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Regulation of spermatogonial stem cell behavior in vivo and in vitro[1]

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Abstract

Spermatogonial stem cells (SSCs) are one of the best studied types of stem cells with respect to cellular aspects and new data on the molecular pathways regulating their behavior now become available frequently. In non-primate mammals, SSCs have been identified as single spermatogonia that either selfrenew, by dividing into two new single spermatogonia, or differentiate by giving rise to a pair of spermatogonia connected by an intercellular bridge. After cell loss, selfrenewing divisions of SSCs are strongly preferred implicating the existence of regulatory mechanisms that govern the ratio of selfrenewal and differentiation. Glial cell line derived neurotrophic factor (GDNF) and fibroblast growth factor 2 (FGF2), both secreted by Sertoli cells, have been found to enhance SSC selfrenewal. The secretion of GDNF is controlled by FSH. The action of GDNF on SSCs is mediated by its receptors c-Ret and GFRalpha1 and involves the transcriptional repressor Bcl6b. Furthermore, Plzf, like Bcl6b a member of the POZ domain containing proteins, is also involved in enhancing SSC self-renewal but along another as yet unknown pathway. Finally, in vitro studies revealed that the Sertoli cell proteins activin A and BMP4 likely stimulate SSC differentiation. Hence, Sertoli cells produce both factors that promote selfrenewal and factors that promote differentiation. The mechanisms that regulate the proper balance in the secretion of these factors with opposing actions by Sertoli cells, are at present unknown.

Keywords: testis, stem cells, spermatogenesis.

Introduction

In general, spermatogonia in the adult testis are distinguished in type A, when no or very little heterochromatin is present in the nuclei, and type B spermatogonia when the nuclei contain a great deal of heterochromatin. In some, but not all animals, an intermediate type is present called Intermediate (In) spermatogonia, accordingly. Spermatogonial stem cells are A spermatogonia and using whole mounts of seminiferous tubules have been identified to be single cells in various rodents, boar and ram. Accordingly, these cells were called A-single spermatogonia (A-s). Upon division, the daughter cells of the A-single spermatogonia can migrate away from each other and become two new stem cells, in which case self-renewal has taken place. Alternatively, the daughter cells can stay together connected by an intercellular bridge, in which case they are called A-paired spermatogonia (A-pr). The formation of a pair of spermatogonia comprises the first differentiation step in the spermatogenic lineage, and these cells are destined to ultimately become spermatozoa. It has been established that the spermatogonial stem cells in rodents divide on the average 2 to 3 times each cycle of the seminiferous epithelium (review: de Rooij and Russell, 2000).

In the differentiating pathway, starting with the A-pr spermatogonia, larger and larger clones are formed as during each division cytokinesis is not complete and hence numbers of cells per clone double with each division. Hence, the A-pr spermatogonia form a chain of 4 so-called A-aligned spermatogonia (A-al) that generally divide another 1 or 2 times to become chains of 8 or 16, sometimes 32, cells. During a particular period of the cycle of the seminiferous epithelium, i.e. stages VII-VIII, most A-al spermatogonia will differentiate into so-called A1 spermatogonia. From A1 spermatogonia onwards the spermatogonia acquire a fixed pattern of proliferative behavior and carry out 6 subsequent divisions to finally become spermatocytes that will enter meiotic prophase. In mice and rats the generations of spermatogonia following A1 spermatogonia are called A2, A3, A4, intermediate (In) and B spermatogonia.

Three important regulatory mechanisms exist to assure a proper cell production in the spermatogonial compartment (Fig. 1). First, stem cell behavior in terms of a proper ratio between self-renewal and differentiation has to be controlled to prevent stem cell depletion, to accomplish recovery after stem cell loss because of for example chemotherapy and to prevent overproduction of stem cells and the formation of a tumor. Second, the differentiation step from A-al to A1 spermatogonia has been shown to be subject to several regulatory mechanisms, involving stem cell factor (SCF) / c-kit receptor (de Rooij *et al*., 1999), the Dazl protein (Schrans-Stassen *et al*., 2001), testosterone levels (Shuttlesworth *et al*., 2000) and retinoic acid (van Pelt and de Rooij 1990; de Rooij 2001). Finally, a third regulatory mechanism exist to regulate cell production quantitatively. It has been found that too many spermatogonia are produced than the Sertoli cells can support and this is corrected at the local level in each

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area of the epithelium by way of apoptosis of the surplus of spermatogonia. This apoptosis usually occurs among A2-A4 spermatogonia (de Rooij and Janssen 1987; de Rooij and Lok 1987).

