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Spermatogonial Stem Cell Biology

Contents

Abstract

Spermatogonial stem cells are at the beginning of the spermatogenic process. In non-primate mammals they are single cells (A_s spermatogonia). Their daughter cells either migrate away from each other and become two new stem cells or stay together, connected by an intercellular bridge. The latter designates these cells to differentiate and ultimately become spermatozoa.

The purification of spermatogonial stem cells is hampered by the lack of specific markers. Present protocols only allow for a purity of 10% at best. Spermatogonial stem cells are difficult to culture in the absence of serum and a feeder layer. Better results have been obtained with co-cultures of Sertoli cells with mouse and bovine spermatogonia. Spermatogonial stem cells can be transplanted to recipient testes the endogenous spermatogenesis of which has been removed which makes it possible to perform functional assays of stem cell capacity of germ cell suspensions.

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The ratio between self renewal and differentiation of spermatogonial stem cells can be regulated as after cell loss self-renewal is increased. A major factor that plays a role in regulating stem cell fate is glial cell line derived neurotrophic factor (GDNF) produced by Sertoli cells which inhibits stem cell differentiation. Furthermore, spermatogonial stem cells are mainly localized to those tubule areas that border on the interstitial tissue. Apparently, also factor(s) from the interstitium, possibly testosterone, inhibit stem cell differentiation. Research on spermatogonial stem cells and the regulation of their differentiation may become facilitated by spermatogonial (stem) cell lines that have recently been developed.

Key-words: spermatogenesis, spermatogonial stem cells, testis, GDNF, transplantation, cell culture, cell lines.

Invited Mini-review

Introduction

Spermatogenesis starts with a series of mitotic divisions of spermatogonia. The last of these divisions renders spermatocytes that go through S phase, then pass through the lengthy prophase of the first meiotic division and subsequently carry out the two meiotic divisions to give rise to haploid spermatids. Initially, spermatids have a round shape but then elongate to become spermatozoa that leave the seminiferous tubules through the tubule lumen (review Russell *et al.*, 1990).

Stem cells are at the basis of spermatogenesis and are both able to selfrenew and to give rise to differentiating daughter spermatogonia. This dual capacity of stem cells ensures the long-lasting ability of the testis to produce spermatozoa. In recent years our knowledge about these important cells is rapidly growing, especially with respect to our understanding of the regulation of the behavior of these cells. The progress in this field will be reviewed.

Spermatogonial Stem Cells in Non-primate Mammals

Spermatogonial stem cells are single cells located on the basal membrane of the seminiferous tubules and are called A-single (A_s) spermatogonia (review de Rooij & Russell, 2000). 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 (Fawcett *et al.*, 1959; Weber & Russell, 1987) (Fig. 1) . In all further divisions, starting with the pair, cytokinesis will also be incomplete, leading to the formation of increasingly large syncytia of germ cells. As 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.

