*^{v} (*c-kit*$^{W/W\text{-}v}$) mouse testis, which lacks spermatogenesis and contains Sertoli cells and a few spermatogonia (Chen *et al.,* 2005). Consistent with these other findings, ERM expression using an IRES-LacZ reporter cassette targeted to the ERM locus showed staining exclusively in Sertoli cells. ERM expression was not seen at 2 weeks of age postnatally and weak staining was seen at 3 weeks. By 4 weeks of age, strong LacZ expression, indicative of ERM production, was apparent in the Sertoli cells, while all germ cells were negative.

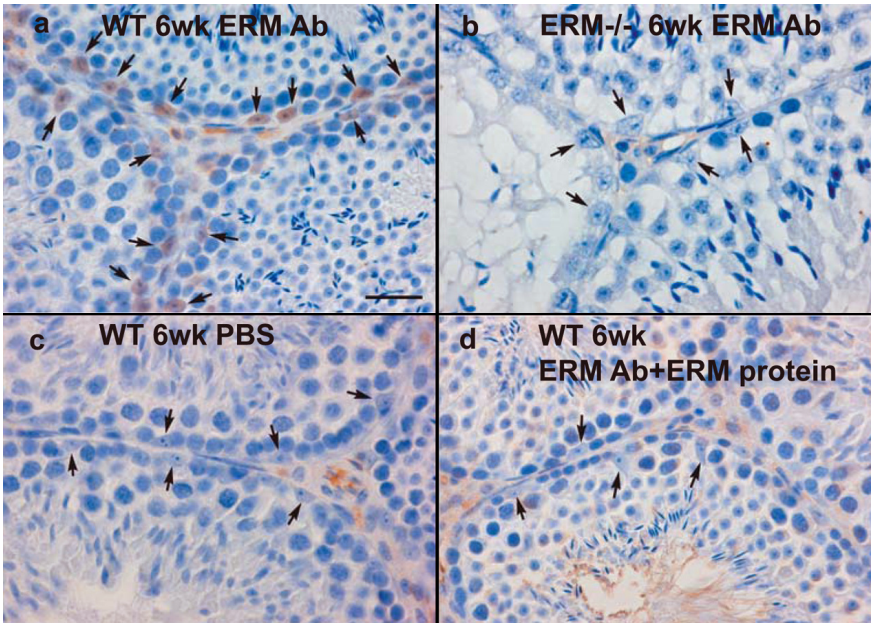

Figure 2. Immunostaining using mAb 3H7 against mouse ERM protein. a) Wild-type (WT) stains specifically in the nucleus of Sertoli cells (arrows); b) ERM-/- testis shows no staining; c) Control in which PBS is substituted for 3H7 Ab; d) 3H7 Ab is neutralized by pre-incubation with purified ERM protein. From Chen et al, Nature 2005; 436: 1030-1034.

Serum concentrations of hormones that normally promote spermatogenesis (testosterone, FSH) were normal (Chen et al., 2005), indicating that the loss of spermatogenesis was not due to deficiencies in spermatogenic hormones, consistent with the observation that the first wave of spermatogenesis occurs in these animals. In addition, GDNF was normal in the ERM-/- mice, indicating that GDNF was not downstream of ERM in Sertoli cells.

These initial data led to the conclusion that ERM deficiency was causing loss of spermatogenesis through an effect in the testis, rather than a systemic endocrine effect. The presence of ERM in juvenile Sertoli cells, but not germ cells, suggested that it was a Sertoli cell protein that like GDNF was somehow necessary for SSC maintenance, although GDNF did not appear to be downstream of ERM. Recent findings from our laboratories suggest that the role of ERM may be more complex than originally realized.