Recently, considerable progress is being made in understanding the regulation of the behavior of spermatogonial stem cells both in vivo and in vivo. This progress will now be the subject of this review.

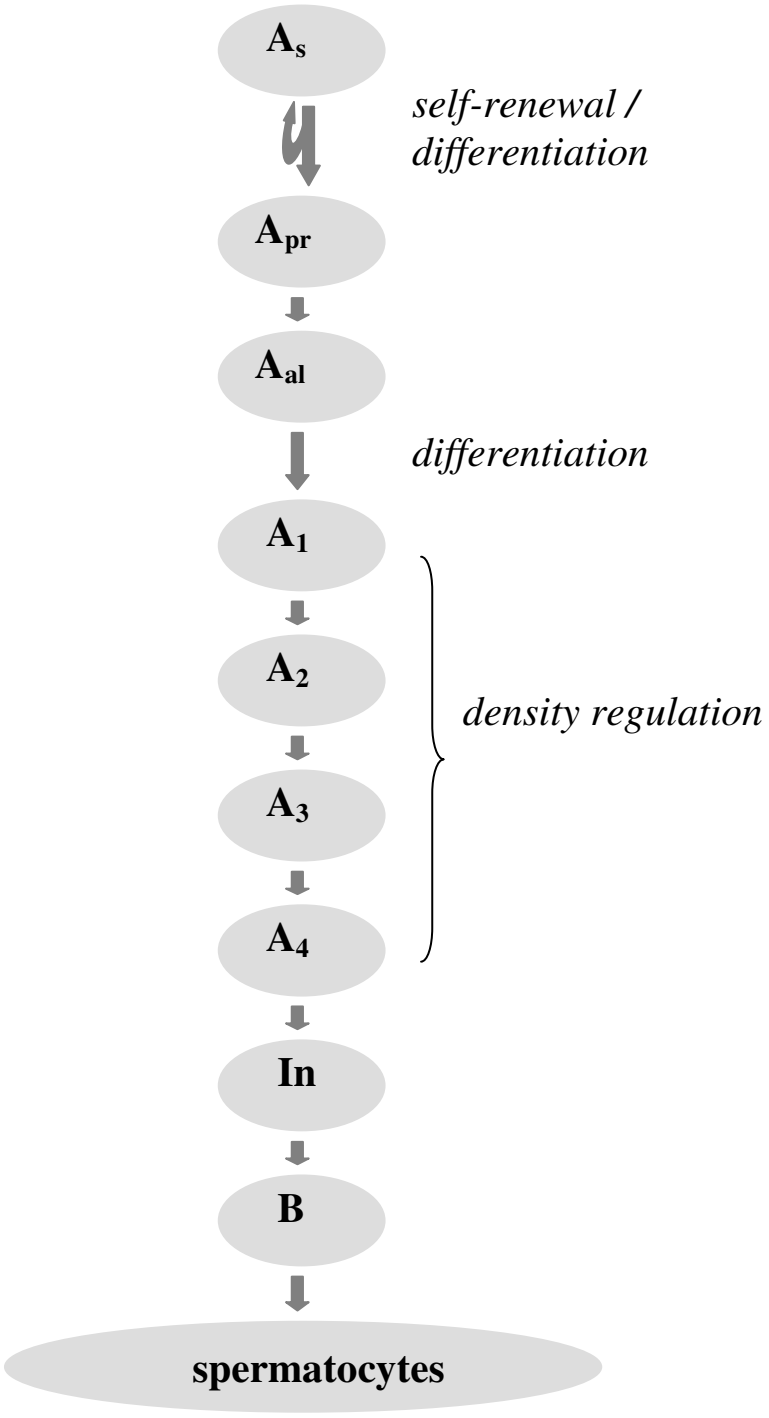

Figure 1. Scheme of spermatogonial proliferation and stem cell renewal in rodents and the regulatory mechanisms that ensure the proper number of stem cells, spermatogonial differentiation and density regulation.

In the normal seminiferous epithelium the ratio between stem cell renewal and differentiation should be about 1:1. When too many stem cells carry out self-renewing divisions, a germ cell tumor will arise as for example is the case in mice over-expressing Glial cell line derived neurotrophic factor (GDNF) which enhances self-renewal (see below). In contrast, when stem cells would prefer differentiation a depletion of the epithelium would occur. Still, the system should be flexible to allow recovery from cell loss, for example caused by toxic agents or radiation. Indeed, it was calculated that after irradiation surviving spermatogonial stem cells almost only self-renew during at least the first 6 divisions (van Beek *et al*., 1990). Hence, there must be a regulatory mechanism that senses a shortage in germ cell production and subsequently acts by stimulating self-renewing divisions of spermatogonial stem cells which will consequently enhance germ cell production.