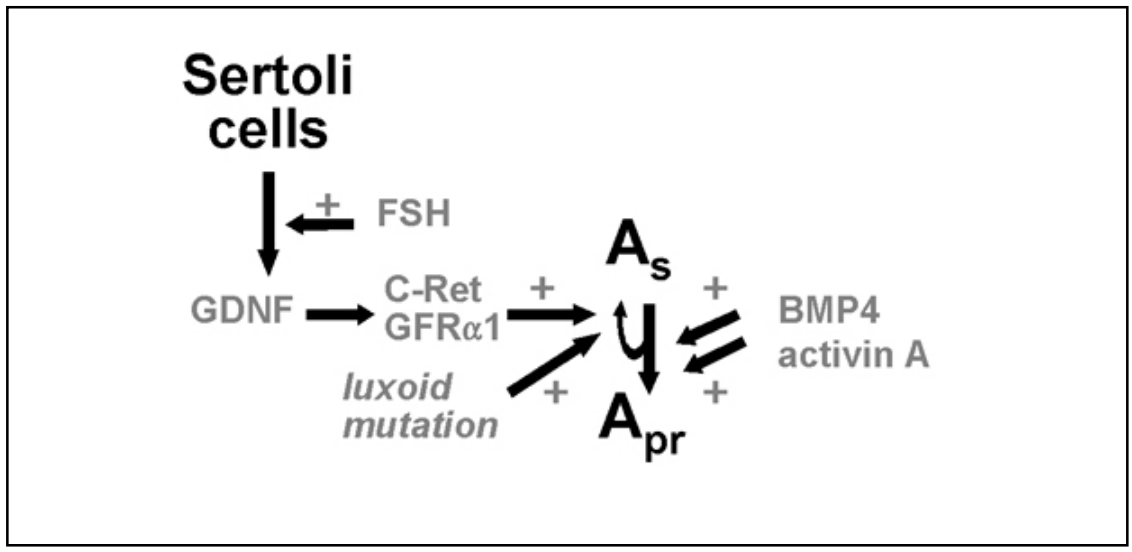

Figure 1. Scheme of spermatogonial stem cell (A$_s$) renewal and differentiation and the present knowledge about the regulatory mechanisms that operate at this early step in the spermatogenic process.

The A_{pr} spermatogonia divide further to form chains of 4, 8, up to occasionally 32 A-aligned (A_{al}) spermatogonia. The A_{al} spermatogonia can go through a differentiation step and become so-called A1 spermatogonia. This differentiation step involves slight morphological changes (Chiarini-Garcia & Russell, 2001) and brings about changes in cell cycle characteristics of the spermatogonia (Huckins, 1971[b,a]; Lok & de Rooij, 1983; Lok *et al.*, 1983). In most non-primate mammals there are six divisions following the formation of A1 spermatogonia, the last of which giving rise to spermatocytes. In total, there are about 10 spermatogonial divisions between the spermatogonial stem cells and the formation of spermatocytes (de Rooij & Russell, 2000).

Purification of Spermatogonial Stem Cells

In the adult mouse testis, there are about 35.000 stem cells which is 0.03% of all germ cells (Tegelenbosch & de Rooij, 1993).Various techniques have been developed to purify the total population of A spermatogonia, achieving a purity varying between 85 to 98% (Bellve *et al.*, 1977; Morena *et al.*, 1996; Dirami *et al.*, 1999). Unfortunately, in the mouse only about 3% of the A spermatogonia are stem cells (Tegelenbosch & de Rooij, 1993) and it will not likely be much different in other animals. Hence, although a 100-fold enrichment of stem cells can be achieved by purifying A spermatogonia, the purity is still very low. To further increase the purity, a method has been developed to isolate spermatogonia from vitamin A deficient animals (van Pelt *et al.*, 1996). In vitamin A deficient rats and mice, spermatogenesis is arrested at the differentiation step of A_{al} into A1 spermatogonia and the testes of these animals only contain A_s, A_{pr} and A_{al} spermatogonia (van Pelt & de Rooij, 1990). Starting from testes of vitamin A deficient animals, theoretically a cell population containing about 10% stem cells can be obtained

(Tegelenbosch & de Rooij, 1993; van Pelt *et al.*, 1996).

Certain biochemical markers have been used to enrich spermatogonial stem cells (Shinohara *et al.*, 1999; Shinohara & Brinster, 2000). Using anti-∃1- and anti-∀6-integrin and negatively selecting for the c-kit receptor, which is not present on spermatogonial stem cells (Schrans-Stassen *et al.*, 1999), a 40-fold enrichment of spermatogonial stem cells from testicular germ cells could be accomplished (Shinohara *et al.*, 1999).

Taken together, the purification of spermatogonial stem cells has not yet reached further than a purity of about 10% at the most. More specific membrane markers for these cells will have to be found to achieve further progress in this field.

Spermatogonial Cultures

Attempts have been made to culture pure populations of stem cells in the absence of serum or a feeder layer. Unfortunately, these attempts have been less successful in that few cells survive one week of culture (Dirami *et al.*, 1999; Creemers *et al.*, 2002[a]). Nevertheless, in a longterm culture of a mixed germ cell suspension, in the presence of serum and on a feeder layer, some spermatogonial stem cells did survive and were able to repopulate a recipient mouse testis after transplantation (Nagano *et al.*, 1998). Recent results indicate that the maintenance of spermatogonial stem cells in culture can be improved by the addition of glial cell line derived neurotrophic factor (GDNF) and that the presence of Sertoli cells, activin A and BMP4 is deleterious to stem cells probably through the induction of differentiation (Nagano *et al.*, 2003) (Fig. 1).