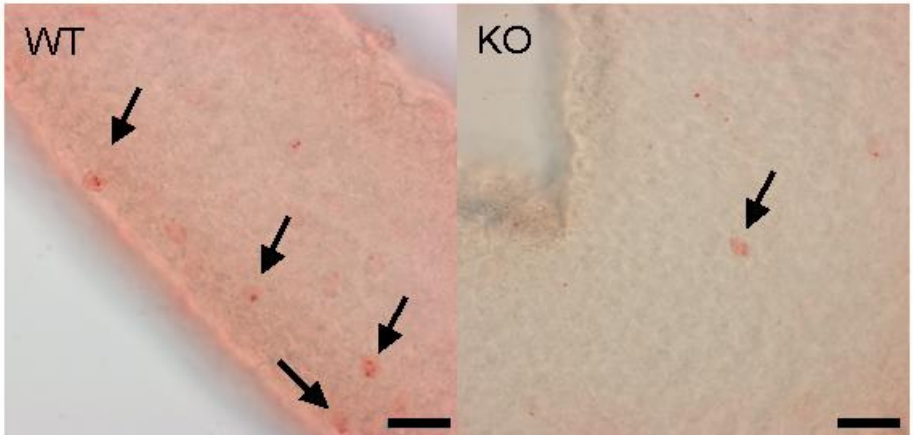

Figure 3. Wholemount immunohistochemistry with an antibody against GFRα1 in 28-day-old testis tubules of wild-type (WT) and ERM$^{-/-}$ (KO) mice. GFRα1 staining is used a specific marker for SSCs, and stained SSCs are indicated by a black arrow. Staining is sharply reduced by day 28 in ERM$^{-/-}$ mice, indicating almost a total loss of SSCs by this age. The space bar shown for both wild-type and ERM$^{-/-}$ mice = 50 μm.

We have recently sought to examine the temporal sequence of SSC loss in ERM$^{-/-}$ mice by using wholemount immunohistochemistry with an antibody against GFRα*1* in testis tubules of wild-type and ERM$^{-/-}$ mice. GFRα*1* is a specific SSC marker, and can be used to visualize and quantitate SSCs. Our results indicate that loss of SSCs begins in ERM$^{-/-}$ mice between 4 and 8 days of age and SSCs disappear over a period of 3-4 weeks (Fig. 3), with all SSCs being lost by 5 weeks. The early onset of SSC loss indicates that the neonatal period is critical for ERM actions in promoting stem cell maintenance.

Our data showing that disappearance of SSCs begins by one week postnatal suggested that ERM may have effects at a younger age than the 3- to 4-week postnatal time period where it was originally detected in Sertoli cells. Based on this, we have recently examined neonatal expression of ERM using immunohistochemistry and QPCR. Our immunohistochemistry results using a specific antibody showed that ERM is expressed both in Sertoli cells as well as in many SSCs from postnatal day 0 up to day 16 (Carnes *et al*., unpublished), when staining was substantially reduced in both cell types. This was consistent with QPCR data showing significant levels of ERM mRNA expression at days 0 and 4 in mouse testes (Tyagi *et al*., unpublished). These results show that ERM is present neonatally in both SSCs and Sertoli cells. This neonatal ERM expression is consistent with our observations that the neonatal period is when SSC loss begins, and is also consistent with the recent report that neonatal mouse SSCs in long-term culture expressed ERM (Oatley *et al*., 2006). These results clearly indicate that a full explanation of ERM actions in seminiferous epithelium requires an elucidation of this transcription factor's role in SSCs, as well as in Sertoli cells (Fig. 4). Experiments are presently underway to determine if ERM in Sertoli cells, SSCs, or both cell compartments is necessary to sustain SSCs during development.

