Spermatogonial stem cells in the bull

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Spermatogoniale stamcellen in het rund

(met een samenvatting in het Nederlands)

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door

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geboren op 12 december 1965 te Anaco, Venezuela

Promotor : Prof.dr. D.G. de Rooij

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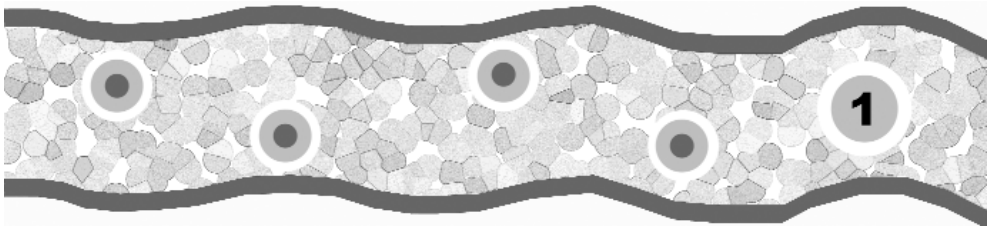

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General structure of the testis

The testis is the male gonad, responsible for producing both sperm and male hormones and it is located outside of the body cavity in most mammals [1]. This organ has an external capsule of connective tissue (tunica albuginea) that in the bovine emits septa inwards to create divisions called lobules. Inside each lobule there is a single highly contorted tube or seminiferous tubule, where sperm formation takes place (Figure 1). Among the seminiferous tubules there is the interstitial space containing loose connective tissue, lymphatic and blood vessels and various types of cells (mainly macrophages and Leydig cells). For a comprehensive review on testis structure see Russell et al., 1990 [1].

Sperm produced in the seminiferous tubules is conveyed to a central net of tubules or rete testis, which is embedded in a centrally located connective tissue area called mediastinum testis. Sperm leave the testis through a series of exit ducts (efferent ducts) and subsequently continue traveling through the ductus epidydimis, ductus deferens and finally the urethra (see Figure 1).

Spermatogenesis

Spermatogenesis is the process of sperm production taking place within the seminiferous tubules. Millions of sperm are produced on a daily basis and the process generates many more than needed, normally one sperm being enough to successfully fertilize an egg as part of the sexual reproduction strategy in higher animals. Spermatogenesis includes a series of cell divisions which begins at the level of the spermatogonial stem cells (SSC) and many subsequent steps, ultimately leading to the formation of spermatozoa, the highly specialized, differentiated gametic cells. The continuous changes taking place in the differentiating cells are rather radical. While differentiating type A spermatogonia, with a morphology similar to that of SSC, have a big round shaped nucleus with one to three conspicuous nucleoli, sperm areterminally differentiated, slim cells. The extreme specialization of spermatozoa includes tight packaging of the chromatin in the nucleus, presence of a motility apparatus, a metabolism suitable for energy production, the capacity to survive autonomously and the ability to penetrate the barriers surrounding the egg upon fertilization. Most steps of spermatogenesis involve complex events and changes and it can be considered as a lengthy process.

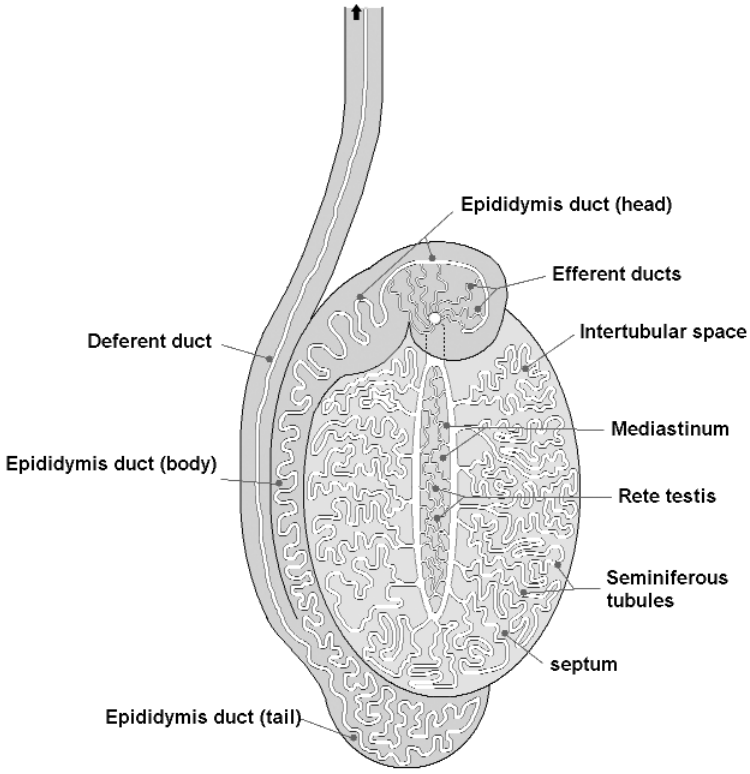

Fig. 1. Schematic view of the bovine testis. Tubular structures and excurrent ducts belonging to the parenchyma of the organs (testis and epidydimis) are shown. The location of stromal elements like septa and mediastinum is shown but not depicted in detail.

Spermatogenesis yields high numbers of spermatozoa and, since the quality of their genetic material is crucial to future generations, the maintenance of DNA integrity is of fundamental importance. Therefore, special mechanisms exist to guarantee that the DNA replicates faithfully and when errors do occur, the affected germ cells are destroyed by entering apoptosis (programmed cell death) [2]. Defective sperm can also be destroyed by macrophages either in the male or in the female genital tract [3]. Clearly, besides quantity, quality is also an important goal in spermatogenesis.

A crucial part in the process of spermatogenesis is meiosis. The main goals of the meiotic divisions are the increase of genetic variation through the exchange of parental genes and the reduction by half of the number of chromosomes. By this mechanism, premeiotic cells, which are

diploid (two sets of chromosomes) become haploid (cells with one set of chromosomes).

Spermatogenesis is an accurate process in terms of time and spatial arrangements. Two concepts are important to understand its underlying mechanisms: *the cycle of the seminiferous epithelium and the spermatogenic wave*. In any given segment of the seminiferous epithelium, a specific combination of one to two generations of particular types of spermatogonia, spermatocytes and spermatids can be seen in a tubular cross-section. Each of these specific arrangements constitutes a reference set, called stage. Spermatogenesis advances in such a way, that these stages succeed one another in a strict order. The sequence of events between the disappearance of a specific cell association in a particular area and its reappearance in the same area has been called the cycle of the seminiferous epithelium [4-7], Figure 2, A. The duration of the cycle should not be confused with the duration of spermatogenesis, which is the time it takes for a SSC to produce a generation of sperm. It takes about 4.5 seminiferous cycles for this to happen in mammals [7, 8]. For example in the mouse, the cycle duration is 8.6 days [9]. Therefore, spermatogenesis can be estimated to last 39.6 days. In the bull, the duration of the spermatogenic cycle is 13.5 days, and spermatogenesis lasts 61 days (61 days / 13.5 days = 4.5) [7].

Furthermore, the subsequent epithelial stages follow each other in sequence along the seminiferous tubules, although sometimes the sequence may reverse in direction. This phenomenon has been called the spermatogenic wave [4, 8], Figure 2, B. Summarizing, cycle is to time what the wave is to space. Seen from a different angle, spermatogonia committed to differentiate start proliferating at different subsequent times along the length of the seminiferous tubules. Taken together the testis contains tubule segments in all epithelial stages at any one point in time allowing a continuous flow of sperm out of the testis.

Spermatogonial stem cells

In general, stem cells are cells that can either divide into daughter cells committed to differentiate or into new stem cells to maintain the original pool size. A stem cell can divide and produce a daughter stem cell and a differentiating daughter cell, an event called an asymmetric division. On the other hand, when one stem cell produces two daughter stem cells while another stem cell generates two differentiating daughter cells, the divisions can be termed symmetric [10]. It is currently not known which of these theoretical mechanisms of stem cell division actually takes place

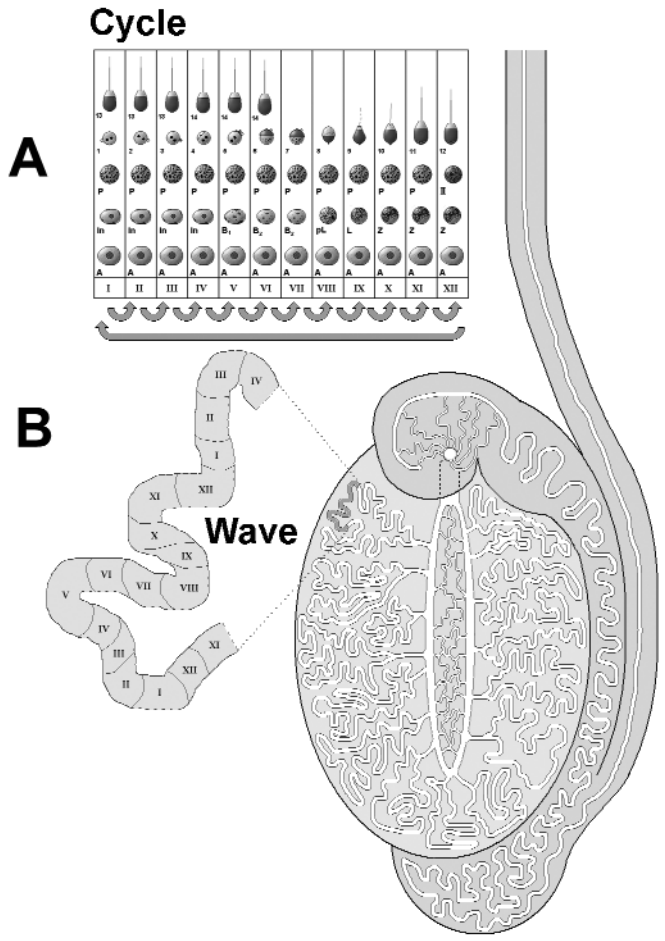

Fig. 2. Spermatogenetic cycle and wave. A. In a given time and area of a seminiferous tubule there will always be one specific combination of germ cell types called stage or association. There are 12 stages in bulls and a given segment of seminiferous tubule will go through the 12 stages and then repeat the process again. This is called the cycle of spermatogenesis. B. Stages proceed one another in an orderly manner in the length of the seminiferous tubule. Twelve stages scheme is modified from Berndtson and Desjardins, 1974 [5].

in tissues. Stem cells are rare and usually located in areas of tissues where nutrients and protection is secured. This special microenvironment is called the niche [10-13]. A broad body of knowledge about this is based on the study of lower organisms because many of the regulating mechanisms of these cells seem to be well conserved [11, 12, 14]. Also important related information comes from several mammalian stem cell systems, the hematopoietic system perhaps being studied the most.

Stem cells in the male germ line are called spermatogonial stem cells (SSC). These cells are considered to be single cells derived from gonocytes at the start of spermatogenesis, which occurs postnatally during the prepubertal period [7, 15]. SSC sustain the male germ-line lineage and are

located in the basal compartment of the seminiferous tubules [1, 7, 15-17]. They are the only stem cells in the male that carry genetic information to next generations [16, 18-20]. As any other stem cells, SSCs cycle slower than non stem cells and do not show many phenotypic characteristics that could serve as a means of identification. However, some morphological characteristics and specific topographic arrangements have been observed for early type A spermatogonia (A_s, A_{pr} and A_{al}) using specific conditions of histological processing [21]. Accordingly, green fluorescent protein labeled, "undifferentiated" spermatogonia have been traced to a preferred area near interstitial blood vessels [22]. Moreover, some markers have been identified which considerably boosted the study of the physiology of SSC. Some of the most important markers are summarized in table 1.

Role of Sertoli cells in spermatogenesis

Sertoli cells are somatic cells that are responsible for sustaining the germ cells. They are a key element in the tight regulation of the highly organized progression of spermatogenesis. Sertoli cells produce factors that affect the balance between differentiation and self-renewal of the SSC pool and the maturation of germ cells during their development to become sperm. In spermatogenesis there exists a complex molecular cross-talk to which germ cells also contribute with substances that feed-back to Sertoli cells [1, 40, 41]. For example, FGF2 is produced by germ cells and targeted to adult Sertoli cells which in turn increase their gene activity to produce FSH receptors [41], plasminogen activator [40, 41] and sulfated glycoprotein 1[41].

FGF receptors are found in Sertoli cells [42, 43]. Furthermore, FGF2 was found in pachytene spermatocytes and conditioned medium from cultures of these cells increased the production of transferrin by Sertoli cells [44]. The germ cells responsible for the induction of Sertoli cells to produce plasminogen activator, seems to be preleptotene spermatocytes. Interestingly, plasminogen activator is involved in the loosening of tight junctions, an important component of the blood-testis barrier, during preleptotene cell translocation to the adluminal compartment of the seminiferous tubule. Another classical example of germ cell signaling to Sertoli cells is the paracrine circuit of sex hormone-binding globulin (SHBG), former androgen binding protein (ABP). Sertoli cells increase the secretion of SHBG when exposed to germ cell conditioned medium with or without FSH [40].

Table 1.Overview of markers used to identify spermatogonial cell types

As and Apr	GFRA1 [23-25]
As, Apr and Aal	PLZF [26,27] OCT4 [28], NGN3* [29], NOTCH-1 [23], SOX3 [30], c-RET [31], CDH1 [32]
A spermatogonia	RBM [33]
Spermatogonia	EP-CAM [34]
Pre-meiotic germ cells	STRA8 [35], EE2 [36]
Cells on basal membrane and interstitium	CD9 [37]
Spermatogonia, spermatocytes and round spermatids	GCNA1 [38]
Premeiotic spermatogonia and postmeiotic spermatids	TAF4B [39]

However, a putative SHBG stimulator factor is yet to be found. Besides the exposure to soluble factors, the physical contact between Sertoli cells and germ cells is also important, to an extent that it can originate physiological regulatory responses. This role of Sertoli cells is facilitated by their extensive contact with germ cells, and Sertoli cell cytoplasm spans the whole radial axis of the seminiferous tubule (from the basal lamina to the lumen) while branching out cytoplasmic extensions from their long axis. Carbohydrate complexes have been found to play an important role in specific germ-Sertoli cell recognition areas, mainly in the form of glycans, and when absent, spermatogenesis becomes impaired [45]. Sertoli cells maintain tight junctions with one another that seal off the more advanced germ cells from the basal compartment, hiding them away from the immune system since these postmeiotic germ cells with recombined genetic material, produce and show surface antigens which are foreign to the body, being therefore autoantigenic [40, 46]. The tight junctions between Sertoli cells constitute the main anatomic basis of the blood-testis barrier [47-49]. Spermatogonia remain in the basal compartment in contact with the basal lamina. For extensive information on Sertoli cell physiology see Griswold, 2005; Hess and Franca, 2005 [50, 51].