In several renewing tissues, stem cells occupy specific areas. For example in the intestine, stem cells reside near the bottom of the crypts and stem cells in the bone marrow also occupy specific niches. Until recently, in the seminiferous epithelium no such niches were found for spermatogonial stem cells. Now it has become clear that most spermatogonial stem cells are preferably present in those areas of seminiferous tubules that border on interstitial tissue (Chiarini-Garcia *et al*., 2001; Chiarini-Garcia *et al*., 2003). Apparently, the interstitial tissue affects stem cell behavior in such a way that differentiation is less likely to occur when stem cells lie close to it. Interestingly, high testosterone levels have been found to prevent spermatogonial differentiation (Meistrich and Shetty, 2003) and in the normal epithelium testosterone levels will be highest in tubule areas bordering on interstitial tissue where Leydig cells reside. In addition, GDNF secretion by Sertoli cells has been found to be under the control of FSH (Tadokoro *et al*., 2002) and levels of this hormone will also be highest in the interstitial tissue.

Regulation of differentiation and self-renewal

Data obtained from transgenic mice and mutations

Like in all other renewing tissues, the seminiferous epithelium is able to react to stem cell loss by enhanced stem cell renewal in order to replace lost stem cells. The question then arises which molecular pathways are involved. Recent data indicate a role for GDNF. Normally, GDNF is secreted by Sertoli cells while a subset of spermatogonia express both receptors for this growth factor, c-Ret and GFR-α1. Ectopic expression of GDNF in spermatogonia or overexpression in Sertoli cells induces the formation of large clusters of single type A spermatogonia, while normal spermatogenesis is suppressed (Meng *et al*., 2000; Yomogida *et al*., 2003). Moreover, in mice overexpressing GDNF in spermatogonia, germ cell tumors that resemble human seminoma, are formed at about one year of age (Meng *et al*., 2001). In heterozygotic GDNF deficient mice, spermatogenesis deteriorates with age as germ cells become depleted (Meng *et al*., 2000). Clearly, GDNF has a role in the regulation of self-renewal and differentiation of spermatogonial stem cells. Too high levels of GDNF inhibit stem cell differentiation and cause an accumulation of stem cells and low levels stimulate differentiation and cause stem cell depletion.

Recently, details about the molecular pathway along which GDNF exerts its regulatory action have been obtained from microarray analysis studies (Oatley *et al*., 2006). SSC cultures were established from 6 days old mice in a GDNF containing medium. Gene expression in SSCs was compared between cultures in which GDNF remained present and cultures at several time points after withdrawal of GDNF, assuming that the withdrawal of GDNF induces the start of differentiation of the SSC present. The expression levels of six genes were found to decrease considerably. One of these genes, *bcl6b* was studied in detail. Bcl6b was necessary for in vitro maintenance of SSCs as checked by the SSC transplantation assay of cells in which Bcl6b mRNA was reduced by siRNA. Furthermore, Bcl6b deficient mice showed a depletion of the seminiferous epithelium. Hence, Bcl6b is involved in the downstream signal transduction pathway of GDNF in SSCs.

Interestingly, Bcl6B is a member of the poxvirus and zinc finger (POZ) family of transcriptional repressors and one other member of this family had already been implicated in the regulation of SSC renewal and differentation, i.e. Plzf (promyelocitic leukemia zinc finger protein). *Plzf* was found to be the disrupted gene in the luxoid mutation in mice (Buaas *et al*., 2004; Costoya *et al*., 2004). Mice having the classical spontaneous mutation *luxoid* exhibit a progressive loss of spermatogonial stem cells. Apparently, the gene involved in this mutation also has a role in the regulation of spermatogonial stem cell renewal and differentiation. The Plzf protein was localized to A-s, A-pr and A-al spermatogonia. Although both Plzf and Bcl6b act to prevent SSCs from differentiation, only Bcl6b was found to be under the control of GDNF (Oatley *et al*., 2006). Apparently, Plzf is involved in a different regulatory pathway. The molecular pathways downstream of Plzf and Bcl6b in SSCs have still to be studied but, through the POZ domain, may involve the corepressors N-CoR and SMRT (Payne and Braun, 2006).