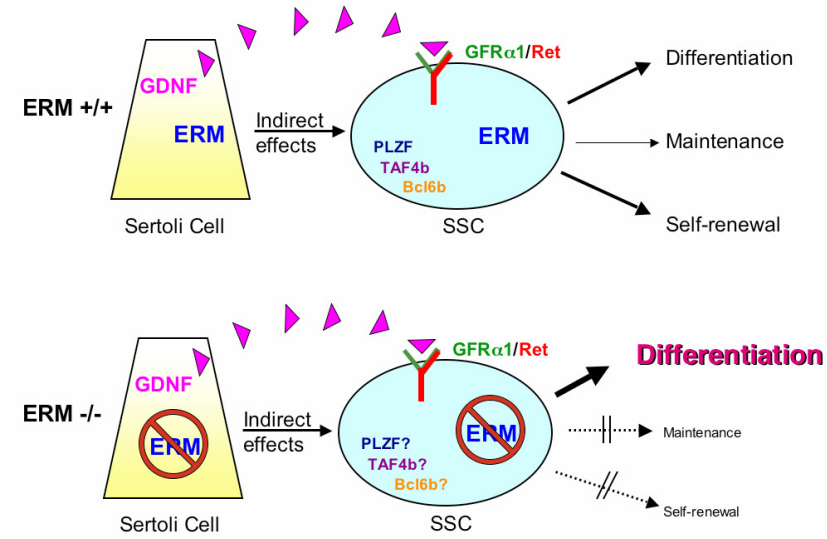

Figure 4. Factors necessary for spermatogonial stem cell (SSCs) maintenance. The transcription factor ERM is essential for maintenance of SSCs. In the WT mouse, ERM is expressed in Sertoli cells, and may alter Sertoli cell communication with SSCs. In addition, preliminary work indicates that ERM is also produced in SSCs themselves, suggesting direct effects of SSC ERM on SSC differentiation and self-renewal. In ERM-/- testis, spermatogenesis is lost due to a failure of SSC self-renewal and/or maintenance, although the relative contributions of Sertoli cell and SSC ERM to this process are presently unclear. Other factors important for spermatogonial stem cell maintenance are GDNF, PLZF, TAF4b and Bcl6b. GDNF is produced by Sertoli cells and signals through its receptors, GFRα1 and RET, on the surface of SSCs. In ERM-/- testis, GDNF levels are not altered, but loss of ERM could influence the receptors of GDNF, thus disrupting the GDNF signaling pathway. Bcl6b was shown to be a downstream target of GDNF and could be affected in the ERM-/- testis. PLZF and Taf4b are expressed in germ cells, but it is not clear what relationship these factors have to the ERM and/or GDNF signaling pathways.

Other factors essential for SSC maintenance/differentiation

In addition to ERM and the GDNF/RET/GFRα*1* signaling pathways, recent work has led to the identification of other molecules necessary for SSC maintenance. Extensive literature indicates that the transcriptional repressor Plzf (promyelocytic leukemia zinc-finger) may be essential for maintaining hematopoietic and other stem cells and inhibit their differentiation (Reid *et al*., 1995). Data reporting that Plzf is important for inhibiting stem cell differentiation in other organs are consistent with two recent papers suggesting that Plzf is vital in regulating testicular SSCs. Plzf is deleted in the naturally occurring luxoid mutation (Buaas *et al*., 2004) and also has been

knocked out (Costoya *et al.,* 2004). Plzf is expressed in the subset of spermatogonia that do not express either cyclin D1 or D2, markers of later stages of spermatogonial development, and cells staining positively for Plzf represent the progenitor-type SSC.

Both the naturally occurring mutant lacking Plzf and Plzf knockout (*Plzf*$^{-/-}$) mice (Buaas *et al*., 2004; Costoya *et al.,* 2004) have progressive losses of spermatogonia with age, including SSCs. In many tubules, all germ cells are eventually lost, including SSCs. This does not appear to result from deficits of hormones regulating spermatogenesis (Costoya *et al.,* 2004). The phenotypic similarities between *Plzf*$^{-/-}$ mice and mice lacking ERM or some component of the GDNF/RET/GFRα*1* signaling pathway suggest that Plzf could be downstream of ERM or GDNF/RET/GFRα*1*, or at least that Plzf deficiency may act through pathways common with ERM and GDNF/RET/GFRα*1* to bring about SSC loss.