As in other stem cell systems, there is also a molecular microenvironment or niche for stem cells in the testis. This niche is provided by Sertoli cells, particularly those in the vicinities of the

interstitial tissue [22] where a delicate equilibrium exists in which SSC are able to maintain their stem cell character [14, 52]. Thus, SSC are localized on the basal membrane, surrounded by Sertoli cells. It has been established that SSC are preferentially situated in those areas of the seminiferous tubules that border on the interstitial tissue but they are intermixed with other similar looking spermatogonia already committed to differentiate [1, 53-55]. The differentiating spermatogonia belong to the so called transit amplifying pool [56].

Comparison of rodent and bovine spermatogenesis

Rodents have always been the classical model to study spermatogenesis in mammals. In rodents, the male germ cell lineage starts at the level of type A-single spermatogonia (A_s) which are the SSCs [16, 29, 57]. Figure 3 shows the developmental path of differentiating germ cells up to the formation of spermatozoa. A-single spermatogonia divide into daughter cells that remain interconnected by cytoplasmic bridges after incomplete cytokinesis and continue to do so after each subsequent division [16, 58, 59] (Figure 3). Assuming no cell death occurs, the total theoretical number of daughter cells arising from one progenitor cell (x), can be calculated by the formula $x = 2^n$, where n is the number of cell divisions taking place during the process. For instance, in mice and rats there are 9 to 10 spermatogonial divisions and two meiotic divisions and hence from one SSC, 4096 spermatozoa can be generated.

Clones from 4 cells or more have the appearance of chains and continue to be joined by cytoplasmic bridges. Therefore, they are called aligned (A aligned or A_{al}) spermatogonia that keep proliferating until a remarkable differentiation step occurs, consisting of a transformation into A_1 spermatogonia without a cell division. A_1 spermatogonia are believed to be irreversibly committed to differentiate (Figure 3) [1, 16] which for A_{pr} and A_{al} spermatogonia remains a matter of debate [57]. A series of six divisions follows the transition of A_{al} into A_1 spermatogonia. Thus, there are several generations of differentiating A spermatogonia (A_1 through A_4) and finally A_4 spermatogonia divide into Intermediate spermatogonia and these into type B spermatogonia. The division of type B spermatogonia into preleptotene spermatocytes marks the end of spermatogonial multiplication being a transit-amplifying phase of cell production in the in the spermatogenic lineage. Preleptotene spermatocytes are the last cells to duplicate their DNA and subsequently enter the process of meiosis [1]. Meiosis is a very complex, not yet fully understood

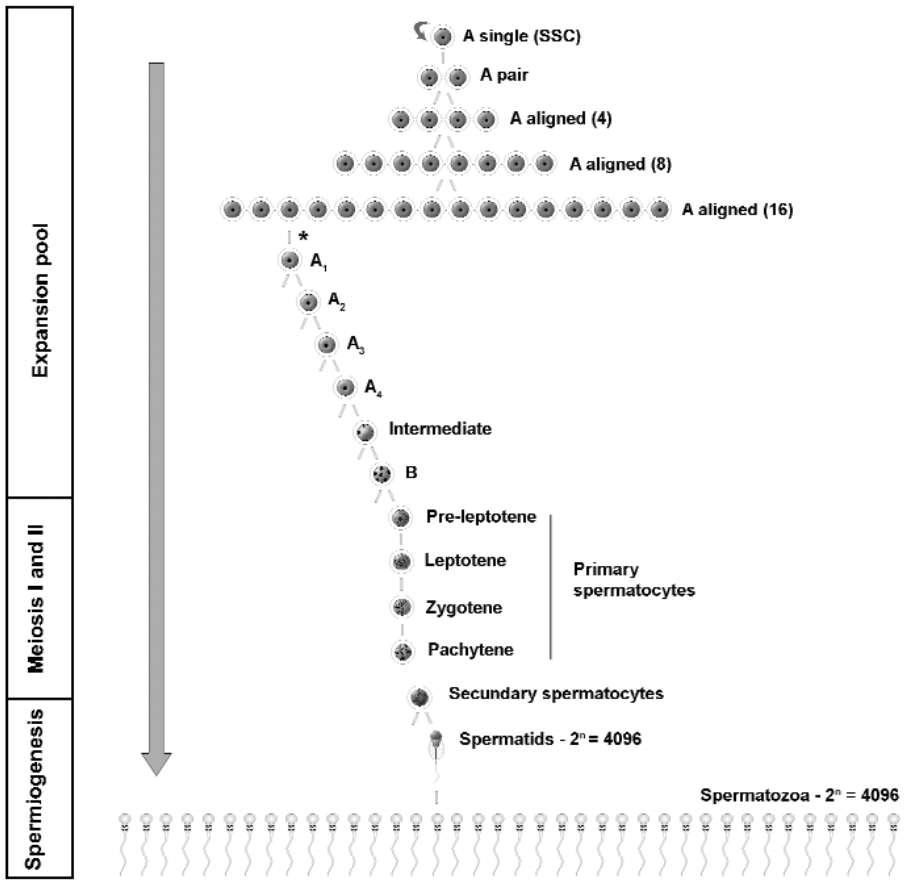

Fig. 3. Schematic progression of cell divisions and steps of differentiation leading to the formation of spermatozoa in the testis. Clones of 32 type A aligned spermatogonia are rarely seen and those of 64 virtually do not exist. (*)= differentiation step (with no cell division).

process. For a comprehensive review on mammalian male meiosis see Kleckner, 1996 [60]. There are two meiotic divisions during spermatogenesis. Meiosis I has a very lengthy prophase, and various developmental prophase steps can be morphologically identified (leptotene, zygotene, pachytene and diplotene). Afterwards, primary spermatocytes enter the first meiotic division and render secondary spermatocytes. The latter cells quickly enter the second meiotic division to produce spermatids, which are at first round-shaped and then undergo a transformation through a process called spermiogenesis, that finally reshapes them into sperm.

At birth the seminiferous tubules only contain Sertoli cells and fetal germ cells, called gonocytes. At some point of postnatal development some gonocytes directly form A_1 spermatogonia (a time-point called the start of spermatogenesis), which proliferate and develop into progressively more differentiated cell types already mentioned above, produce spermatocytes that carry out meiosis, give rise to spermatids and eventually the first sperm is formed at puberty (for a general review on the morphology of germ cells, see Russell et al., 1990 [1], for specific details on the morphology of bovine germ cells see Berndtson and Desjardins, 1974; Abdel-Raouf, 1960; Curtis and Amann, 1981 [5, 61, 62]). From this moment onwards, the cycle of the seminiferous epithelium becomes normally established and continues steadily, increasing its efficiency as the animal becomes sexually mature. In the prepubertal bull the start of spermatogenesis also seems to originate from gonocytes that become A_1 spermatogonia [55]. Although the kinetics of spermatogonial multiplication in bovine species have been described [55], more studies involving seminiferous tubule whole mount preparations should be carried out, as the bovine model will not likely be fundamentally different from that in the ram, a ruminant species as well. In the ram spermatogonial multiplication and stem cell renewal is very similar to that in rodents [63].

One available bovine model postulates three types of spermatogonial precursors: basal spermatogonial stem cells (BSC), aggregated spermatogonial precursor cells (ASPC) and committed spermatogonial precursor cells (CSPC) [55]. BSCs are stem cells equivalent to A_s. They can either renew themselves through low frequency divisions all along the cycle of the seminiferous epithelium or continue into the differentiating path, rapidly dividing to produce ASPCs during stages I through IV while remaining together as clusters. ASPCs are the equivalent to A_{al} spermatogonia in the rodent model and represent the transit amplifying cells among the early spermatogonial population. At the end of this propagating phase, ASPC cells separate from each other and appear intermixed with differentiating spermatogonia ($A_1 – A_4$) and intermediate spermatogonia. ASPC cells are negative for proliferation markers at this time and rather go through a growing phase (V through VIII) to transform (stages VII, VIII and starting of I) into A_1 spermatogonia that are irreversibly committed to differentiate and are subsequently called CSPCs (committed spermatogonial precursor cells). These authors postulate as well, that there is a flexible pattern of bovine type A spermatogonia propagation because A_1 formation is not strictly synchronized in a given area of seminiferous tubules. Hence, there can be 2 to 3 divisions of differentiating spermatogonia

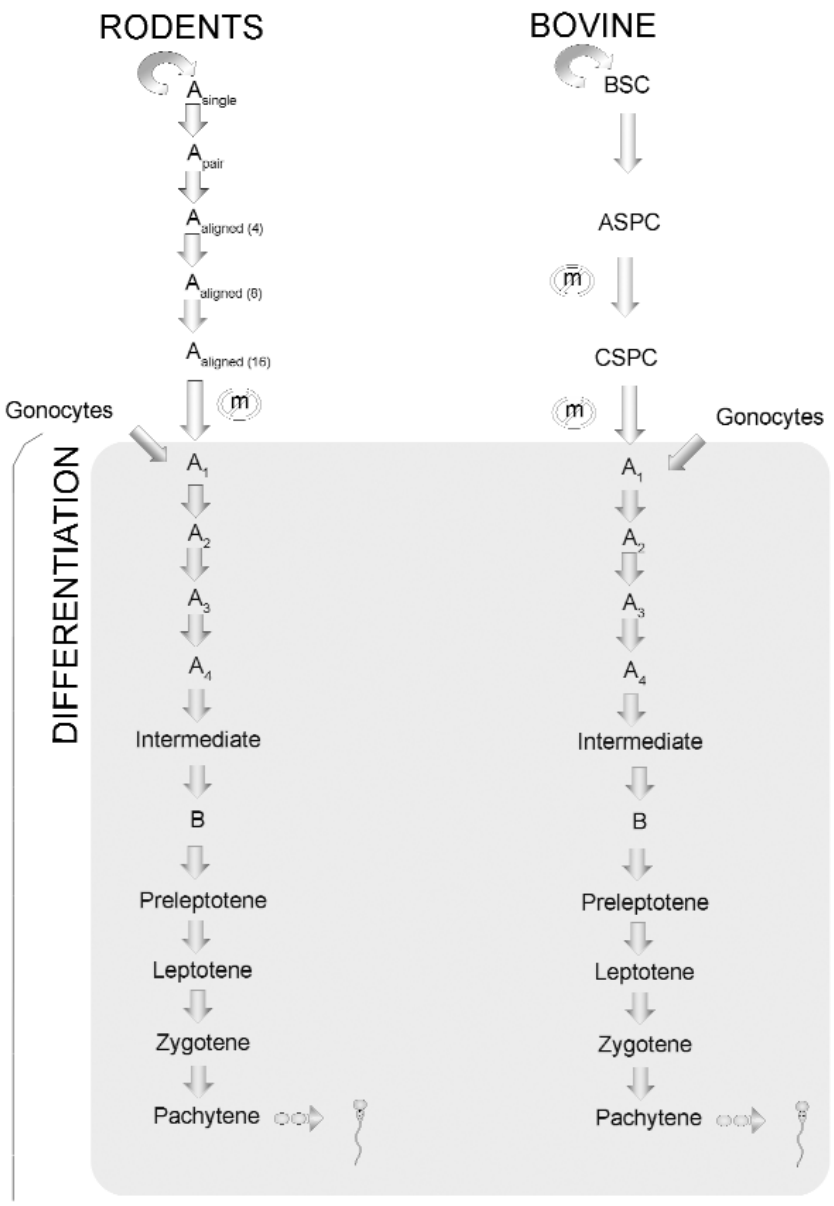

Fig 4. Comparison of the developmental paths in the progression of spermatogenesis in rodents and bovine species (rodent scheme [17, 57]; bovine scheme[55]).

(CSPC) during one cycle of the seminiferous epithelium, depending on the duration of cell division of A_1 to produce either A_2 or A_3 [64]. In prepubertal bulls, BSC divide directly into CSPC, similarly as in the mammalian general model. (Figure 4) [55].

In vitro systems for spermatogonial stem cell expansion

Short term culture of primary spermatogonial stem cells

So far, two factors have hindered the initial efforts to culture spermatogonial stem cells. First, the lack of specific markers to identify spermatogonial stem cells, and second, the limited knowledge on nutritional and growth factor requirements for this specific cell type. Attempts at isolation and culture of testicular cells were already conducted during the 1970's [65-70]. Two types of culture systems were used in the development of a protocol for spermatogenesis in vitro, namely testis organ culture and culture of isolated cell suspensions. Although the first seemingly is the most attractive one because the testis cytoarchitecture is preserved and physiological conditions are emulated [71-73], it has the inconvenience of providing a highly complex environment in which the effects of factors on individual cells remain difficult to interpret. Consequently, the approach of culturing isolated germ cells has been more widely used.

Initial attempts of isolating spermatogonial stem cells have started with the isolation of type A spermatogonia (including spermatogonial stem cells) by enzymatic digestion of testicular tissue [74-77]. These efforts have been strengthened with different combinations of techniques such as the use of testes missing more advanced germ cell types and hence, enriched in stem cells (testes from prepubertal [76, 78], cryptorchid [79-81] and vitamin A deficient animals [77, 82]), and techniques to purify A spermatogonia (discontinuous density gradient centrifugation [77], differential plating [24, 76], fluorescent activated cell sorting (FACS) [83, 84] and magnetic activated cell sorting (MACS) [20, 81, 85, 86]). The typical purity of type A spermatogonia at the start of the culture using these methods is 50 to 90 %.

Cryptorchid testes contain only type A spermatogonia as germ cells and it is possible that SSCs can reach concentrations of 1:200, which represents a SSC activity 25-fold higher than in the wild type testis [79]. Enrichment for SSCs can be even more efficient in the cryptorchid model by using FACS, provided appropriate markers are used. For instance, positive selection for α-integrin pro-

vides an enrichment of SSC of 166-fold [83]. Moreover, in experiments using FACS and positive selection for EPCAM in normal rat germ cell suspensions, fractions were produced with 1:13 and 1:8.5 SSCs in suspensions originating from neonates and pups respectively [84].

