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Spermatogonia: origin, physiology and prospects for conservation and manipulation of the male germ line

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Abstract. In recent years, the scientific community has become increasingly interested in spermatogonia. Methodological breakthroughs, such as germ cell transplantation and spermatogonial culture combined with novel germ line transfection strategies, have provided interesting new opportunities for studying the physiology of spermatogonial stem cells and their interaction with the stem cell niche. Furthermore, intense research into pluripotent and adult stem cells has generated new insight into the differentiation pathway of germ line stem cells and has opened new perspectives for stem cell technologies. The present review briefly introduces the physiology of spermatogonial stem cells and discusses future directions of basic research and practical approaches applicable to livestock maintenance and animal reproduction.

Origin of spermatogonia in ontogenesis

Primordial germ cells can be detected at a very early stage of ontogenesis owing to morphological and biochemical differences between somatic and germ cells. Primordial germ cells can be first detected in embryo-associated but extra-embryonic tissues during early ontogenesis. In mice, the primordial germ cells are located in the yolk sac and, only after nearly all other vital tissues of the embryo have been formed, the primordial germs cells start to migrate into the area of the genital ridge (McLaren 2003; Fig. 1). After migration into the undifferentiated gonads, the primordial germs cells differentiate into female or male germ cell precursors, depending on the sexual gonadal differentiation (Capel 2000). A non-reversible decision is made when these cells either enter meiosis to become oocytes or when they arrest mitotically while the formation of the seminiferous cord is initiated to form a testis. Cord formation appears to be the most decisive event allowing primordial germ cells to differentiate into male germ line stem cells. The germ cells resume proliferative activity at later stages of testicular development and give rise to millions of differentiating germ cells throughout adulthood.

Fig. 1. Migration of the primordial germ cells (PGC) in the mammalian embryo at different developmental stages. (Reprinted with permission from Fioroni 1987.)

Initiation of spermatogenesis from stem cells

In the testis of adult mammalian species, spermatogonial stem cells maintain their numbers by self-renewal and give rise to differentiating germ cells. Most of the diploid germ cells are differentiating spermatogonia undergoing several rounds of mitotic divisions before entering meiotic prophase. In all mammals, undifferentiated spermatogonia maintain a stable population and derive differentiating spermatogonia, which finally enter meiosis and give rise to spermatocytes. Basic features of spermatogonial subtypes and their proliferation have been described for most species. These data were obtained mainly from histological cross-sections. The behaviour of spermatogonial stem cells has been analysed in whole mounts of testicular tissue. In non-human primates, seven different types of spermatogonia have been identified (Clermont and Leblond 1959; Clermont 1969; Clermont and Antar 1973; Kluin *et al*. 1983; Fouquet and Dadoune 1986; Zhengwei *et al*. 1997): the reserve stem cell A_{dark} spermatogonium, the renewing stem cell A_{pale} spermatogonium, the intermediate $A_{transition}$ spermatogonium and four generations of B spermatogonia, namely B_1, B_2, B_3 and B_4. Although the morphological descriptions of undifferentiated and differentiating spermatogonia have long been presented, only our

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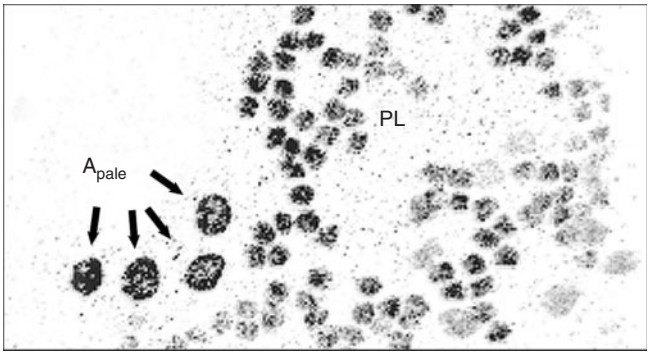

Fig. 2. Bromodeoxyuridine (BrdU)-positive A_{pale} spermatogonia (A_{pale}) and preleptotene spermatocytes (PL) in stage VII of the seminiferous epithelium of an adult rhesus monkey (image converted from a fluorescent micrograph taken from a whole mount of a seminiferous tubule).