The testicular phenotype of TAF4b knockout (*TAF4b*$^{-/-}$) mice suggest that this protein could also be essential for SSC maintenance. The testis expresses specific transcription factors not found in other tissues. In addition, the testis has specialized components of the basal RNA polymerase II complex that are also major regulators of testis-specific gene expression. In the mouse, TAF4b is a gonad-specific component of the RNA polymerase complex and plays an important role in transcriptional regulation.

TAF4b$^{-/-}$ mice initially make sperm and are transiently fertile (Falender *et al*., 2005). However, after this first wave of spermatogenesis, these animals undergo progressive losses of the spermatogenic lineage and by 3 months of age, seminiferous tubules are devoid of SSCs and all germ cells. The loss of SSCs does not appear to result from decreases in hormones normally associated with spermatogenesis.

TAF4b is expressed exclusively in germ cells; expression is high in spermatogonia, with some expression in spermatids. Impairments produced by lack of TAF4b reflect germ cell deficits exclusively and Sertoli cells of *TAF4b*$^{-/-}$ mice can support spermatogenesis, as shown by restoration of spermatogenesis following transplantation of WT spermatogonia into *TAF4b*$^{-/-}$ testes. TAF4b$^{-/-}$ mice show a loss of SSCs after the first wave of spermatogenesis that parallels that seen in *ERM*$^{-/-}$ mice, but it is not known whether Taf4b has any relationship to the ERM or GDNF/RET/GFRα*1* pathways of action.

A number of other factors have been recently identified that are essential for spermatogonial differentiation, and may represent proteins that regulate slightly later developmental decisions during spermatogenesis. For example, Sohlh1 is a basic helix-loop-helix transcription factor that is expressed in prespermatogonia and Type A spermatogonia (Ballow *et al*., 2006). The absence of Sohlh1 disrupts spermatogonial differentiation into spermatocytes, similar to the knockout of another transcription factor, Sox3 (Raverot *et al*., 2005). The absence of either Sohlh1 or Sox3 alters expression of neurogenin 3 (Ngn3), which is itself involved in the early differentiation decisions of spermatogonia (Yoshida *et al*., 2006). All of these factors, Sohlh1, Sox3 and Ngn3, regulate aspects of fate determination in spermatogonia, but so far no data has linked these factors with other factors that regulate stem cell maintenance, such as ERM, Plzf, Taf4b or the GDNF/RET/GFRα*1* pathway.

How is ERM regulated in the Sertoli cell?

Based on the critical role of ERM normally in the maintenance of SSCs, it is obviously of great interest to determine the factor(s) that control ERM expression in the Sertoli cell. Initial work describing the role of ERM in the testis indicated that fibroblast growth factor (FGF) 1, 2 and 9 (but not FGF 7 or 10) regulated expression of ERM mRNA in the TM4 Sertoli cell line (Chen *et al.,* 2005). These results were consistent with previous work indicating that various members of the FGF family regulate ERM expression in developing organs (Liu *et al*., 2003). Other data has implicated the PKC pathway in the regulation of ERM in lymphocytes (T'Sas *et al*., 2005). In addition, in vitro work has shown that ERM is regulated by the MAPK and protein kinase A signaling cascades (Janknecht *et al*., 1996). This work in other cell types indicates that there is likely to be multifactorial effects on ERM production by Sertoli cells, and we are presently addressing this question.

FGF2 stimulates ERM mRNA (Chen *et al.,* 2005), but other factors controlling ERM and the mechanism of FGF2 effects on ERM are not well understood. We observed that FGF2 caused a dose-dependent increase in ERM mRNA expression, with maximum stimulation of 8.3-fold at 25 ng/ml, consistent with the 8- to 9-fold increase in ERM expression reported in the initial work. Western blotting showed an increase of similar magnitude in ERM protein, suggesting that FGF2 effects on ERM are regulated transcriptionally. Our results indicated that FGF2 acted through both the mitogen activated protein kinase (MAPK) and phosphatidyl inositol 3-kinase (PI3K) signaling cascades to regulate ERM (Simon *et al.*, unpublished). FGF2 stimulated a 5-fold increase in mRNA expression for GDNF; this is the most potent regulator of GDNF identified in Sertoli cells. Finally, we determined that other classic endocrine regulators of Sertoli cells, FSH, testosterone and activin A, did not affect ERM mRNA expression. These results indicate that FGF2 regulates two Sertoli cell proteins, ERM and GDNF, that are essential for spermatogonial stem cell maintenance and self-renewal and that effects of FGF-2 effects are partially mediated through the MAPK and PI3K pathways. Although classical hormones known to regulate Sertoli cells (FSH, testosterone, activin A) do