The congenital malformation called Apert syndrome has been shown to be mainly of paternal origin in such a way that the chance of fathers to sire a child with this malformation increases with age. It was found that the syndrome involves a gain of function mutation of the fibroblast growth factor receptor 2 (FGFR2) gene. It could be deduced that the mutation

arises in an SSC of the father and this event then gives this SSC an advantage above the neighbouring SSCs in that it propagates itself by enhanced self-renewal, replacing more and more normal SSCs and ultimately leading to the production of more and more mutated sperm with increasing age (Goriely *et al*., 2005). The FGFR2 receptor was indeed found to be present in a SSC cell line (Goriely *et al*., 2005). This suggests that the molecular pathway downstream of the FGFR2 is also involved in regulating the balance between self-renewal and differentiation of SSC. As FGF2 is produced by Sertoli cells, this may well be another way in which these cells regulate SSC behavior.

Data from SSC in culture

Generally, various growth factors are added to cultures of SSCs. Nagano *et al*. (2003) found improved maintenance of SSC when GDNF was added to the culture, while bone morphogenic protein 4 (BMP4) and activin A reduced stem cell numbers. It was assumed that BMP4 and activin A enhance SSC differentiation. Intriguingly, both BMP4 and activin A are proteins secreted by Sertoli cells.

Recently, very successful protocols for the longterm culture of SSCs have become available, allowing propagation of SSC for at least two years without losing the ability of these cells to repopulate a recipient mouse testis (Kanatsu-Shinohara *et al*., 2003; 2005; de Rooij, 2006; Kanatsu-Shinohara *et al*., 2006). Growth factors added to the media in these cultures include GDNF, FGF2, epidermal growth factor (EGF) and leukemia inhibiting factor (LIF). As described above there is good reason to believe that GDNF and FGF2 enhance self-renewal of SSCs but the possible roles of EGF and LIF in SSC cultures are still unclear.

Conclusions

Figure 2 shows the present state of the art about the knowledge on the molecules and molecular pathways that regulate SSC self-renewal and differentiation. So far there are two pathways and probably three, via Plzf, that promote self-renewal and two that enhance SSC differentiation. Apparently, Sertoli cells secrete both factors that enhance self-renewal, GDNF and FGF2, and factors enhancing differentiation, BMP4 and activin A. Obviously, the next question then is how the secretion of these factors by Sertoli cells is regulated. In this respect an important finding was done by Tadokoro *et al*. (2002), who found that FSH stimulates GDNF secretion by Sertoli cells. One can speculate that this finding provides a rationale for the increased FSH levels often seen in human patients with a less optimally functioning seminiferous epithelium (Zitzmann *et al*., 2006). Increasing stem cell numbers would provide the best basis for a recovery to a more normal epithelium. Clearly, this will have to be studied in further detail.

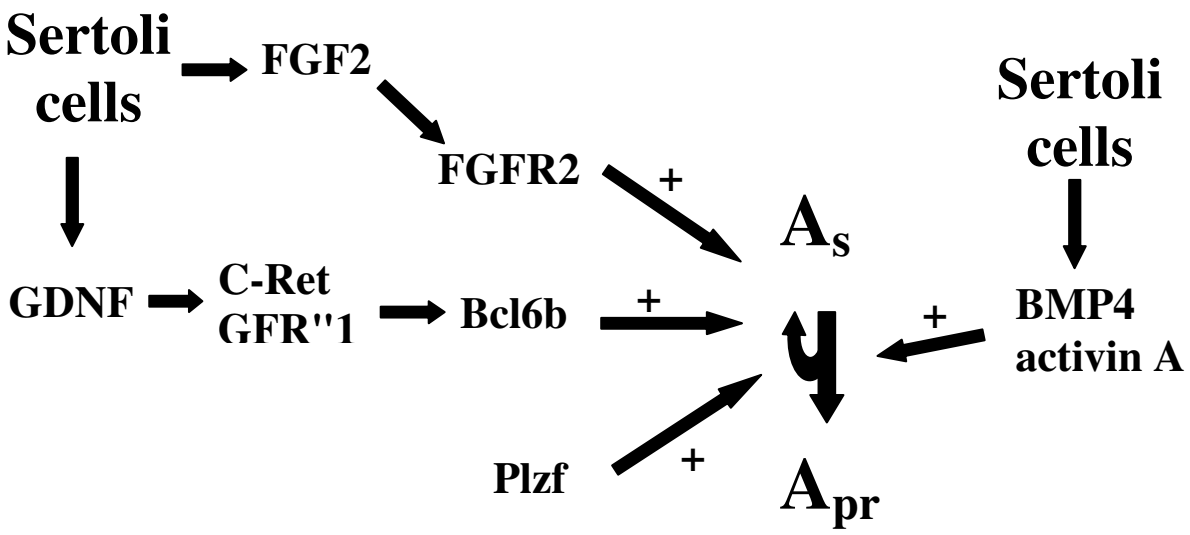

Figure 2. Molecular pathways regulating the ratio between self-renewing and differentiating divisions of spermatogonial stem cells.

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