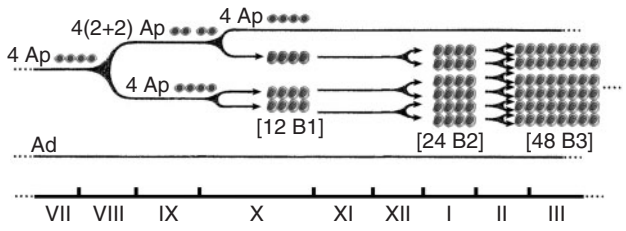

Fig. 3. Expansion of spermatogonial clones from spermatogenic stages VII–III in the adult rhesus monkeys.

own recent studies in non-human primates have demonstrated unequivocally that proliferating spermatogonia are arranged in clones that are fully synchronised and connected by cytoplasmic bridges (Fig. 2). Every 10.5 days, the renewing A_{pale} spermatogonia form small clones that split after this initial mitotic event (Ehmcke *et al*. 2005*a*). In contrast, the clones of differentiating spermatogonia maintain their clonal integrity during the subsequent divisions. Thus, premeiotic germ cells form increasingly larger clones. Finally, a large number of clonally arranged preleptotene spermatocytes enters meiosis (Ehmcke *et al*. 2005*a*, 2005*b*; Fig. 3). These data show that the expansion of stem cells proceeds in a clonal growth pattern that cannot be easily explored in tissue sections. So far, the description of spermatogonial stem cells, as well as the proliferative pattern of premeiotic germ cells, of domestic species has been described almost exclusively in tissue sections. Therefore, more basic research is needed to answer some of the relevant questions, including: what are the exact mechanisms of premeiotic germ cells expansion in domestic species? How often do the differentiating spermatogonia divide? How often do stem cells turnover? Which cell type is the true stem cell and which is the proliferating precursor? Answering these questions is difficult because astonishing differences exist between even closely related species, indicating that the mechanisms of stem cell turnover and the kinetics of pre- and post-meiotic germ cell development are quite adaptable for each species.

Spermatogonia: target for animal conservation?

As a clinical diagnosis, complete depletion of testicular stem cells is defined as Sertoli cell only syndrome (SCO). A partial depletion of testicular stem cells induces a focal SCO. Spermatogenic recovery after irradiation follows an all-or-nothing pattern in primates (de Rooij *et al*. 2002; Schlatt *et al*. 2002*a*; Kamischke *et al*. 2003). This finding indicates a critical role for spermatogonial stem cells in the restoration process. Functional proof for this is provided from germ cell transplantation experiments in mice and monkeys revealing that continuous recolonisation of remaining or reintroduced testicular stem cells leads to reinduction of spermatogenesis (Nagano and Brinster 1998; de Rooij *et al*. 2002; Kamischke *et al*. 2003). Therefore, spermatogonia are considered the selective target cells for the preservation of testis tissue and function, as well as for the development of novel options for fertility preservation in oncological patients (Nordhoff and Schlatt 2003; Orwig and Schlatt 2005). In the future, spermatogonial stem cells may be reintroduced into the testis of oncological patients to re-induce spermatogenesis (Radford *et al*. 1999; Radford 2003; Wallace and Thomson 2003). However, in domestic species, the prime interest with regard to testicular stem cell technology is not a cure of infertility of breeding stock or to explore the effects of environmental factors on male fertility. It is of prime importance to develop novel strategies to conserve valuable germ line cells and to introduce genomic changes in the male germ line (Hill and Dobrinski 2006).