Short-term cultures, have been carried out with a typical duration of 7 days, in which maintenance (survival and/or proliferation) of A spermatogonia [74, 82, 87], or more specifically SSCs [75, 80, 81, 88], could be achieved, as demonstrated by the transplantation assay, which is the functional test currently available to estimate SSC activity [89, 90]. In this procedure, donor germ cells are transplanted into the testes of a recipient mouse, the endogenous spermatogenesis of which has been depleted either because of the Wv/Wv mutation, by treatment of the mice with the alkylating agent busulfan, or alternatively by local testicular X-irradiation [91]. After transplantation, the donor spermatogonial stem cells repopulate the seminiferous epithelium of the recipient mice in focal areas called colonies each of which is assumed to be started by one SSC. Thus, the number of colonies is correlated with the number of original SSC transplanted [92].

Spermatogonial stem cells proliferate during the first week of culture [73, 80, 81, 88, 93, 94], specially during the first two days [82, 94]. Regretfully, in most if not all of these cases, either type A spermatogonia or SSCs, dropped in numbers. Therefore, maintenance but not expansion of the SSC population was possible under general conditions used, such as the presence of medium with serum (FCS or FBS) ranging from 1 to 20%, but more commonly 10% [75, 80-82, 88, 93, 94], temperature of 32 or 37°C in an atmosphere of 5% CO_2 in a humid chamber. Additionally, most systems included some form of a feeder layer to support the germ cells in culture.

Progress to overcome the problem of the decrease in SSC numbers was achieved by considering additional factors favorable for spermatogonial stem cell renewal [20, 81, 93]. Kanatsu-Shinohara et al., introduced an improved culture system based on a commercial medium (StemPro-34® SFM, Invitrogen) and supplementation with various agents, hormones and growth factors including ß-estradiol, progesterone, epidermal growth factor (EGF), fibroblast growth factor 2 (FGF2), leukemia inhibitory factor (LIF) and glial cell line-derived neurotrophic factor (GDNF) [93]. Only when these conditions were met, did mouse germ cells proliferate and form colonies during the first week. Subsequently, after continuous sub-culturing during 4 to 5 months, they achieved an expansion of SSC in the order of 10^{12} fold (see below).

Kubota et al., [20, 81] enriched SSC from cryptorchid mice by fluorescent activated cell sorting (FACS) and/or magnetic activated cell sorting (MACS), using THY1 as a specific marker and these authors cultured the cells in MEM-α medium in the presence of serum, a feeder layer of STO cells, and selected growth factors [81]. They found a positive but not significant effect of GDNF on spermatogonial stem cell proliferation and concluded that probably the serum was masking the effect of the growth factors tested. Thus, they set up a serum free system, and when testing GDNF they managed to maintain spermatogonial stem cells in culture but not to expand the population [20]. The breakthrough came when they additionally included soluble GFRA1 (receptor for GDNF) and Fibroblast Growth Factor 2 (FGF2) achieving an expansion of the spermatogonial stem cell population in the short term (8 to 10 days) [20].

Several other growth factors have been postulated to have a role in spermatogonial stem cell renewal in vitro. However, well sustained evidence from independent laboratories indicate that many of these factors actually have a neutral effect or even reduce the numbers of SSCs. Increasing numbers of SSCs during culture indicates self-renewal. A steady state might suggest survival while a decline in number can be interpreted as stem cell death and/or differentiation (see Table 2).

Long term culture of primary spermatogonial stem cells

To propagate SSC, it is necessary to culture the cells for a long period of time and perform serial sub-culturing. Nagano et. al., [75] showed that testicular cells from neonatal and adult mice could be maintained in culture for as long as 4 months. When cultured on STO feeders and undergoing one passage per month, these cells were able to generate spermatogenesis following transplantation. This indicates the presence of SSCs even after 4 months of culture. Jeong et al., further improved the protocol by adding KITLG, LIF, FGF2, IGF1, IL11, Oncostatin M (OSM) and PDGF to the medium and found the formation of germ cell colonies [94]. After three months of culture and eight passages, these colonies were still able to colonize a recipient testis after transplantation, suggesting the presence and propagation of spermatogonial stem cells.

Kanatsu-Shinohara et al., [93] successfully cultured neonatal mouse testicular cells for periods of five months and more. The germ cells formed colonies during culture that could be passaged and resulted in germline stem cell lines (GS cells) which kept the ability to colonize a recipient

Table 2. Effect of growth factors and other agents on spermatogonial stem cells in culture

Enhancement	Steady state	Decline
Presence of serum		
GDNF (100 ng/ml) [80] GDNF (40 ng/ml) [88] GDNF (10 ng/ml) [80] GDNF (1–100 ng/ml) [81]	KITLG [80] LIF [80] FGF2(20 ng/ml) [80] FGF2(1 ng/ml) [81]	FLK2L [80] Activin A [80] BMP4 [80] FGF2 (10 - 100 ng/ml) [81]
Serum free		
GDNF (40–100 ng/ml) [20] IGF1 [20]	KITLG [20] LIF [20] NOG [20] EGF [20]	

The effect refers to the number of spermatogonial stem cells in culture as demonstrated by their property to form colonies after transplantation to seminiferous tubules of mice devoid of endogenous spermatogenesis. Serum components can exert unknown effects on spermatogonial stem cells biology that could mask the effect of the growth factors studies. GDNF = glial derived cell line neurotrophic factor; KITLG = KIT ligand, or former SCF, stem cell factor (Steel Factor); IGF-I = insulin-like growth factor; LIF = leukemia inhibitory factor; FGF2 = basic fibroblast growth factor; Noggin is an antagonist of bone morphogenetic proteins; EGF = epidermal growth factor; Flk-2L is structurally related to KITLG; BMP4 = bone morphogenetic protein 4; NOG = noggin

testis after transplantation. They even achieved an exponential increase in the number of cells that could restore fertility of congenitally infertile recipient mice following transplantation. The GS cells showed a 5 x 10^{12} fold expansion after 27 passages in 134 days [93].

It is not yet clear whether serum in the culture has a positive or negative effect on SSC. One of the most common arguments against the use of serum is the presence of unknown and variable factors that may hinder the interpretation of the results [81]. However, Kanatsu-Shinohara

et al., [95] used 1 % serum in their medium and still obtained enhancement of stem cell renewal, while Kubota et al., successfully introduced a serum free culture system [20]. Long-term spermatogonial culture systems have also been developed for species other than the mouse. Primary isolated bovine spermatogonia were cultured without adding feeder cells while the somatic cells present in the isolated cell suspension formed a confluent monolayer a few days after the initiation of the culture [78]. Furthermore, colonies were formed after each passage. Although most of the colonies underwent differentiation, transplantation assays revealed the continued presence of SSC within these cultures.

Scope of this thesis

Bovine species represent a key element in the food industry all over the world. As the world population grows, the optimization of the processes to obtain animal proteins for human consumption becomes more and more important. In the production chains of the cattle industry, the search for reproductive technologies involving the isolation, preservation and in vitro multiplication of the bovine male germ line becomes an obvious challenge because it potentially provides a means to reduce the normally long time required to achieve genetic improvement in this species. Therefore this thesis explores the basic aspects of the behavior of bovine SSC in vitro.

First, we studied the postnatal development of the testis in cattle breeds of commercial use in the tropics and contrasted this knowledge with the more extensively studied European breeds (chapter 2). Then, we analyzed the kinetics of early bovine spermatogonia during short (chapter 3) and long-term culture (chapter 4), starting from previous experiences in our laboratory as a base-line [76, 78, 96]. To this aim, quantitative approaches were introduced. We also gained insight into the formation of SSC colonies in vitro and the effect of growth factors on this process (chapters 3 and 4).

Furthermore, we sought specific growth factors to induce SSC renewal activity in vitro (chapters 3 and 4). The presence of SSCs was demonstrated by a functional assay consisting of the transplantation of germ cell suspensions into nude mouse recipient testes. Additionally, we have set up a specialized bovine culture system to successfully propagate bovine SSCs which included a highly enriched medium (Stem cell medium) supplemented with growth factors (GDNF, LIF, EGF and FGF2) and the use of long-term and serial subculturing (chapter 4).

Finally, we evaluated a semisolid medium based on agarose in order to attempt to limit the movement of type A spermatogonia with the expectation of developing an in vitro SSC assay in the near future (chapter 5).

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Testicular development in Brahman bulls

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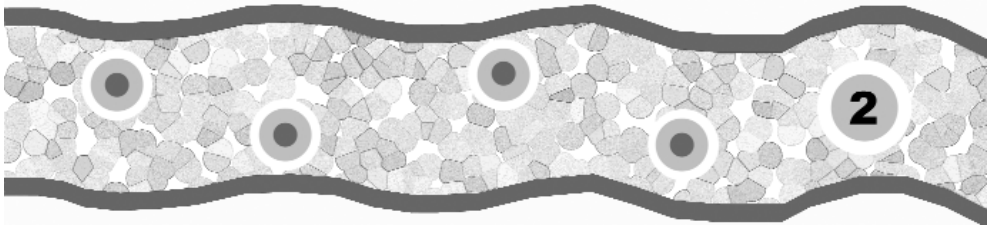
Chapter



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Abstract

Brahman breed bulls (*Bos indicus*) are widely used to introduce environmental resistance traits into meat-producing herds. However, their reproductive development is slower than European breeds (*Bos taurus*). The objective of this study was to assess the development of the seminiferous epithelium in Brahman bulls. Twenty-three prepubertal bulls were castrated and testicular samples taken for histological processing. Light microscopic images were digitized and cells of the seminiferous epithelium were assessed. Immature Sertoli cells gradually decreased in numbers and were no longer detected after approximately 14 months of age; concurrently, the numbers of mature Sertoli cells increased from 10 to 14 months. Spermatogenesis started during the ninth month; prior to that, only gonocytes and immature Sertoli cells were observed. Type A spermatogonia, spermatocytes, round spermatids, elongated spermatids and spermatozoa were first detected at 9.5, 11, 11, 13 and 16 months of age, respectively. The delay in the onset of puberty in Brahman bulls with respect to *B. taurus* was attributed to a longer duration of the prepubertal period (interval from start of spermatogenesis to puberty) and a later start of spermatogenesis.

Introduction

The *Bos indicus* group of cattle is comprised of different breeds with origins that can be traced to India, where they have developed into animals of a considerable strength, and with resistance to many diseases, parasites and extreme weather conditions. Brahman breed cattle, which belong to this group, are widely used in tropical areas, mainly as a source of meat, under many different farm management conditions. Unfortunately, Brahman bulls possess several reproductive disadvantages when compared to European breeds (*Bos taurus*), including lower sexual activity [1], a higher incidence of sperm abnormalities in adult bulls [2] and late puberty [3–7]. In addition, slower testicular growth [4] and a smaller scrotal circumference [8,9] have also been reported for Brahman bulls.

In young bulls, the beginning of the prepubertal period is characterized by the start of the spermatogenesis, ultimately leading to the formation of spermatozoa, under the influence of gradually increasing pulses of LH [10]. Spermatogenesis is initiated when fetal germ cells (gonocytes) divide to produce adult type spermatogonia, which in turn develop into increasingly dif-

ferentiated cell types [10–13]. When spermatozoa, the most differentiated germ cells, appear for the first time in the lumen of the seminiferous tubules, the animal reaches puberty [6,10,14].

The temporal pattern of histological changes within the seminiferous epithelium during prepubertal development is well known for *B. taurus* breeds [10,12,15,16], but no analogous information is available for the Brahman bulls. The present study was undertaken to describe qualitative and quantitative aspects of the cellular dynamics within the seminiferous epithelium in developing prepubertal *B. indicus* bulls, particularly the Brahman breed, with special emphasis on the timing of the appearance of specific germ cell types, the start of spermatogenesis and puberty.

Materials and methods

Bulls

Twenty-three Brahman bulls ranging from 8.5 to 19 months of age were castrated and testicular samples were processed for histological study. There were 3, 1, 2, 2, 3, 1, 2, 3, 3, 2 and 1 bulls that were 8.5, 9.5, 10.0, 11.0, 12.0, 14.0, 15.0, 16.0, 17.5, 18.5 and 19.0 months, respectively. These individuals were randomly chosen from the general population bulls on commercial cattle farms located in the Barinas State, South Western Venezuela. This area is located in the tropical dry forest life zone, 200 m above sea level, mean temperature 26.3°C, relative humidity 81% and average precipitation of 1700 mm/year. Standard health and sanitation programs were utilized on these farms, including vaccination against rabies, blackleg (and other clostridial diseases), pasteurellosis, and foot and mouth disease and periodic deworming. The bulls consumed locally introduced grass species (i.e.*Cynodon sp.* and *Echinochloa polystachya*) in paddocks under a stocking rotation system, and had continuous access to water and mineral salts. Physical examination revealed no apparent anomalies of the reproductive organs. Body weight and scrotal circumference were determined for each bull.

Castration and histological sampling

Age at castration was recorded (based on individual birth certificates). Immediately prior to castration, the bulls were sedated with 0.25 mL/50 kg of xylazine (Rompun®; Bayer, Caracas, Venezuela) and the testes were locally infiltrated with 2% lidocaine (Biofarmacia C.A., Maracay, Venezuela) at the level of the spermatic cord. One excised testis from each bull was randomly chosen for perfusion (via the testicular artery) of normal saline (0.9%), followed by a fixative (glutaraldehyde 2%, v/v in 100 mM cacodylate buffer (Electron Microscopy Sciences, Hatfield, PA, USA). These solutions were injected under moderate pressure with a 20 mL hypodermic syringe.

Testicular biometry

Prior to fixation, the spermatic fascia and epididymis were rapidly separated from the testis and the total testis mass was measured by weighing the testis with its Tunica albuginea still asso-

ciated. The latter structure was dissected from the testis after fixation and weighed. The mass of the testicular parenchyma (MTP) was obtained by subtracting the weight of the tunica albuginea from the total testis mass. The volume of the testicular parenchyma was estimated by dividing MTP by the parenchymal specific gravity (PSG) [17–20]. The PSG was calculated by weighing one piece of testicular parenchyma in the air and submerged in distilled water; it is equal to the weight value in air divided by the weight value submerged in water, subtracted by the weight value in air. No correction was made for mediastinum weight.