not regulate ERM, FGF2 is emerging as a key regulator of Sertoli cell proteins that control SSCs (Simon *et al.*, unpublished).

Fertility, infertility and ERM

ERM is clearly obligatory for SSC maintenance and fertility in mice, but a crucial question is whether it has a similar role in humans and other animals. Murine ERM is a 510 AA protein (58 kD) (Chotteau-Lelievre *et al*., 1997). Human and murine ERM are about 95% identical at the amino acid level mouse (Monte *et al*., 1994; Chotteau-Lelievre *et al*., 1997). Furthermore, the crucial ETS domain and the N-terminal acidic domain involved in transactivation are identical in human and mouse (Monte *et al*., 1994; Chotteau-Lelievre *et al*., 1997), suggesting both evolutionary pressure on these portions of the molecules as well as significant roles for ERM in both species. Spermatogenesis is highly conserved among mammalian species. Since ERM is highly conserved between mouse and human and essential for mouse SSC maintenance, ERM likely plays a similar role in humans. There is minimal information available regarding ERM structure and function outside of the rodent and human literature, but the close similarities between mouse and human ERM suggests extensive conservation across mammalian species and the likelihood that ERM is essential for SSC maintenance in all mammals.

Although ERM expression is ubiquitous in humans (Monte *et al*., 1994), there is no information on its expression or function in human testis. However, some infertility in humans and animals may involve ERM or its downstream signaling pathway, and understanding ERM signaling may facilitate efforts to treat some types of infertility. Elucidation of the ERM signaling pathway also may provide information leading to development of new contraceptive methodologies, in that components of the Sertoli cells/SSC signaling pathway could be targets for contraceptive development. In addition, since ERM effects on stem cells may be unique to testis, this suggests the possibility of targeting this pathway in testis without deleterious effects on other stem cells. The difficulty in human male contraceptive development is to halt spermatogenesis without inhibiting androgen production/action and causing side effects such as loss of libido. ERM production by Sertoli cells appears to have no connection to androgen status, further reinforcing the concept that inhibiting ERM's downstream targets may hold promise for male contraceptive strategies.

Conclusion

Our present knowledge of the factors that regulate SSC maintenance, self-renewal and differentiation is still in an early stage. The ERM and GDNF/RET/GFRα*1* signaling pathways appear to be major factors in these processes. Other proteins such as Plzf and TAF4b also appear to be critical for SSC maintenance, and whether these are somehow involved in the ERM and/or GDNF/RET/GFRα*1* signaling pathways is presently unclear. The rapid and accelerating progress in this field promises major mechanistic insights in the years to come into critical processes such as how SSC maintenance, self-renewal and differentiation are regulated, and should lead to identification of additional proteins that regulate SSCs as well as integrative models of how the proteins presently known to be involved in this process interrelate.

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References

Aponte PM, Soda T, van de Kant HJ, de Rooij DG. 2006. Basic features of bovine spermatogonial culture and effects of glial cell line-derived neurotrophic factor. *Theriogenology*, 65:1828-1847.

Ballow D, Meistrich ML, Matzuk M, Rajkovic A. 2006. Sohlh1 is essential for spermatogonial differentiation. *Dev Biol*, 294:161-167.

Braydich-Stolle L, Nolan C, Dym M, Hofmann M-C. 2006. Role of glial cell line-derived neurotrophic factor in germ-line stem cell fate. *Ann NY Acad Sci*, 1061:94-99.