The clonal expansion of differentiating male germ cells and the recolonisation of spermatogenesis from stem cells indicates that the testicular stem cell and not the differentiating germ cells can be the target cell population when the male germ line is to be conserved or when genomic changes are inserted in the germ line. However, it may be possible to also target other potentially pluripotent cells and/or germ line cells, because it was shown recently in the mouse that even primordial germ cells isolated from the embryonic epiblast or teratocarcinoma cells have the potential to colonise the testis and generate male germ cells (Chuma *et al*. 2005; Nayernia *et al*. 2005). These findings indicate that the testicular microenvironment offers unique niches to germ line cells. Exposure of competent cell types of various origins appears to initiate morphogenetic changes guiding the responsive cells into the male differentiation pathway. Obviously, one of the most important steps in this differentiation pathway is the generation of a new type of stem cell: the spermatogonium. It appears that only after germ line cells have passed the spermatogonial stem cell stage are they able to generate male progeny entering meiosis and differentiating into sperm. The stem cell step is also necessary to colonise the

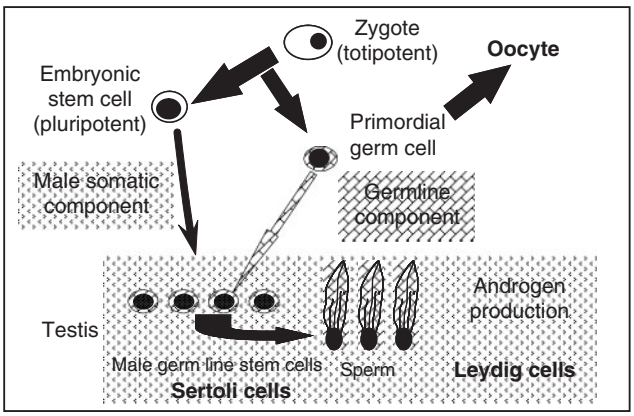

Fig. 4. Importance of the testicular microenvironment for male germ line stem cells. Primordial germ cells appear early during development. When they are exposed to the testicular microenvironment, they differentiate into male germ line stem cells. Otherwise, they give rise to female germ cells.

seminiferous tubules and thereby form sufficient progenitors for the generation of high quantities of male gametes. The testicular microenvironment is unique in allowing germ line cells to enter this differentiation pathway. Figure 4 shows the dependence of the male germ line on entering the testicular microenvironment to initiate the male differentiation pathway.

As was shown previously, primordial germ cells in culture dishes are capable of forming oogonia and follicle-like structures (Hubner *et al*. 2003). Interestingly, *in vitro* maintenance of premeiotic male germ cells alone or in coculture with feeder cells does not lead to generation of elongating male gametes, although, in some studies, several markers indicate some degree of meiotic progression (Creemers *et al*. 2002; Feng *et al*. 2002; van Pelt *et al*. 2002; Nagano *et al*. 2003; Geijsen *et al*. 2004). However, transplantation of these germ cells back into the testis allows the generation of male gametes (Nagano *et al*. 2003; Toyooka *et al*. 2003). We conclude from these findings that a variety of different totipotent and germ line cells are capable to enter meiosis under *in vitro* conditions but that male germ cell differentiation occurs exclusively in the intact testicular microenvironment. Preliminary experiments using mouse embryonic germ cells that were carrying the green fluorescent protein (GFP) transgene under control of the Oct4 promoter (the same Oct4-positive cells that were used in the study of Hubner *et al*. 2003) in coculture with immature mouse Sertoli cells revealed that these undifferentiated germ cells can obtain a male phenotype (Fig. 5). Initially, the embryonic germ cells only survive in contact with the Sertoli cells under conditions of serum-free and growth factor-free media. The germ cells grow in small clusters, often located on top of the Sertoli cell feeder layer (Fig. 5*a*). After several days, a few GFP-positive germ cells migrate from these clusters into the cord-like structures that are formed by Sertoli cells after prolonged culture periods (Fig. 5*b*; Schlatt *et al*. 1996). After the embryonic germ cells have migrated into the cord-like structures, they colonise large areas of the culture dish (Fig. 5*c*). When the culture dishes are coated with extracellular matrix, the embryonic germ cells show a different migration and localisation pattern. Under these culture conditions, they are observed exclusively at the periphery of the cord-like structures (Fig. 5*d*). We interpret these findings to indicate the ability of embryonic germ cells to respond to a new microenvironment. Interestingly, when these Sertoli cell–germ cell fragments were grafted into nude mice after several weeks of culture, we found that they generated teratocarcinomas, indicating that the transformation of cells into spermatogonia has not been completed in all cells or that the pluripotent differentiation pathway has not been terminated under these culture conditions. We conclude from these experiments that Sertoli cells provide specific niches to which a subfraction of embryonic germ cells can respond. The responsive cells then differentiate into spermatogonial-like cells. Once the germ cells pass through the bottleneck of recognising the new microenvironment, they can expand and colonise all niches offered in this tissue.