Sample collection and histology

Cubic pieces of testicular parenchyma (about 1 cm^3) were collected from an area located caudo-medial-centrally in the testis, adjacent to the corpus epididymis [21]. Other points in the testis are histologically similar [22,23]. Prior to processing, pieces were stored (at 5 °C) in 2% glutaraldehyde (v/v) in 100 mM of cacodylate buffer. Samples were cut smaller (0.5 cm^3) and post-fixed in osmium tetraoxyde (Electron Microscopy Sciences) with cacodylate buffer, dehydrated in alcohol and included in EMbed 812 (Electron Microscopy Sciences). Sections (3 μm thick) were prepared with a Porter-Blum MT2-B ultramicrotome (Sorvall Inc., Newtown, CT, USA), stained with toluidine blue and studied with light microscopy. Other sections (10–20 μm thick) were used for nuclear diameter determination.

Quantification of cellular types

Cells were studied and counted on images captured and digitized through a video camera and digital software (Professional Pixera®; Pixera Corporation, Los Gatos, CA, USA) attached to a light microscope (Nikon Eclipse E400; Nikon Inc., Melville, NY, USA) to yield the average percentage of seminiferous epithelium cells in round cross-sections. The final magnification on the computer monitor was 1400X. Forty round cross-sections were counted per bull in random movements (though X and Y) spaced approximately 250 μm apart to count different tubules [24]. Cell types studied included: immature Sertoli cells, mature Sertoli cells, gonocytes, spermatogonia (A, intermediate and B), spermatocytes (including preleptotene, leptotene, zygotene and pachytene stages) and spermatids (round and elongated). Criteria for the identification of these cell types were previously described [15,25,26]. Although spermatozoa were not quantified, their presence in the seminiferous tubules was recorded for each bull. All cells in each of the 40 round

tubule cross-sections were counted and the average percentage of each cell type per tubule was calculated. Each of these values was corrected for several factors (thickness, diameter, nuclear volume density, volume density of tubules containing germ cells and frequency of stages of the cycle of the seminiferous epithelium; the latter was done in those that had reached puberty). The absolute number of cells of each specific type per testis was estimated using the following formula (modified from Okwun et al., [20]):

number of cells per testis = % cells x $V_{tubules}$ x CF / nuclear volume

In this equation, $V_{tubules}$ is the stereologic estimation (volume density) of the total volume of the testis occupied by seminiferous tubules, CF the factors to be corrected and nuclear volume is the theoretical volume of one cell.

The volume density ($V_{tubules}$) is equal to V_{testis} x $Vv_{(tubules,testis)}$, where V_{testis} corresponds to the volume of the testicular parenchyma, $Vv_{(tubules,testis)}$ is the volume fraction of seminiferous tubules within the testis and represents the volume proportion of the testicular parenchyma occupied by seminiferous tubules [26]; this was estimated through a test system consisting of 480 random points laying over the digitized images randomly generated by stereological software (Stesys 2.0 Institute of Physiology, Academy of Sciences, Czech Republic). The component includes several corrections as expressed by the sub-formula CF = CF_1 x CF_2 x CF_3 x CF_4. In this equation, the first term (CF_1) corrects cell counts for thickness and nuclear diameter and was estimated through a formula presented by Weibel and Paumgartner [27]:

$$CF_1 = 2 / \{[(2 + P^2)(1 - P^2)^{1/2}] + 3g\}$$

where P = r_0/R (r_0 is the radius of the smallest nucleus of the specific counted cell type and R is the average radius of the counted cellular type); g is the relative thickness = T/d (T is the thickness of the section and d is the average diameter of the counted cell type). The average nuclear diameter was obtained by measuring 40 nuclei of each cell type in 20 μm sections [20] stained with toluidine blue. Images were captured and digitized when the largest area of each nucleus was detected by adjusting the micrometric control of the microscope [20]. Measurements were performed on a personal computer using the UTHSCSA Image Tool program, developed at the University of Texas Health Science Center at San Antonio, TX, USA, and available from the Internet (http://ddsdx.uthscsa.edu/ dig/itdesc.html).

Since Sertoli cells have an irregular nucleus, their diameter was calculated as the average length of their major and minor axes [28]. The nuclear diameter of elongated spermatids was calculated in the same way. CF_2 or $Vv_{(tubulesGC, Vtubules)}$, corresponded to the volume density of tubules containing germ cells with respect to the total volume of seminiferous tubules in the testis and it was estimated similarly to $Vv_{(tubules,testis)}$. $Vv_{(tubulesGC, Vtubules)}$ (CF_2) was the volume fraction of seminiferous tubules containing germ cells within the testis and represented the volume proportion of the complete seminiferous epithelium tubular system with presence of germ cells. This factor (CF_2) excluded the tubules containing only Sertoli cells; it was estimated through a test system consisting of 480 random points laying over the digitized images generated by Stesys 2.0 software and counting the points falling over the profiles of tubules that contained any kind of germ cells. CF_3 is the volume density of nuclei located inside the seminiferous tubules (volume occupied by nuclei) in relation to general tubule volume ($Vv_{(nuclei, tubules)}$) This was performed with the digital stereological layout previously described, by counting points falling over any nucleus within the seminiferous tubules cross-sections. CF_4 corresponds to the frequency of the stages (associations) of the seminiferous epithelium where the specific counted cell type appeared and was calculated only for the bulls with fully functional spermatogenesis, i.e. pubertal.

The volume of the nucleus for each specific cell type was estimated by the volume formula for the sphere: $V_{sphere} = 4/3\pi\ r^3$. The radius (r) was previously calculated in CF_1. A correction for histological shrinkage was not carried out, since the procedures used to process the samples (glutaraldehyde and osmium tetroxyde fixation, embedding in epoxy resin) resulted in limited shrinkage [20].

Definition and determination of various other end points

The time of the start of spermatogenesis was estimated from the regression curve of type A spermatogonial number with age as the independent variable (see Section "Statistical analysis"), as the time when this cell type first appears in the seminiferous epithelium. The onset of puberty was assessed on the basis of the age at which spermatozoa were observed in the lumen of the seminiferous epithelium for the first time. The germ cell/Sertoli cell ratio was estimated by dividing the absolute total number of germ cells per testis by the absolute total number of Sertoli cells (immature and mature type). Tubule diameter was measured in 20

round cross-sections of seminiferous tubules per bull (on digitally captured images) and the average calculated.

Spermatogenic efficiency

Estimation of the spermatogenic efficiency within the seminiferous tubules of prepubertal animals was performed through the following indexes: intermediate spermatogonia:spermatocytes, intermediate spermatogonia:spermatids, spermatocytes: spermatids. These indexes were calculated by dividing the number of more advanced cell type (numerator) by that of the younger generation cell type (divisor). Counts of type A spermatogonia were not used in the divisor of the formula of the first two ratios (due to the difficulty to morphologically determine the number of generations of this cell type).We did not use the counts of type B spermatogonia; according to the model of kinetics of the bull seminiferous epithelium proposed by Hochereau de Reviers [29], these cells undergo two mitotic divisions.

Statistical analysis

All statistical analysis was carried on SPSS 11.5 for Windows software. Regression analysis was performed on the variables body weight, testicular weight, scrotal circumference, seminiferous tubule cross-sectional diameter, absolute numbers of somatic and germ cell types per testis, germ cell/Sertoli cell ratio and spermatogenic efficiency indexes (ratio intermediate spermatogonia:spermatocytes, ratio intermediate spermatogonia: spermatids and ratio spermatocytes:spermatids), using age (in months) as the independent variable. Correlation analysis (Pearson) was used on selected variables. Scatter graphs were generated and curves fit with Sigma Plot 8.0 for Windows.

Results

Body and testicular growth

Body weight increased 1.8-fold from 8.5 to 19 months of age, while testis weight increased 6.4-fold (Figure 1). Scrotal circumference increased nearly 1.7-fold during the same period. Testicular growth was particularly accelerated between 11 and 16 months. Seminiferous tubule diameter steadily increased with age (94.8–197 μm) according to the regression model (Figure 1), and was highly correlated with testicular weight and scrotal circumference (for both, $r = 0.9$, $P < 0.01$).

Histology

Between 8.5 and 10 months of age, lumen formation (in the seminiferous tubules) began as small lacunae that appeared near the center of the tubules. The basal lamina was very thick and surrounded the cell types present at that stage, i.e. immature Sertoli cells and gonocytes. Nuclei of immature Sertoli cells had an oval shape with a regular border; these cells were arranged at different heights in the radial axis of the tubule. At 8.5 months, gonocytes were the only germ cells present. They were big round cells with a pale cytoplasm and one conspicuous nucleolus. At that age, most of them had relocated and made contact with the basal lamina (Figure 2A), although some remained in the central area of the tubule until about 9 months. Oval type A spermatogonia are the daughter cells of gonocytes entering mitosis during the start of spermatogenesis [11]; a few were detected at 9.5 months and thereafter (Figure 3), these cells were present in all bulls. Therefore, spermatogenesis started at 9.5 months in these bulls. At 11 months, the basal lamina of the seminiferous tubules was still thick but became thinner from 12 months of age onwards. The lumen was already formed and devoid of cells.

Sertoli cells were now more evenly distributed and their nuclei located near the basal lamina. Gonocytes coexisted with type A spermatogonia in the basal compartment of the seminiferous epithelium (Figure 2). Also at 11 months, spermatocytes started to appear (Figure 3). Late spermatocytes (zygotene and pachytene) were present in 95% of the seminiferous tubules of animals from 11 months of age onwards. Round spermatids were present in only 15% of tubules at 11 months, but increased in frequency from 13 months onwards (Figure 3). Before 14 months of

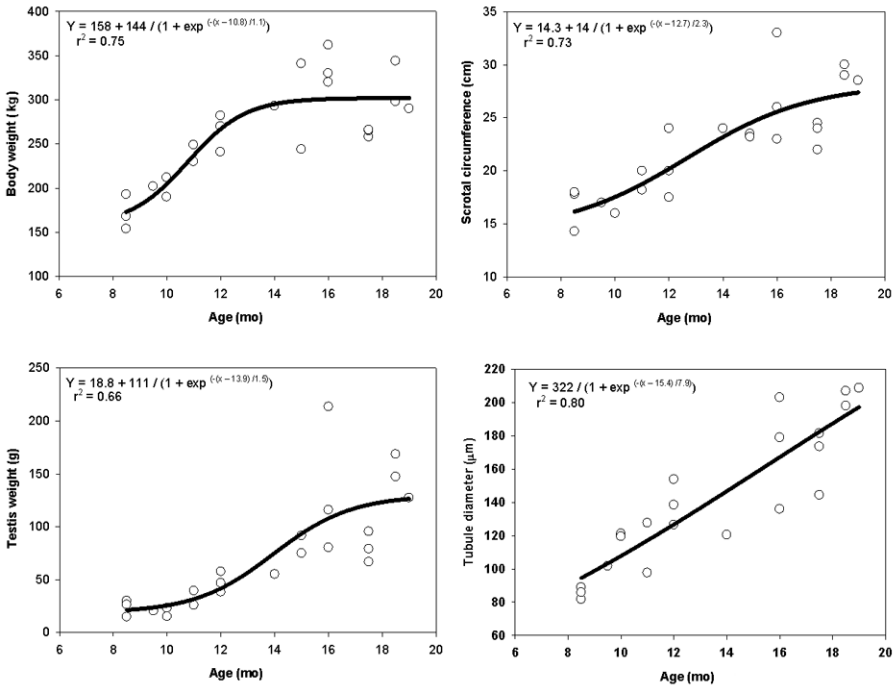

Fig. 1. Body weight, testis weight, scrotal circumference and seminiferous tubule cross-section diameter in relation to age (regression) in prepubertal Brahman bulls.

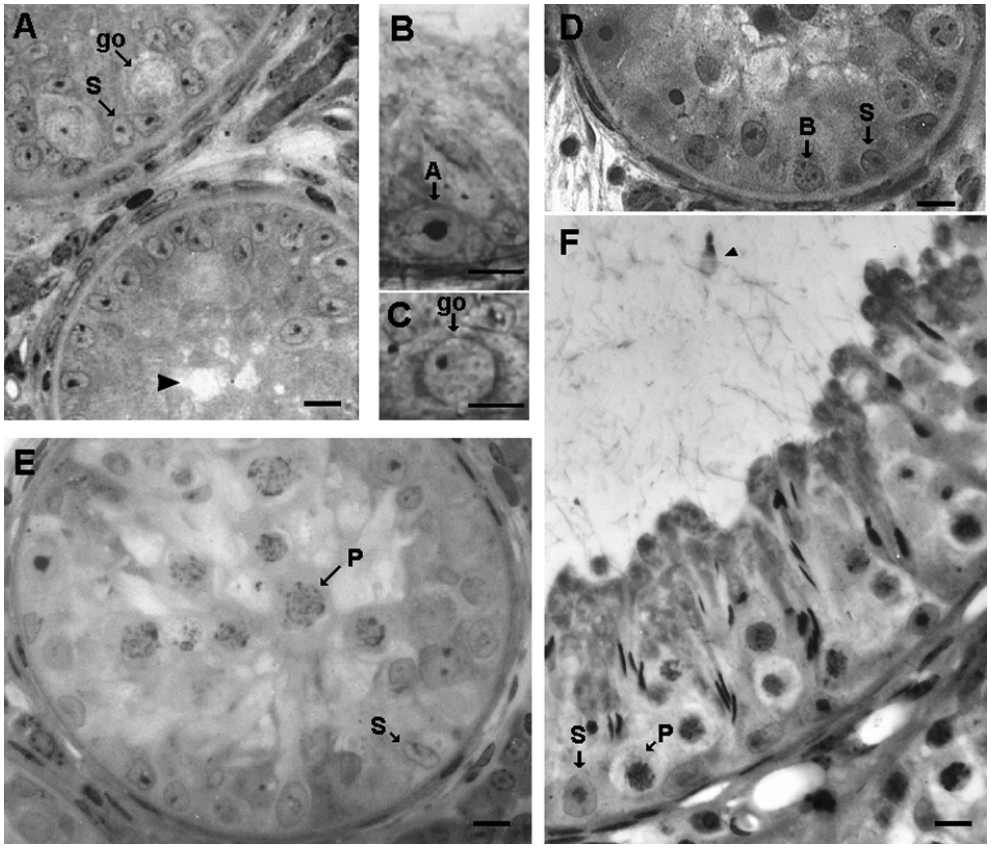

Fig. 2. Tubules from Brahman bull testes at different stages of postnatal development. (A) 8.5 months of age. Immature Sertoli cells (S) are often accommodated in two layers within the seminiferous tubules. Most gonocytes (go) contact the basal lamina. Small lacunae show the incipient lumination (arrowhead); (B and C) 11 months of age. Gonocytes (go) and type A spermatogonia (A) coexist at this time; (D) 12 months of age. Differentiating spermatogonia like B type (B) can be seen among some already mature Sertoli cells (S); (E) 14 months of age. Spermatocytes, i.e. pachytene (P) are observed towards the lumen of the tubule; (F) 16 months of age. Spermatogenesis is complete. Spermatozoa (arrowhead) are already being shed into the lumen, bar = 10 μm.