Long-term survival (25 days) and proliferation of mouse spermatogonia cultured with Sertoli cells and in the presence of serum has also been achieved (van der Wee *et al.*, 2001). Recently, a bovine spermatogonia/Sertoli cell co-culture system was developed allowing survival, proliferation and differentiation up to cells showing characteristics of spermatids, during at least 4 months (Izadyar *et al.*, 2003). In these cultures, 2 types of spermatogonial colonies were formed, one consisting of only single stem cells and a mixed type in which, besides stem cells, also pairs and chains of cells were formed. Intriguingly, these data suggest the existence of two types of stem cells, one prone to differentiate and one only capable of self-renewal without the proper stimulus.

Spermatogonial Stem Cell Transplantation

The presence of functional spermatogonial stem cells can be checked by the spermatogonial stem cell transplantation technique (Brinster & Avarbock, 1994; Brinster & Zimmermann, 1994). In this technique, germ cells of one mouse are transplanted into the testes of a recipient mouse, the endogenous spermatogenesis of which is depleted because of the *Wv/Wv* mutation or by treatment of the mice with the alkylating agent busulfan. An alternative method to kill the endogenous spermatogonial stem cells in the recipient mice uses fractionated X-irradiation (local testicular doses of 1.5 and 12 Gy, 24

hr apart). The latter protocol reliably causes more than 95% depletion (Creemers *et al.*, 2002[b]). After transplantation, the donor stem cells repopulate the seminiferous epithelium of the recipient mice. Interestingly, also rat spermatogonial stem cells are able to repopulate the mouse testis and produce normal rat spermatogenesis in the mouse (Clouthier *et al.*, 1996; Russell & Brinster, 1996)! However, stem cells from other species transplanted into mouse testes either produce defective spermatogenesis (hamster: Ogawa *et al.*, 1999) or initiate repopulation by spermatogonia only as these cells apparently fail to develop further (rabbit and dog: Dobrinski *et al.*, 1999; bull: Izadyar *et al.*, 2001).

Recently, the possibility of carrying out spermatogonial stem cell transplantation in larger domestic animals has been shown for the pig, goat and bull (Honaramooz *et al.*, 2002; Izadyar *et al.*, 2002; Honaramooz *et al.*, 2003; Izadyar *et al.*, submitted).

Regulation of Stem Cell Renewal and Differentiation

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. After a high dose of irradiation surviving spermatogonial stem cells almost only self-renew during at least their first 6 divisions, leading to a rapid recovery of stem cell numbers (van Beek *et al.*, 1990). This indicates that there are mechanisms that can inhibit stem cell differentiation and/or enhance self-renewal in situations of cell loss.

In several renewing tissues, stem cells occupy specific areas. For example in the intestine, stem cells reside near the bottom of the crypts (Potten, 1998) and stem cells in the bone marrow also occupy specific niches (Schofield, 1983). 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 present in those areas of seminiferous tubules that border on interstitial tissue (Chiarini-Garcia *et al.*, 2001). 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 (Shuttlesworth *et al.*, 2000; Shetty *et al.*, 2001; Tohda *et al.*, 2001). Possibly, testosterone levels, which will be the highest in the ares bordering on the interstitial tissue, also have a role in regulating stem cell behavior. However, germ cells do not possess androgen receptors so that testosterone can only indirectly affect spermatogonia via peritubular myoid cells or (more likely) Sertoli cells that both express this receptor.

The ratio between self-renewal and differentiation of spermatogonial stem cells being under the control of regulatory mechanisms, the question arises which molecular pathways are involved. Recent data indicate that glial cell line derived neurotrophic factor (GDNF) is involved. Normally, GDNF is secreted by Sertoli cells (Trupp *et al.*, 1995) while a subset of spermatogonia express both receptors for this growth factor, Ret and GFR-alpha1 (Meng *et al.*, 2000). Ectopic expression of GDNF in spermatogonia induces

the formation of large clusters of single type A spermatogonia, while normal spermatogenesis is suppressed. 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). GDNF deficient mice die during the first postnatal day (Pichel *et al.*, 1996) whereas heterozygotes survive. In heterozygotes, spermatogenesis deteriorates with age as germ cells become depleted (Meng *et al.*, 2000). It was concluded that GDNF has a role in the regulation of self-renewal and differentiation of spermatogonial stem cells (Fig. 1). 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.

Another interesting recent finding in this field is that in the classical spontaneous mouse mutant *luxoid*, adult males exhibit a progressive loss of spermatogonial stem cells (Braun *et al.*, 2001). Apparently, the as yet unknown gene(s) involved in this mutation also has a role in the regulation of spermatogonial stem cell renewal and differentiation (Fig.1).