It remains to be investigated which other types of germ line and pluripotent cells other than spermatogonia (primordial germ cells, embryonic stem cells, embryonic germ cell, teratocarcinoma stem cells) are able to initiate spermatogenesis in the testicular microenvironment. Recent findings in the female mouse indicate that even adult stem cells isolated from bone marrow may be competent to enter the germ line and generate gametes (Johnson *et al*. 2005). In the future, the isolation of germ line-competent cells from sources other than the testis may render it possible to even use female cell preparations as donor cell preparations for germ cell transplantation and the re-initiation of spermatogenesis. If this becomes a valid new option, a selective generation of female offspring may be achievable from a breeding male that carries exclusively XX-containing germ line stem cells.

As an alternative to germ cell transplantation, testicular grafting has been shown to be a successful approach for the initiation of spermatogenesis and to generate sperm after dissection of testicular tissue from immature donors (Honaramooz *et al*. 2002; Schlatt *et al*. 2002*b*, 2003). This technique has already been successfully combined with prior cryopreservation of immature testicular tissue (Schlatt *et al*. 2002*b*; Shinohara *et al*. 2002; Orwig and Schlatt 2005). Testicular grafting is applicable to domestic species and provides an interesting option for conservation of the male germ line (Dobrinski *et al*. 2000; Dobrinski 2005). In principle, this technique offers a preservation of the male germ line stem cell together with its niche. Because the tissue is able to recover in the host up to the level of full spermatogenesis, grafting offers an easy tool to maintain the germ line of valuable livestock before it reaches puberty or generates sperm.