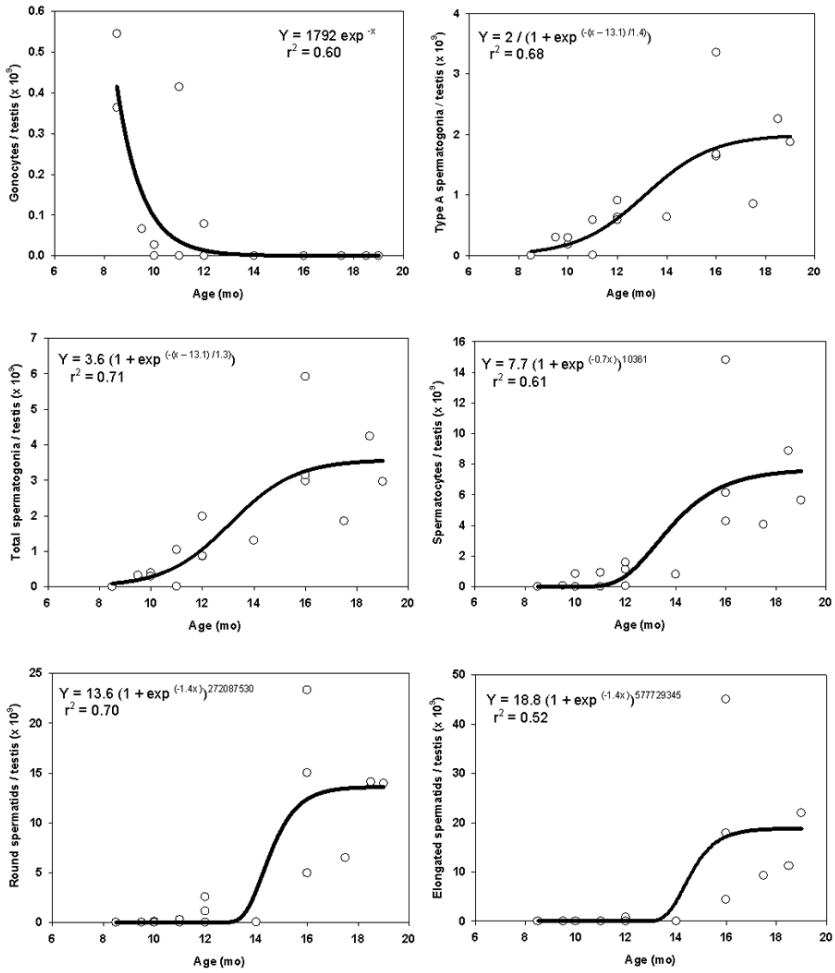

Fig. 3. Numbers of various types of germ cells in relation to age (regression) in prepubertal Brahman bulls.

age, only two bulls (age 12 months) had elongated spermatids (in 7.5 and 17.0% of the tubule cross sections, respectively). These cells were detected shortly after round spermatids (Figure 3).

At 16 months of age, round and elongated spermatids represented nearly 30% of all germ cells and appeared in about 94% of tubules. By 16 months of age, all bulls were producing spermatozoa (as indicated by the presence of spermatozoa in the tubule lumen; Figure 2F). Hence, this was the age of puberty onset. Full spermatogenesis was established in all bulls between 17

and 19 months (spermatozoa were found in the lumen of the tubules in every bull). The total number of Sertoli cells increased 2.5-fold between 8 and 14 months of age (Figure 4). Immature Sertoli cells, which proliferated in young bulls and differentiated into the mature type, gradually decreased in numbers after 9 months of age and totally disappeared in bulls after 14 months. Concurrently, the mature type increased considerably from 9.5 to 14 months of age. In bulls from 12 to 14 months of age, most Sertoli cells had the terminally differentiated mature phenotype (irregular shaped nucleus with a characteristic nucleolus). The number of Sertoli cells stabilized at approximately 14 months of age; at that time, the most common germ cells were spermatocytes, which tended to fill the entire adluminal compartment and protrude into the lumen (Figure 2E). Testicular cellular dynamics during prepubertal development were reflected in the growth of the testis; testis weight was strongly correlated with the total number of Sertoli cells ($r = 0.81$, $P < 0.01$) and germ cells ($r = 0.97$, $P < 0.01$). However, germ cells contributed more to the increase in testicular weight than Sertoli cells, as the germ/Sertoli cell ratio increased from 0.2 to 3.5 between 8.5 and 19 months of age (Figure 4).

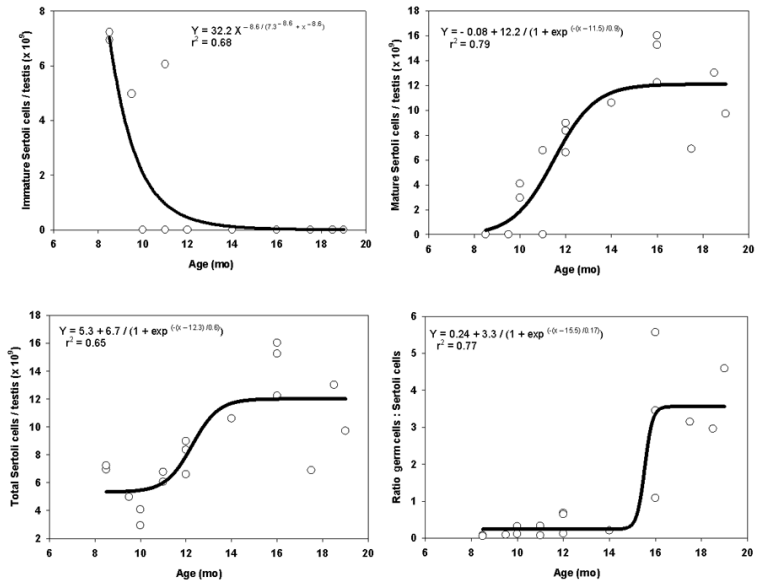

Fig. 4. Sertoli cell numbers and ratio germ cells/Sertoli cells (G/S) in relation to age (regression) in prepubertal Brahman bulls. G/S is the ratio of the total number of germ cells with respect to the total of Sertoli cells (germ cells accommodated by one Sertoli cell).

Efficiency of the spermatogenic process

The three ratios used for the evaluation of the efficiency of spermatogenesis during the prepubertal period in Brahman bulls included intermediate spermatogonia:spermatocytes, intermediate spermatogonia:spermatids and spermatocytes:spermatids. All three ratios were based on the efficiency of production of an advanced cell type by a precursor cell, under the form: (one unit precursor cell/number of advanced cells produced). Therefore, these indexes demonstrated the degree of efficiency on the process they span as compared with the theoretical physiological yield of one specific cell type, based on known cell kinetics. All indexes progressed from a basal level (lag phase) to a steep increment (rapid growth phase) around 15 months of age (Figure 5). Shortly before 16 months, the curve reached a stationary phase at its highest value (Figure 5). Initial basal levels for the indexes (before 15 months of age) were 1:2.41 (intermediate spermatogonia:spermatocytes); 1:4.07 (intermediate spermatogonia:spermatids) and 1:0.54 (spermatocytes:spermatids). Top plateau values (after 16 months) were 1:7.36 (intermediate spermatogonia:spermatocytes); 1:29.37 (intermediate spermatogonia:spermatids) and 1:4.38 (spermatocytes:spermatids).

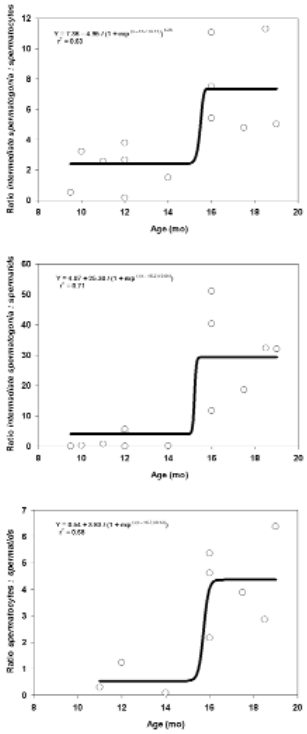

Fig. 5. Spermatogenic efficiency during the prepubertal period. Ratios including: intermediate spermatogonia:spermatocytes; intermediate spermatogonia:spermatids and spermatocytes:spermatids. The y-axis shows the second component of the specific ratio (1:y), that is, the number of advanced type cells (y) produced by one precursor cell. Therefore, higher values indicate higher efficiency.

Discussion

The present study was the first qualitative and quantitative description of testicular development in the Brahman breed. Previous work on testicular development in other breeds of *B. indicus* have relied on qualitative descriptions and the recording of the time of appearance of various cell types within the seminiferous epithelium [6,7,30]. The prepubertal period in cattle is characterized by rapid increases in both body and testicular weight. In the testis, this is associated with cell proliferation; adult type germ cells arise from gonocytes as progenitor cells, regardless of breed [3,4,9,10,15]. There were important differences between *B. indicus* and *B. taurus* subspecies that clearly reflected differences in the timing of reproductive events. Gonocytes were the only germ cells present at 8.5 months in Brahman bulls. Their relocation to the basal compartment of the seminiferous tubules occurred at different ages (approximately 9 months of age versus 2–4.5 months) and corresponding tubule diameters (99 μm versus 50–80 μm) in Brahman versus *B. taurus* [16] bulls, respectively. The division of gonocytes into type A spermatogonia is an important event that marks the start of spermatogenesis. This occurred at 9.5 months of age in Brahman bulls, which was similar to that in the African *B. indicus* breed Angoni (9 months) [7], but much later than in the Brazilian Nelore breed (6 months) [30] (another *B. indicus* breed). Spermatogenesis started much later in Brahman than in *B. taurus* bulls (9.5 months versus 2–4 months, respectively) [10,12,15]. There might be differences among *B. taurus* breeds in the onset of spermatogenesis. For example, in Holstein bulls (dairy) it occurs around 4 months [10,12] whereas in Swedish Red bulls (beef), it occurs as early as 2 months of age [15]. In Brahman bulls, Sertoli cell differentiation began around the onset of spermatogenesis and extended for a period of approximately 2.5 months onwards. In *B. taurus* bulls, Sertoli cells also differentiate after the start of spermatogenesis, but interestingly, they do so earlier than in Brahman bulls, i.e. between 5 and 7 months of age [10]. Spermatocytes begin to appear at 11 months of age, but it is only between 12 and 16 months of age that their numbers increased considerably. This wave occurred when the tubule diameter ranged from 127 to 167 μm, similar to *B. taurus* (120–180 μm) [16], but in Brahman bulls it occurred about 7 months later. Apparently, spermatocytes appeared faster after the start of spermatogenesis in Brahman bulls than in European cattle. The interval from the start of spermatogenesis to the first appearance of spermatocytes in Brahman bulls was 2 months, while the same interval lasted 4–4.5 months in *B. taurus* bulls [12,15]. The emergence of haploid

cells had a different timing as well. The time between the start of spermatogenesis and the first appearance of round spermatids in Brahman bulls was 4 months, compared to 5–5.5 months in European cattle [12,15]. Remarkably, the interval between the appearance of spermatids and appearance of spermatozoa was longer in Brahman bulls than in *B. taurus* (2.5 versus about 1 month, respectively).

Regardless of apparent differences among *B. indicus* breeds, the onset of puberty in these animals occurs later in life than in *B. taurus*. The seminiferous tubules of Brahman bulls contained sperm from 16 months onwards, consistent with other studies that used first detected sperm in an ejaculate as a means of estimating the onset of puberty [3,14,31], as well as studies using histological techniques (15 16 months) [6,7,30] in various *B. indicus* breeds. In contrast, European breeds had spermatozoa in the lumen of the seminiferous tubules at 8 months of age [10,12,15,16], 1.5 months before spermatogenesis started in Brahman bulls. In conclusion, puberty was delayed 8 months in Brahman versus *B. taurus* bulls.

Sertoli cells nourish and provide an adequate environment for the development of germ cells. In the present study, a germ cell/Sertoli cell (G/S) index was calculated, indicating the number of germ cells accommodated by a single Sertoli cell. A period of very fast increase in the index occurred between 15 and 16 months (approximately a 14-fold increase), reaching a plateau value of 3.5 afterwards. Clearly, this period of exponential growth (15–16 months) coincided with the onset of puberty in Brahman bulls. Similarly, Berndtson and Igboeli [32] calculated an average G/S index of nearly three in pubertal beef bulls (*B. taurus*).

Brahman bulls had relatively low spermatogenic efficiency from the start of the process (9.5 months) to 15 months of age, presumably due to apoptotsis in the seminiferous epithelium. The enhanced apoptosis of germ cells in the front of developing spermatogenic cell type resembled that reported in mice [33,34]. Efficiency improved after 15 months of age, presumably with lower levels of apoptosis and achievement of optimal numbers of germ cells accommodated by each Sertoli cell.

The ratio intermediate spermatogonia:spermatocytes indicated the efficiency of the process of generating spermatocytes from intermediate spermatogonia. In this specific developmental step, there was a sharp increase in efficiency between 15 and 16 months of age (from 30 to 92 %),

which corresponded with the period of steeply increasing spermatocyte numbers (Figure 3). A steady, high efficiency value was reached shortly before puberty (16 months; Figure 5). The low efficiency values for this index before 15 months of age might have been due to spermatocyte losses. The ratio intermediate spermatogonia: spermatids reflected the efficiency of the process of production of spermatids, starting from intermediate spermatogonia. Therefore, it is logical that this index increases in parallel with the growth phase of spermatid numbers (Figs. 3 and 5); it started on a basal level (12.7%), and subsequently increased to 91.8% after 15 months of age. Similarly and with the same implications, the ratio spermatocytes:spermatids reached peak efficiency after spermatocytes and spermatids were present in great numbers (approximately 15 months), suggesting that there are few cell losses in spermatid generation (and by extension in the integrity of the meiotic process). The efficiency of the generation of spermatids from spermatocytes changed from 13.5% (at the start of spermatogenesis) to about 100% shortly before puberty. This very high pre-pubertal values (1:4.32 or 100% efficiency) seemed close to those of 3-year-old *B. taurus* bulls (Holstein breed), in which cell losses occur at meiosis (5%), but are minimal during spermiogenesis [21]. Furthermore, cell death increases as these bulls sexually mature [21]. On the other hand, adult *B. indicus* bulls (Nellore breed, 4–6-year-old) had a ratio of spermatocytes:spermatids of 1:3 (75% efficiency) [35]. The ratio between the numbers of spermatocytes and spermatids sometimes exceeded the maximum possible. This was attributed to either underestimation of the number of spermatocytes or overestimation of the number of spermatids; the latter might have occurred because elongated spermatids were difficult to count due to their close proximity to each other, especially near the time of puberty. If this is the case, then the ratio intermediate spermatogonia:spermatids should also be somewhat lower than presented in this study.