Brinster RL. 2002. Germline stem cell transplantation and transgenesis. *Science*, 296:2174-2176.

Brinster RL, Zimmermann JW. 1994. Spermatogenesis following male germ-cell transplantation. *Proc Natl Acad Sci USA,* 91:11298-11302.

Buaas FW, Kirsh AL, Sharma M, McLean DJ, Morris JL, Griswold MD, de Rooij DG, Braun RE. 2004. Plzf is required in adult male germ cells for stem cell self-renewal. *Nat Genet*, 36:647-652.

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. 2005. ERM is required for transcriptional control of the spermatogonial stem cell niche. *Nature*, 436:1030-1034.

Chotteau-Lelievre A, Desbiens X, Pelczar H, Defossez PA, de Launoit Y. 1997. Differential expression patterns of the PEA3 group transcription

factors through murine embryonic development. *Oncogene*, 15:937-952.

Chotteau-Lelievre A, Dolle P, Peronne V, Coutte L, de Launoit Y, Desbiens X. 2001. Expression patterns of the Ets transcription factors from the PEA3 group during early stages of mouse development. *Mech Dev*, 108:191-195.

Clermont Y. 1962. Quantitative analysis of spermatogenesis of the rat: a revised model for the renewal of spermatogonia. *Am J Anat*, 111:111-129.

Costoya JA, Hobbs RM, Barna M, Cattoretti G, Manova K, Sukhwani M, Orwig KE, Wolgemuth DJ, Pandolfi PP. 2004. Essential role of Plzf in maintenance of spermatogonial stem cells. *Nat Genet*, 36:653-659.

Falender AE, Freiman RN, Geles KG, Lo KC, Hwang K, Lamb DJ, Morris PL, Tjian R, Richards JS. 2005. Maintenance of spermatogenesis requires TAF4b, a gonad-specific subunit of TFIID. *Genes Dev*, 19:794-803.

Fuchs E, Tumbar T, Guasch G. 2004. Socializing with the neighbors: stem cells and their niche. *Cell,* 116:769-778.

Guan K, Nayernia K, Maier LS, Wagner S, Dressel R, Lee JH, Nolte J, Wolf F, Li M, Engel W, Hasenfuss G. 2006. Pluripotency of spermatogonial stem cells from adult mouse testis. *Nature*, 440:1199-1203.

Hamra FK, Chapman KM, Nguyen DM, Williams-Stephens AA, Hammer RE, Garbers DL. 2005. Self renewal, expansion, and transfection of rat spermatogonial stem cells in culture. *Proc Natl Acad Sci USA*. 102:17430-17435.

Hamra FK, Schultz N, Chapman KM, Grellhesl DM, Cronkhite JT, Hammer RE, Garbers DL. 2004. Defining the spermatogonial stem cell. *Dev Biol*, 269:393-410.

Hofmann MC, Dym M. 2005. Long-term cultures of mammalian spermatogonia. *In*: Skinner MK, Griswold MD (Eds.). *Sertoli cell biology*. London: Elsevier Academic Press. pp.449-470.

Hofmann MC, Braydich-Stolle L, Dym M. 2005. Isolation of male germ-line stem cells; influence of GDNF. *Dev Biol*, 279:114-124.

Huckins C, Clermont Y. 1968. Evolution of gonocytes in the rat testis during late embryonic and early post-natal life. *Arch Anat Histol Embryol*, 51:341-354.

Janknecht R, Monte D, Baert JL, de Launoit Y. 1996. The ETS-related transcription factor ERM is a nuclear target of signaling cascades involving MAPK and PKA. *Oncogene*, 13:1745-1754.

Kanatsu-Shinohara M, Miki H, Inoue K, Ogonuki N, Toyokuni S, Ogura A, Shinohara T. 2005. Long-term culture of mouse male germline stem cells under serum- or feeder-free conditions. *Biol Reprod*, 72:985-991.