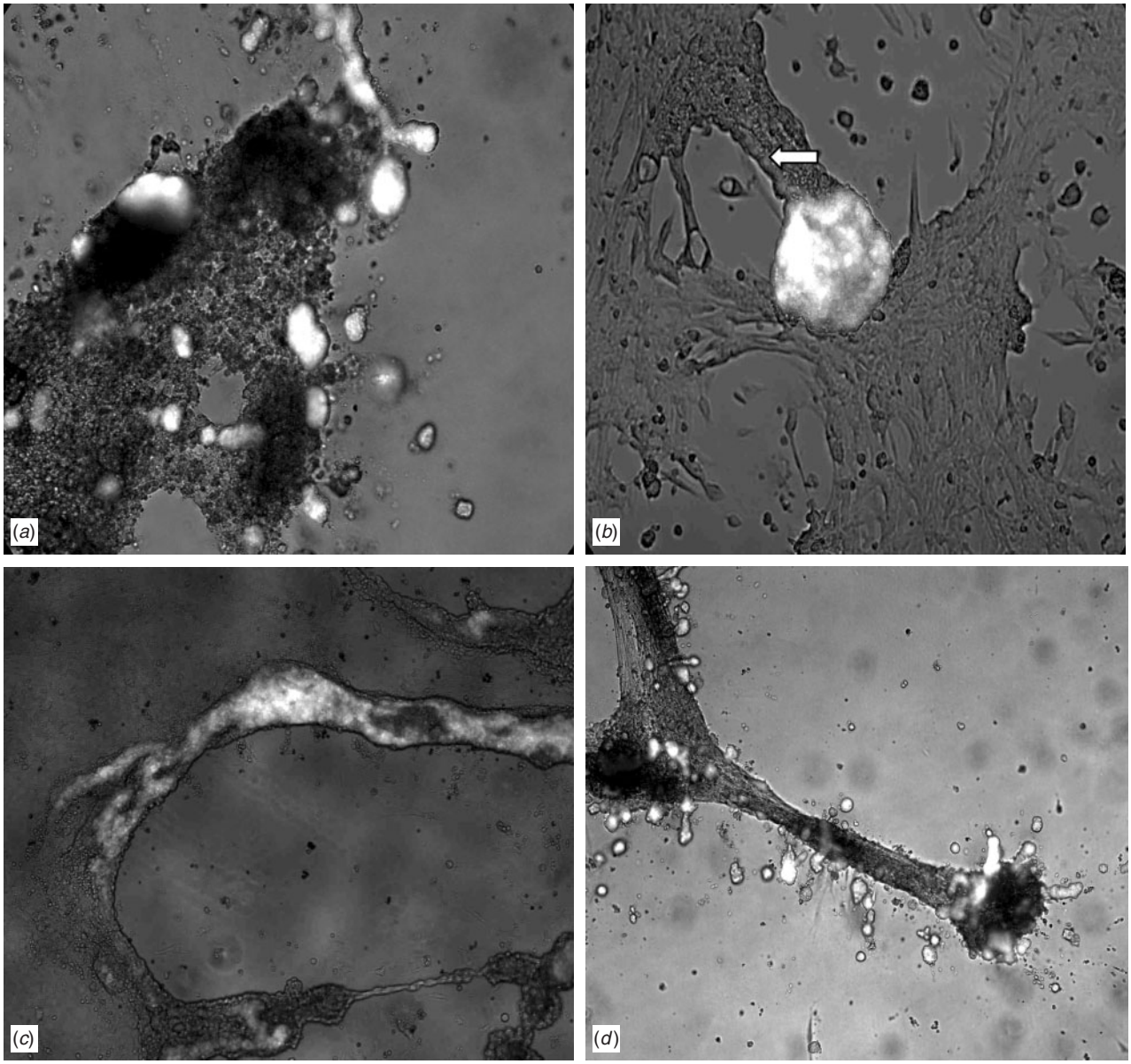

Fig. 5. Male germ line differentiation of embryonic germ cells in coculture with testicular stem cells. The germ cells express green fluorescent protein (GFP) under control of the Oct4 promoter (bright signal), allowing them to be identified as long as they maintain their pluripotent or germ line status. The embryonic germ cells were cocultured with Sertoli cells from 5-day-old standard outbred mice. (*a*) The Oct4-positive germ cells form small clones in contact with Sertoli cells during the first days of culture. (*b*) Germ cells migrate out of the clusters (arrow) and start colonisation of the cord-like structures formed by Sertoli cells after several days of culture. (*c*,*d*) Embryonic germ cells colonise most of the tubule-like structures after several weeks of culture. The localisation pattern of germ cells is either internal when the cells are grown on laminin-coated coverslips (*c*) or at the periphery of the cord-like structures when the cells are cultured in the presence of extracellular matrix (*d*).

In summary, spectacular new findings on pluripotent cells and their capacity to transform and differentiate into different cells of the germ line offer unexpected new opportunities for germ line conservation and manipulation. In the near future, these findings will be combined with optimised cell culture and transfection strategies that may lead to new pathways for the introduction of genes into the germ line and improved strategies for the selection of targeted germ line cells. New strategies, such as germ cell transplantation and testicular grafting, generate a wide spectrum of scenarios by which gonadal cells and germ line cells can be preserved and restimulated to generate sperm. Although the safety and implications of any procedure that converts the DNA obtained from somatic cells or pluripotent stem cells into gametes has

to be explored, these tools open completely novel scenarios for the generation of gametes and may have enormous impact on the future development of animal production.

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