Environmental factors affect sexual development in bulls; season of birth may affect the interval between puberty and sexual maturity in Brahman bulls, due to photoperiodic changes throughout the year [36]. However, in the tropics, the photoperiod is constant and Brahman bulls are especially well adapted to climatic conditions [37]. Another important environmental influence on the onset of puberty is nutrition [38,39]; low nutritional planes may delay the onset of puberty in *B. indicus* bulls [40]. Although the Brahman bulls in the present work came from one specific location, this area is representative of the tropical dry forest life zone and these bulls had optimal

nutrition and appropriate general management conditions. Thus, apparent differences in testicular development between the Brahman bulls in the present study and *B. taurus* bulls in previous studies was not obviously attributable to differences in environmental or nutritional conditions.

Considering the characteristics of prepubertal testis development, we can account for the 8 months delay in the onset of puberty in Brahman bulls in comparison with European breeds. First, Brahman bulls start spermatogenesis at least 5.5 months later. Second, the interval from the start of spermatogenesis to the onset of puberty is 0.5 months longer in Brahman bulls than in European bulls, probably due to a slightly longer spermiogenic process in Brahman bulls. Therefore, we inferred that while the cellular events in the establishment of spermatogenesis in prepubertal Brahman bulls (*B. indicus*) were similar to those in *B. taurus* bulls, the later onset of puberty in the former was mainly caused by a belated start of spermatogenesis. In addition, Sertoli cell differentiation occurs later in this species. The differences probably arise by different regulatory mechanisms between the two bovine types, e.g. different endocrine pulses, as suggested by Wildeus et al., [41].

In future studies, it will be interesting to characterize the small window of initiation of puberty in terms of the efficiency of spermatogenesis (i.e. daily production of spermatozoa from testicular spermatid reserves) [42] yielding values of sperm per gram of testis. This should prove useful for comparisons with existing data on *Bos taurus*. It will also remain important to clarify specific traits involved in the regulation of the establishment of spermatogenesis in young *Bos indicus* bulls, e.g. gonadotrophin pulse patterns and intratesticular milieu (hormones, autocrine and paracrine factors).

In conclusion, testicular development in Brahman bulls was delayed relative to *Bos taurus* bulls, due to a later onset of spermatogenesis. However, once started, the spermatogenic process unfolds at about the same speed in both types of bulls and high levels of efficiency are reached at approximately the same time.

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Basic features of bovine spermatogonial culture and effects of glial cell line-derived neurotrophic factor

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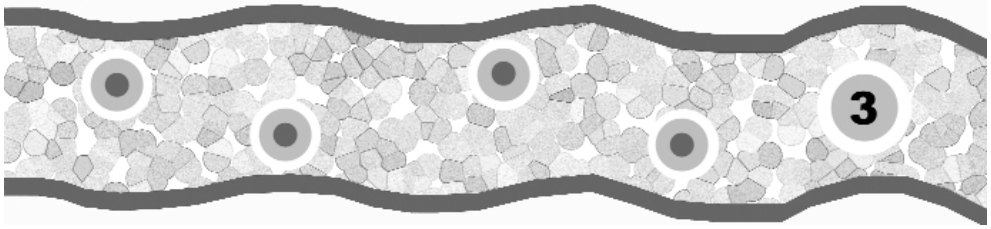
Chapter



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Abstract

Spermatogonial stem cells (SSC) are a small self-renewing subpopulation of type A spermatogonia, which for the rest are composed of differentiating cells with a very similar morphology. We studied the development of primary co-cultures of prepubertal bovine Sertoli cells and A spermatogonia and the effect of glial cell line-derived neurotropic factor (GDNF) on the numbers and types of spermatogonia, the formation of spermatogonial colonies and the capacity of the cultured SSC to colonize a recipient mouse testis. During the first week of culture many, probably differentiating, A spermatogonia entered apoptosis while others formed pairs and chains of A spermatogonia. After 1 week colonies started to appear that increased in size with time. Numbers of single (A_s) and paired (A_{pr}) spermatogonia were significantly higher in GDNF treated cultures at Days 15 and 25 ($P < 0.01$ and 0.05, respectively), and the ratio of A_s to A_{pr} and spermatogonial chains (A_{al}) was also higher indicating enhanced self-renewal of the SSC. Furthermore, spermatogonial outgrowths in the periphery of the colonies showed a significantly higher number of A spermatogonia with a more primitive morphology under the influence of GDNF ($P < 0.05$). Spermatogonial stem cell transplantation experiments revealed a 2-fold increase in stem cell activity in GDNF treated spermatogonial cultures ($P < 0.01$). We conclude that GDNF rather than inducing proliferation, enhances selfrenewal and increases survival rates of SSC in the bovine spermatogonial culture system.

Introduction

Stem cells are capable of both self-renewal and the production of differentiating cells and are present in almost every tissue in the body. Starting from spermatogonial stem cells (SSC) that reside on the basal membrane of the seminiferous tubules in the testis, spermatogenesis takes place as a highly coordinated and efficient process, which produces millions of terminally differentiated spermatozoa a day. SSC, as other stem cells, are found in very low numbers. For example, in the mouse testis, only 0.03% of all germ cells are stem cells [1]. Furthermore, the morphological appearance of SSC is very similar to that of early spermatogonia that already are committed to differentiation and have lost stem cell properties. To make progress in understanding the regulation of self-renewal and differentiation of SSC and to characterize these cells at the molecular level

it is essential to develop methods for their culture and purification. In cell culture systems, stem cells can be exposed to selected growth factors, so that the complexity of the regulatory mechanisms that govern spermatogenesis in vivo, involving a myriad of different signals, can be simplified. In this respect, progress has been made in other stem cell systems like hemopoiesis in which several cytokines and growth factors were found that induce hemopoietic stem cells to proliferate in vitro without undergoing differentiation [2]. Within the testis, the renewal mechanism of SSC is still not completely understood. One important factor, which enhances SSC renewal, is glial cell line-derived neurotrophic factor (GDNF) [3] that originally was found in nervous tissue where it can promote the survival of neurons [4]. GDNF is also involved in non-neural tissues like the kidney, where it plays an essential role in early developmental stages [5]. During the past years, GDNF has been associated with SSC renewal in vivo [3,6] and in vitro situations [7,8]. Normally, in the seminiferous epithelium Sertoli cells secrete GDNF and the receptors for this growth factor, GFR-alfa 1 and c-ret, are present on SSC [3,9,10]. Furthermore, FSH was found to stimulate GDNF production by Sertoli cells [10]. We now have studied the development of primary co-cultures of bovine Sertoli cells and A spermatogonia and we quantified the effects of GDNF on the numbers of SSC and spermatogonial clones, the formation of spermatogonial colonies and stem cell activity by way of transplantation techniques. Our system uses a monolayer of autologous Sertoli cells to provide germ cells an environment that resembles that in vivo as closely as possible [11– 13]. We used 4.5–5-month-old calf testes, an age at which type A spermatogonia are the predominant type of germ cells, with low numbers of type In and B spermatogonia and preleptotene spermatocytes [14,15]. We hypothesized that GDNF increases the number of SSC in vitro, by stimulating their proliferation.

Materials and methods

Cell isolation and purification

Testes were collected from calves between 4 and 6 months of age at commercial slaughterhouses and kept on ice for a maximum of 2 h until processing started. The tunica vaginalis and epididymis were excised and testis weight registered. The testis was gently washed with deionized water and transferred to sterile Petri dishes. About 20 g of testis material was used for each cell isolation. Histological samples were collected and fixed in Bouins fluid or 4% buffered formalin. After removing the tunica albuginea, the testes were minced into small pieces and suspended in minimum essential medium (MEM; Gibco, Invitrogen corporation, Paisley) supplemented with 0.1275% NaHCO3 (Sigma, St. Louis, MO), 4 mM L-glutamine, 0.1 mM single-strength non-essential amino acids, 100 IU/mL– 100 mg/mL penicillin–streptomycin, 40 mg/mL gentamicin and 15 mM Hepes (all from Gibco), subsequently referred to as MEM. Spermatogonial cells were isolated as described by van Pelt et al., [16] with modifications [17,18]. Briefly, this procedure consisted of two enzymatic digestions, filtration, overnight differential plating and discontinuous Percoll density gradient centrifugation. Fractions containing between 50 and 80% of type A spermatogonia were washed, counted and used. Purity was assessed with the help of a Nomarski interference microscopy, Axioskop (Zeiss, Germany). In 22 cell isolations performed, the average purity was 72.1 ± 2%. Hence, 27.9 ± 2% of the cells were contaminating cells, mostly Sertoli cells, as shown by their post-enzymatic morphological appearance (disrupted radial cytoplasmic projections). Viability of the primary isolated cells was evaluated using LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR, USA) consisting of calcein AM and ethidium homodimer-1 according to the manufacturer protocol and thereafter the cells were counted under a fluorescence microscope (Nikon Eclipse TE 200). The total number of A spermatogonia in the suspension was determined with a hemocytometer.

Cell culture

Cells were seeded in MEM with 2.5% FCS and fungizone (1:1000), and plated in culture wells at a concentration of 100 cells/μL. GDNF human recombinant (Sigma, St. Louis, MO, USA)

was added to some of the cultures. Cells were cultured at 37°C, in a humidified atmosphere with 5% CO2. The cultures were refreshed twice a week and lasted 4–25 days depending on the experiment. During the first week of culture, a monolayer of Sertoli cells developed and germ cells could proliferate on top of it. At the end of the culture cells were fixed in Bouins fluid or 4% buffered formalin.

Quantitation of cell numbers

During culture, a colorimetric assay was used to quantify cell numbers, based on the cleavage of the tetrazolium salt 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-tetrazolio]-1,3- benzene disulfonate by mitochondrial dehydrogenases in viable cells. Cultures were grown in a 96-well plate and supplemented with varying concentrations of GDNF (0, 1, 10 and 100 ng/mL), and incubated with WST-1 (Molecular Probes Inc. CA, USA) every 4 days, for a total period of 21 days. The assay was performed in triplicate, taking care of using alternating rows of the plate for each WST-1 assay to ensure cultured cells were completely washed after previous exposure to the reagent. In detail, medium was taken out of each well (100 mL) and 10 mL of WST-1 was pipetted in. Some control wells included medium without cells, with or without GDNF. An incubation period of 2 h followed and the formazan dye was quantified using a scanning multiwell spectrophotometer (ELISA reader, BioRad, model 680, microplate reader, Japan). Afterwards, the medium in the cultures was changed according to the specific treatments and placed back to the incubator.

Immunocytochemistry

For immunostaining, cells were plated in 4-well chamber slides (Lab-Tek, Nalge Nunc Int., IL, USA) each chamber containing 500 mL of cell suspension (100 cells/mL). The treatments included GDNF at concentrations 0 and 100 ng/mL. Cultures were stopped and fixed after 7, 15 or 25 days. Some slides were exposed to 5-bromodeoxyuridine (BrdU) (Sigma, St. Louis,MO) for 2 h under the incubation conditions already described, to detect S phase cells. Type A spermatogonia were identified through DBA (Dolichos biflorus agglutinin) immunohistochemistry, as described by Ertl and Wrobel [19]. Briefly, sections were rehydrated, treated with 3% (v/v) H_2O_2 (Sigma, St. Louis, MO) for 10 min in the dark to inhibit endogenous peroxidase and rinsed in PBS. Unspecific-site blocking was done with 5% BSA in PBS for 15 min and then sections

were washed in HEPES buffer (1% Hepes buffer, Sigma, Paisley, Invitrogen Corporation, 0.1 M NaCl in distilled water). Subsequently, the samples were incubated with biotinylated DBA (Vector Laboratories, Inc., Burlingame, CA) at 1:500 in HEPES for 1 h at 37 °C in a moist chamber. After incubation with DBA, the samples were rinsed with HEPES and subsequently washed three times in PBS. The horseradish peroxidase–biotin complex reaction was performed using an ABC-Kit (Vector Laboratories), reagents A and B, 1:500 each in PBS. Lectin binding was visualized by using 25 mg of 3,39-diaminobenzidine tetrahydrochloride (DAB) (Sigma, St. Louis, MO), as chromogen substrate, and 17 μL of 35% (v/v) H_2O_2 per 50 mL in PBS for 1 min. The samples were rinsed thoroughly in distilled water, counterstained with haematoxylin (Sigma diagnostics, USA), dehydrated in graded alcohols, cleared in xylol and mounted with Pertex (Histolab, Göteborg, Sweeden). Negative control sections were incubated in 1% BSA in PBS without lectin. A similar procedure was used for vimentin staining in 4% formaldehyde fixed cells with the aim of identifying Sertoli cells. After deparafination and rehydration, preparations were treated for endogenous peroxidase inhibition, washed in PBS and treated with Triton X-100 (Sigma–Aldrich, Steinheim, Germany) at 0.5% for 30 min. Unspecific-site blocking was done with NGS (Vector Laboratories, Burlingame, CA, USA). The first antibody was mouse-anti-Vimentin, non-hematopoietic (Biogenex, San Ramon, CA, USA) at concentration 1:50. The second antibody was IgM anti-mouse (Vector Laboratories, Burlingame, CA, USA). ABC and DAB steps were done as described for the DBA immunostaining. All inter-step PBS washes, except those after ABC treatment, were followed by one wash with 0.1% Tween (Merck-Schuchard, Honenbrunn, Germany) in PBS. Also the blocking and antibody steps were performed on this solution.