Spermatogonial Cell Lines

Rat spermatogonial stem cell lines have been established by immortalizing isolated type A spermatogonia by transfection with pSV3-neo (containing the SV40 large T-antigen; van Pelt *et al.*, 2002). The cell lines express germ cell characteristics, have the appearance of A spermatogonia but lack c-kit expression. Through over a 100 passages the cell lines proved to be stable. Furthermore, the cell lines were able to colonize a host mouse testis after transplantation, indicating their stem cell capacity.

Another recently described cell line is one developed by Feng *et al.* (Feng *et al.*, 2002). In this case A spermatogonia were immortalized by telomerase transfection. This cell line is c-kit positive and can be induced to differentiate up to spermatids by adding stem cell factor to the culture. It still has to be established whether or not this cell line has stem cell properties as several reports indicate that spermatogonial stem cells are c-kit negative (Schrans-Stassen *et al.*, 1999; Shinohara *et al.*, 2000).

Recently, a fetal gonocyte cell line has been established by cotransfection of rat gonocytes with pSV3-neo and LTRp53cGg (containing a temperature sensitive p53; Hofmann *et al.*, 1994). This cell line shows molecular characteristics of fetal male germ cells and at the permissive temperature is able to differentiate as seen by morphological nuclear changes (van Pelt *et al.*, unpublished; Fig. 2).

Concluding Remarks

While until recently the emphasis of spermatogonial research was more on the regulation of the A_s, A_{pr} and A_{al} spermatogonia as a group, now specific data on the molecular regulation of spermatogonial stem cell behavior are rapidly emerging. GDNF and its receptors, and the gene involved in the *luxoid* mutation, seem directly involved in the regulation of stem cell renewal and differentiation. Furthermore, testosterone clearly

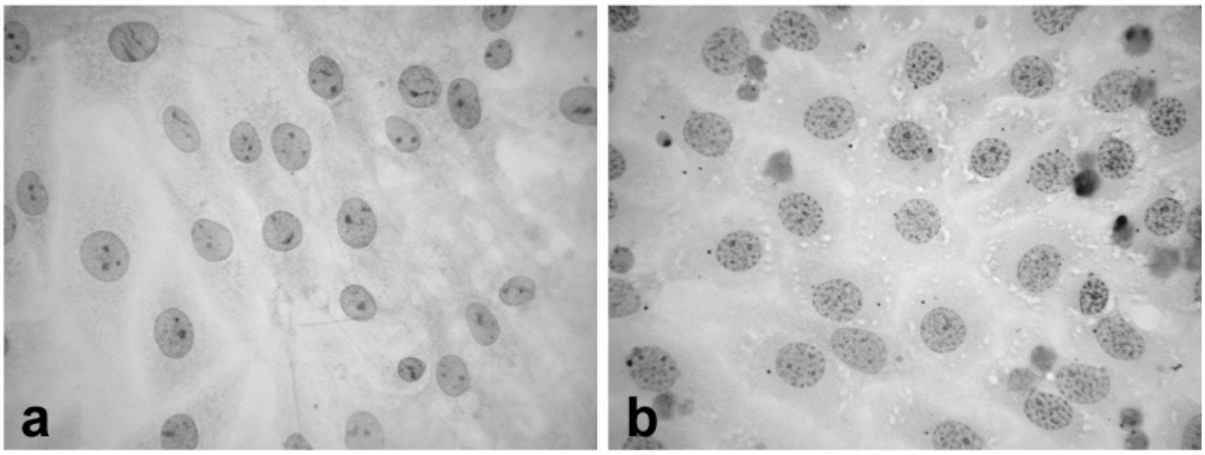

Figure 2. Pas/hematoxylin staining of a gonocyte cell line before (a) and after differentiation (b). Note the changes in the nuclear morphology of the cells.

has an indirect role and may even be responsible for the intriguing fact that spermatogonial stem cells are preferably present in those areas of seminiferous tubules that border on interstitial tissue. These findings together with the possibility to do functional tests for stem cell potential by way of the spermatogonial stem cell transplantation technique, as well as the progress that is recently made in developing culture techniques open this field for new approaches to establish the regulatory mechanisms that govern spermatogonial stem cell renewal and differentiation.

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