Kanatsu-Shinohara M, Ikawa M, Takehashi M, Ogonuki N, Miki H, Inoue K, Kazuki Y, Lee J, Toyokuni S, Oshimura M, Ogura A, Shinohara T. 2006. Production of knockout mice by random or targeted mutagenesis in spermatogonial stem cells. *Proc Natl Acad Sci USA*, 103:8018-8023.

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. 2004. Generation of pluripotent stem cells from neonatal mouse testis. *Cell*, 119:1001-1012.

Kubota H, Avarbock MR, Brinster RL. 2004. Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. *Proc Natl Acad Sci USA*, 101:16489-16494.

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

Li L, Xie T. 2005. Stem Cell Niche: Structure and Function. *Annu Rev Cell Dev Biol*, 21:605-631.

Liu Y, Jiang H, Crawford HC, Hogan BL. 2003. Role for ETS domain transcription factors Pea3/Erm in mouse lung development. *Dev Biol*, 261:10-24

Martin GR, Evans MJ. 1974. The morphology and growth of a pluripotent teratocarcinoma cell line and its derivatives in tissue culture. *Cell*, 2:163-172.

Meng X, de Rooij DG, Westerdahl K, Saarma M, Sariola H. 2001. Promotion of seminomatous tumors by targeted overexpression of glial cell line-derived neurotrophic factor in mouse testis. *Cancer Res*, 61:3267-3271.

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. 2000. Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. Science, 287:1489-1493.

Monte D, Baert JL, Defossez PA, de Launoit Y, Stehelin D. 1994. Molecular cloning and characterization of human ERM, a new member of the Ets family closely related to mouse PEA3 and ER81 transcription factors. *Oncogene*, 9:1397-1406.

Naughton CK, Jain S, Strickland AM, Gupta A, Milbrandt J. 2006. Glial cell-line derived neurotrophic factor-mediated RET signaling regulates spermatogonial stem cell fate. *Biol Reprod*, 74:314-321.

Oatley JM, Avarbock MR, Telaranta AI, Fearon DT, Brinster RL. 2006. Identifying genes important for spermatogonial stem cell self-renewal and survival. *Proc Natl Acad Sci USA*, 103:9524-9529.

Raverot G, Weiss J, Park SY, Hurley L, Jameson JL. 2005. Sox3 expression in undifferentiated spermatogonia is required for the progression of spermatogenesis. *Dev Biol*, 283:215-225.

Reid A, Gould A, Brand N, Cook M, Strutt P, Li J, Licht J, Waxman S, Krumlauf R, Zelent A. 1995. Leukemia translocation gene, PLZF, is expressed with a speckled nuclear pattern in early hematopoietic progenitors. *Blood*, 86:4544-4552.

Ryu BY, Orwig KE, Kubota H, Avarbock MR, Brinster RL. 2004. Phenotypic and functional characteristics of spermatogonial stem cells in rats. *Dev Biol*, 274:158-170.

Shinohara T, Avarbock MR, Brinster RL. 1999. beta1- and alpha6-integrin are surface markers on mouse spermatogonial stem cells. *Proc Natl Acad Sci USA*, 96:5504-5509.

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. 1998. Embryonic stem cell lines derived from human blastocysts. *Science,* 282:1145-1147.

T'Sas F, Brenner C, Mauen S, Putmans P, Monte D, Van Lint C, Moser M, Baert JL, de Launoit Y. 2005. Expression of the Ets transcription factor Erm is regulated through a conventional PKC signaling pathway in the Molt4 lymphoblastic cell line. *FEBS Lett*, 579:66-70.

Yamashita YM, Fuller MT, Jones DL. 2005. Signaling in stem cell niches: lessons from the Drosophila germline. *J Cell Sci*, 118:665-672.

Yoshida S, Sukeno M, Nakagawa T, Ohbo K, Nagamatsu G, Suda T, Nabeshima Y. 2006. The first round of mouse spermatogenesis is a distinctive program that lacks the self-renewing spermatogonia stage. *Development*, 133:1495-1505.