BrdU detection

To determine cell proliferation, 5-bromodeoxyuridine incorporation was studied by immunostaining of the fixed cells. After 2 h of incubation with BrdU, cells were fixed in Bouins solution and kept in 70% alcohol. Samples were rehydrated and incubated with periodic acid (1%) at 60 °C for 30 min. Then, they were placed in running tap water for 10 min and washed in PBS. Unspecific-site binding was prevented by blocking with 5% BSA in PBS for 30 min. Afterwards, the samples were washed three times in PBS. The samples were then incubated with anti-BrdU (Becton Dickinson, San Jose, CA) 1:80 in 1% BSA in PBS for 1 h at room temperature in a moist chamber. As a negative control, a sample was incubated with mouse IgG at the same concentra-

tion of the antibody. After three washes in PBS, incubation with the second antibody was performed. Horse raised anti mouse reagent (included in ABC kit) was used for this purpose, at a concentration of 1:100 in PBS, for 1 h at room temperature and then three PBS washes followed. At this moment, endogenous peroxidase inhibition was done with 3% (v/v) H_2O_2 for 10 min, followed by three PBS washes and ABC incubation. This reaction was performed similarly as in DBA histochemistry. DAB reaction lasted from 40 s to 1 min for optimal results. The remaining steps are the same as in DBA histochemistry.

Quantification of the various cell types

In chamber slides, DBA positive cells as well as Sertoli cells, were counted in 200 random fields in each chamber (800 fields per slide). The cell groups counted were single, pairs and clusters of 3–64 cells. Spermatogonial clones were counted on Days 7, 15 and 25, and Sertoli cells on Days 15 and 25. Spermatogonial colonies that appeared in the cultures were quantified at Days 15 and 25. Colonies were defined as aggregations of more than 64 DBA positive cells in close association with Sertoli cells. Morphologically abnormal or apoptotic DBA positive cells were excluded from the counts. BrdU positive cells were counted to obtain the BrdU incorporation labeling index (spermatogonia and Sertoli cells).

Colony characterization

Colonies were defined as blob-like structures as in previous descriptions in mice [20,21]. All colonies per well were counted on Days 15 and 25. The area of these structures was measured using the UTHSCSA Image Tool program, developed at the University of Texas Health Science Center at San Antonio, Texas, USA, and available from the Internet at http://ddsdx.uthscsa.edu/dig/itdesc.html. For this procedure, we avoided including scattered DBA cells which radiate from the colonies because these cell fields intercross with others coming from nearby colonies in very complex patterns. Spermatogonial outgrowths on the surface of the colonies were evaluated for presence and number of constituent spermatogonia in 138 randomly chosen colonies arising in 25 days old control and GDNF treated cultures. Also in 30 randomly selected colonies from control and GDNF treated cultures, the percentage of spermatogonia in the outgrowths showing morphological signs of apoptosis was calculated.

Spermatogonial stem cell transplantation assay

Cells obtained after 7 days of culture in the presence of GDNF were transplanted into the seminiferous tubules of six nude mice (nu/nu) testes via the efferent ducts as previously described [22,23] (three mice control and three GDNF). One testis per animal was successfully transplanted with 10 mL of cell suspension containing 10^5 cells and the contralateral testis was left undisturbed to be used as a negative control. Both testes of the recipient mice were irradiated (doses of 1.5 and 12 Gy, 24 h apart) to destroy endogenous spermatogenesis, 1 month previous to transplantation as described before [23]. Testes were collected 2 months after transplantation and fixed in Bouins fluid. The experimental protocol of this study followed the guidelines of the care and use of laboratory animals and was approved by the animal care and use committee of Utrecht University. Serial sections were made (5 μm) and every 10th section was used for DBA lectine staining to determine bovine spermatogonia. About 1500 seminiferous tubule round cross-sections were studied per testis to determine the percentage of tubules containing at least one DBA positive cell.

Statistics

General statistical analysis was performed using the independent-samples Student's T test. The presence of type A spermatogonial cells in colonies arising in control and GDNF treated cultures was assessed by a Chi-squared test of independence and the number of these cells in the positive colonies further analyzed using a Student's t-test. All calculations were performed using SPPS software for Windows1 v11.5. 3.

Results

Germ cells

The total number of cells in our bovine co-culture system constantly increased during the first 20 days as evidenced by the WST-1 assay (Figure 1(A)). GDNF affected this growth rate in a dose-dependent manner, 100 ng/mL GDNF inducing a higher rate of cell proliferation than lower concentrations (1 and 10 ng/mL) (Figure 1(A)). Therefore, in further experiments 100 ng/mL of GDNF was used. Throughout the culture DBA positive cells, i.e. A spermatogonia, were present in the form of small clones and, later on, colonies. Small clones comprised of single cells (A_s), pairs (A_{pr}) or chains (A_{al}) of A spermatogonia (3–64 cells) (Figure 2). Some spermatogonial clones aggregated into clumps of spermatogonia in which no cytoplasmatic bridges could be discerned. Colonies were defined as groups of more than 64 DBA positive cells intermingled with somatic cells. GDNF directly affected the numbers of A spermatogonia during the first 25 days of culture (Figure 1(B)). Thus, DBA positive small clones in GDNF treated cultures outnumbered those in controls (Figure 1(B)). This effect was significant ($P < 0.05$) for singles and pairs after both 15 and 25 days of culture (Figure 1(B)). The same could also be seen as a numerical tendency in the case of the chains of A spermatogonia. Single A spermatogonial numbers decreased with time, but this was much less pronounced when GDNF was added to the culture (Figure 1(C)). In every culture, between Days 4 and 10, many spermatogonia (scattered and within the colonies) showed morphological signs of apoptosis as evidenced by a very high number of shrunken and picnotic nuclei. Colonies started to form around Day 6. At Day 7, they were small low density cell masses not firmly attached to the monolayer and orientated upwards in control cultures (Figure 3(A)), while in GDNF treated cultures, they appeared more compact and flattened in the horizontal plane (Figure 3(B)). As days went by, spermatogonial colonies increased in size (Figure 3(C and D)), but colony growth, in general, was not affected by GDNF at Day 15 ($P > 0.05$) (Figure 4(A)). At Day 15 of culture, cells in the colonies looked bigger and less interconnected in the GDNF treated cultures, than in controls (Figure 3, insets). Furthermore, the number of colonies in GDNF cultures remained similar to that in controls at Day 15 ($P > 0.05$) (Figure 4(B)). In all cases, colonies contained DBA positive cells (spermatogonia) (Figure 5(A)) with some of their nuclei being smaller and elongated in comparison to the strongly DBA

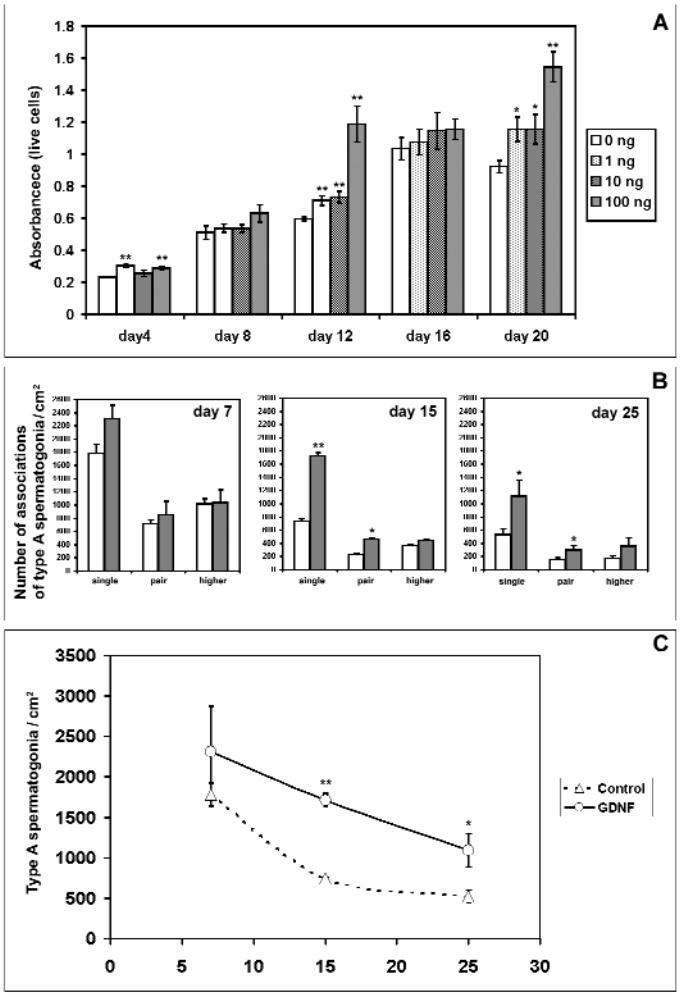

Fig. 1. Behavior of bovine spermatogonial clones in culture. (A) Proliferation activity of bovine type A spermatogonia cultured in medium containing various concentrations of GDNF, assessed by optical density of the tetrazolium salt. All comparisons are made against the control at each specific time point in three separate experiments (n = 3) (*P < 0.05); (**P < 0.01). (B) Number of type A spermatogonial clones, cultured with GDNF (100 ng/mL) vs. control (0 ng/mL), evaluated through DBA lectin staining at Days 7, 15 and 25. n = 3, (*P < 0.05); (**P < 0.01). (C) Numbers of bovine single type A spermatogonia co-cultured with Sertoli cells with GDNF (100 ng/mL) vs. control (0 ng/mL) during 25 days of culture. Comparisons are made against the control at each specific time point. (*P < 0.05); (**P < 0.01).

positive, round shaped singles and pairs of A spermatogonia scattered throughout the cultures. In some colony cells the DBA staining was weak and spermatogonial nuclei smaller, oval and even slightly distorted, which could be associated with differentiation. Bigger colonies found after 15 days had an irregular shape and consisted mainly of a spherical compact aggregation towards the center. These blob-like structures tended to protrude upward rather than spreading over the surface of the culture (Figures 3C and D; 5A). In some cases they had a more irregular shape. In general, colony sizes ranged from 1700 to 360000 μm^2. The distribution pattern of the sizes of the colonies was not different between control and GDNF treated cultures (not shown). Overall, three size groups were distinguished (small colonies of less than 50000 μm^2; intermediate colonies, 50000–100000 μm^2; big colonies, over 100000 μm^2). Small colonies dropped from 97 to 82% between Days15 and 25 of culture while intermediate and big colonies increased from 2 to 13% and 0 to 4.3%, respectively, during the same period.

A second type of colonies was large, flat and had a round shape and was seen only rarely (Figure 5(B)). These colonies showed a weak DBA positivity and also their frequency and size did not increase with the addition of GDNF ($P > 0.05$; data not shown). Very frequently, A spermatogonia were seen on the outer side of the colonies (radial and round) (Figure 5(A and B)). These outer cells appeared as singles and more often pairs in control cultures while bigger clumps were typically seen in GDNF treated cultures. Accordingly, a Chi-square test of independence was performed to examine the relation between the addition of GDNF to the cultures and the presence of type A spermatogonial outgrowths on the surface of the colonies. The number of colonies appearing in the cultures with and without spermatogonial outgrowths in relation to the addition of GDNF is shown in Figure 4(C). Although the number of colonies with outgrowths was higher in cultures with GDNF, the difference was not significant but there were significantly more A spermatogonia in these outer clusters in GDNF treated cultures than controls ($P < 0.05$) (Figure 4(D)).

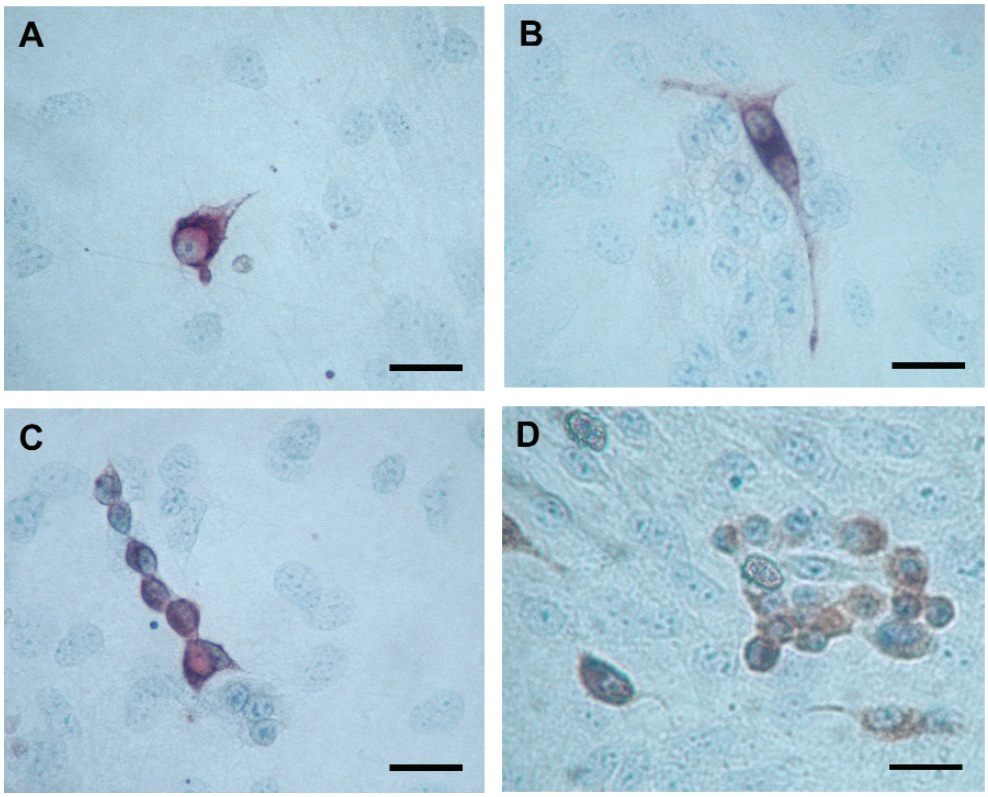

Fig. 2. Composition of A spermatogonial clones as demonstrated by DBA lectin staining. (A) A single (A_s). (B) A pair (A_{pr}). (C) A aligned (A_{al}). (D) Cluster of spermatogonia. Bar = 20 μm.

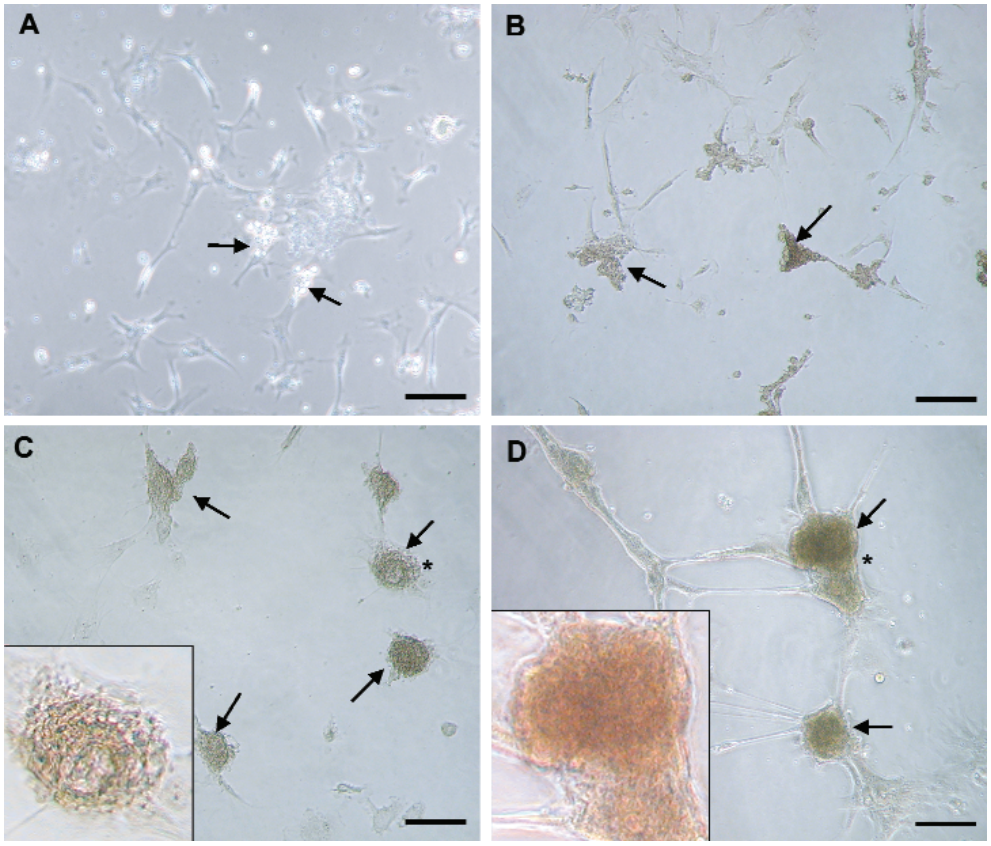

Fig. 3. Bovine type A spermatogonia co-cultured with Sertoli cells with GDNF (100 ng/mL) vs. control (0 ng/mL) at Days 7 and 15. Phase contrast micrographs. (A) Control culture at Day 7. (B) GDNF culture at Day 7. (C) Control culture at Day 15. (D) GDNF culture at Day 15. Colonies appear from 7 days onwards and gradually grow in size. Although there are similar number of colonies with similar sizes in control and GDNF treated cultures, cells look more compactly arranged in colonies appearing in control cultures (inset C) Arrows point to colonies. Insets show details of the colonies marked with an asterisk. Bar = 1 mm.

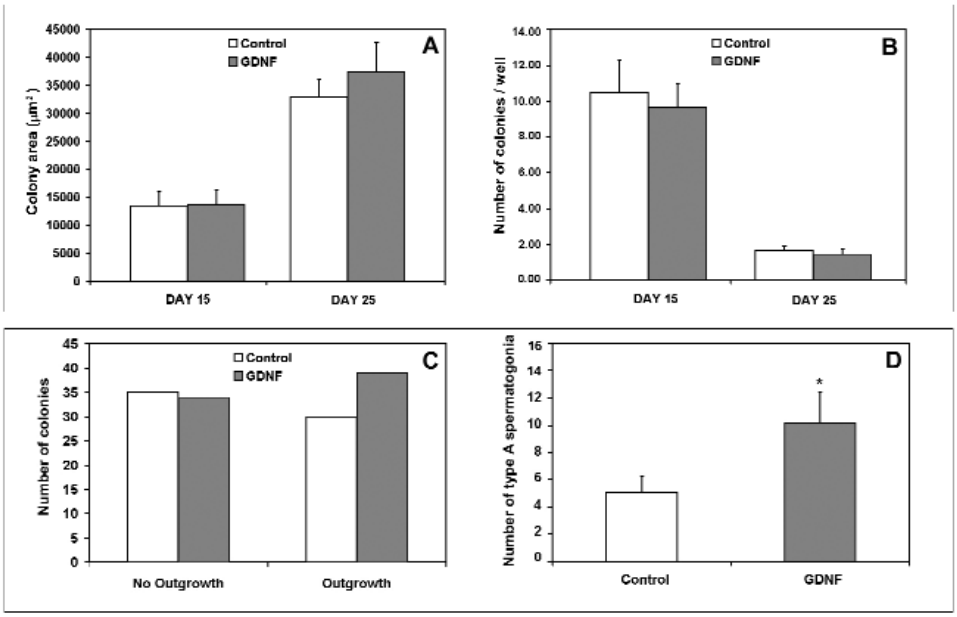

Fig. 4. Colony development in culture. Number (A) and size (B) of colonies (clusters of more than 64 germ cells) in control and GDNF treated cultures at Days 15 and 25. n = 3. (C) Number of colonies with type A spermatogonial outgrowths on their surface at Day 25. No association was detected between GDNF treatment and the presence of outgrowths, according to a Chi-square test of independence, χ^2 (1, N = 138) = 32.5, P > 0.05. (D) Number of cells (type A spermatogonia) on the colony outgrowths on control and GDNF treated cultures. *P < 0.05.

Using a differential interference contrast microscope, we noticed the presence of apoptotic cells in the core of the colonies. This was more evident in colonies in control cultures than in GDNF exposed ones. Indeed, there were 24.9 ± 5.5% apoptotic DBA positive cells in the colonies appearing in control at Day 15 while in GDNF cultures this percentage was 12.5 ± 2.4%. Also at Day 15, the colonies contained many BrdU positive cells (Figure 5(D)). The percentage of BrdU positive cells in the general population of scattered clones was not significantly different in control and GDNF treated cultures (9.7 ± 0.7 and 8.9 ± 0.6%, respectively). The major proliferative activity occurred in small clones and colonies. Unfortunately, a specific labeling index for cells within the more mature colonies could not be determined because of the very high density of the cells. Therefore, the BrdU incorporation was recorded in the periphery of the colonies, where individual cells were clearly discernable and in smaller less dense colonies (Table 1).

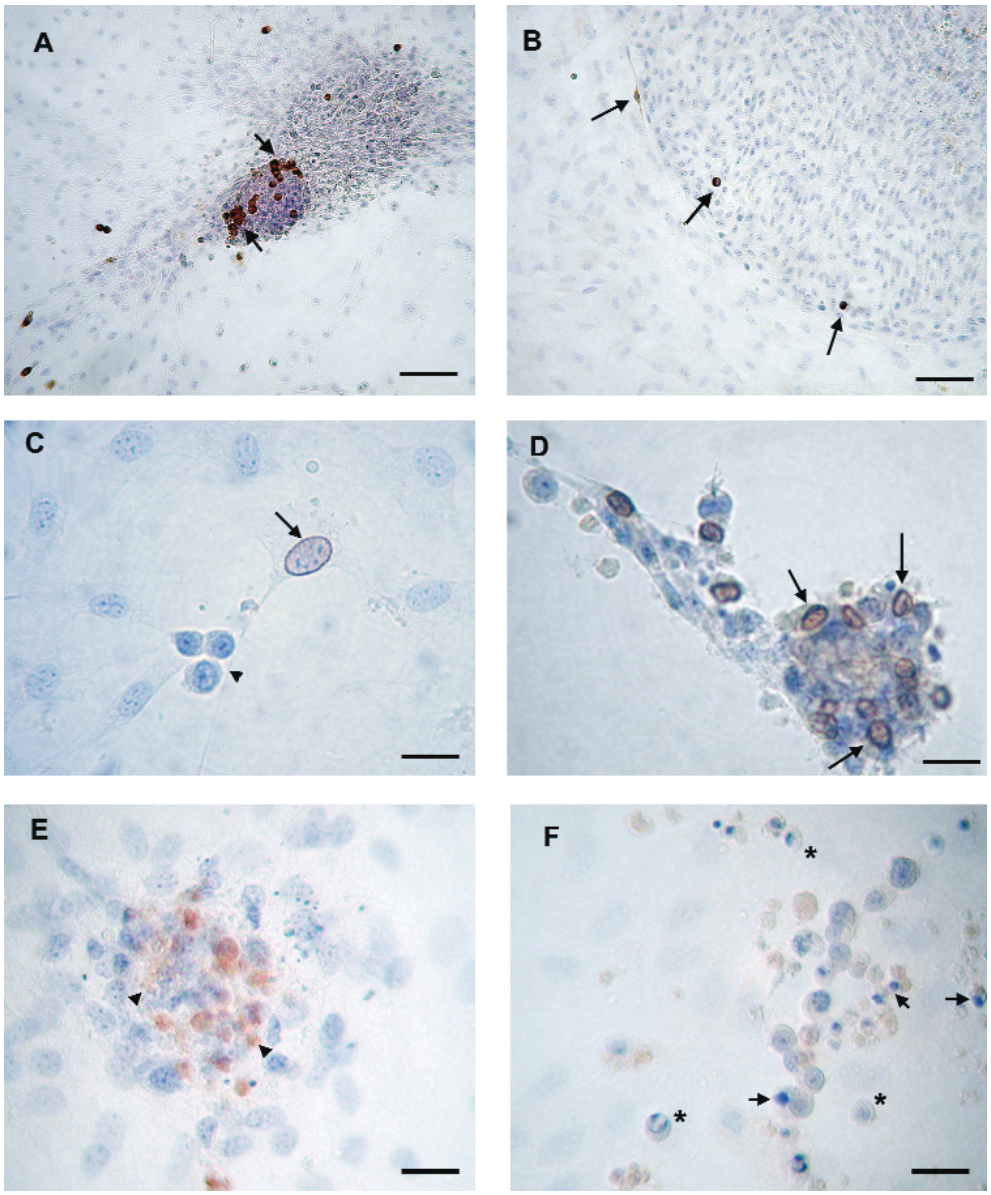

Fig. 5. Bovine type A spermatogonia co-cultured with Sertoli cells. (A) More frequently present radial colony stained with DBA lectin (Day 30). Notice central protrusion and round strongly DBA positive cells on the periphery (arrows). (B) Rarely appearing round colonies. They are weakly stained by DBA lectin and in most cases they show strongly positive round spermatogonia attached to their outer limit (arrows). (C) Sertoli cells positive to BrdU are frequently found in the monolayer (arrow) while type A spermatogonia resting on top are mostly in a quiescent state (arrowhead) (Day 15). (D) BrdU positive cells within the structure of the radial colony (arrows) (Day 15). (E) Dispersing colony with cellular debris (arrowheads) and probably in an initial stage of disorganization (Day 15). (F) Colony in disorganization process with loose cells (asterisks) and picnotic nuclei (arrows) (Day 15). Bar (A, B) = 80 μm; bar (C, D, E, F) = 20 μm.

Table 1. Labelling index (% BrdU incorporation) of spermatogonial clones in culture. Effect of GDNF.

	Treatment	
Clone	*Control*	*GDNF*
As	0.36 ± 0.16	0.17 ± 0.01
Apr	0.04 ± 0.04	0.02 ± 0.01
General	9.68 ± 0.72	8.91 ± 0.63

In general, BrdU positive cells at the rim of the colonies were single spermatogonia. The labeling indexes for scattered A_s and A_{pr} spermatogonial clones were not significantly different in control and GDNF treated cultures (Table 1). Important differences were found in the distribution of clonal sizes in culture (Table 2). GDNF affected the ratio between the numbers of single A spermatogonia and those of larger clones during the first 15 days of culture. For example, against 100 A_s spermatogonia there were 60.5 and 51.3 chains of A spermatogonia at Days 7 and 15, respectively, in control cultures, while these values were 38.2 and 25.5 in GDNF treated cultures ($P < 0.05$ and $P < 0.01$) (Table 2). However, this effect was not present at Day 25 (Table 2).

Table 2. Proportion of spermatogonial clones in culture (clonal indexes)

	Day 7	*Day 15*	*Day 25*
Control	100:40.3:60.5*	100:31.2:51.3**	100:28.6:1
GDNF	100:40.3:38.2*	100:31.2:25.5**	100:28.6:1

Effect of GDNF. Syntax: a:b:c, where a, single A spermatogonia, b, paired A spermatogonia and c, clones of three or more spermatogonia. Single type A spermatogonia were normalized to 100 cells. Numbers of A pairs against 100 singles were not significant in any time point, therefore, values shown are means. * $P < 0.05$. ** $P < 0.01$.

At Day 25, colonies remained DBA positive and turned into an even more compact mass of not individually distinguishable cells. Although there was proliferative activity in these colonies, as demonstrated by BrdU immunocytochemistry, it was not possible to determine a labeling index because of the high cell density. Around 15 days of culture some colonies were in the process of disappearing and dispersing as they showed numerous apoptotic cells and debris (Figure 5(E and F)). Colony numbers decreased approximately 6.5-fold between 15 and 25 days, both in control and GDNF treated cultures) while their size increased 2.6 times (Figure 4(A and B)).

Transplantation essay

Cells obtained from control and GDNF treated cultures were injected into mouse testes devoid of endogenous spermatogenesis. After collecting the testis (2 months posttransplantation) and staining sections for DBA to identify bovine spermatogonia, we found about two times more tubules with positive cells in the GDNF treatment (7.8 $\pm$ 0.6% versus 15.5 $\pm$ 1.1% in control and GDNF treatments, respectively; $P < 0.01$).

Monolayer cells

Sertoli cells were the main constituents of the monolayer, as evidenced by their nuclear morphology (Figure 5(C)). Furthermore, the great majority of the cells in the monolayer were positive for the Sertoli cell marker vimentin (Figure 6(A)) There was no apparent effect of GDNF on the monolayer. In general, Sertoli cells lost their long cytoplasmatic extensions after enzymatic digestion and transformed into round cells with crenated edges. Between Days 1 and 4 of culture they progressively produced extensions, flattened and attempted to make contact with other cells. At Day 4 they showed heavy proliferative activity as evidenced by the presence of numerous mitotic figures in the monolayer and a relatively high BrdU labeling index (41.2 $\pm$ 3.6%). With time, higher levels of confluency were gradually achieved. At Day 15 Sertoli cells were still proliferating, and no significant differences were found comparing control and GDNF treated cultures (i.e. 112.03 $\pm$ 27.46 and 142.10 $\pm$ 25.43% BrdU positive cells in control and GDNF treated cultures, respectively) (Figure 7). Around Day 25, Sertoli cell confluency was high (Figures 5A, B and 6B) and some of these cells could still be found in the S phase of the cycle (i.e. 5.43 $\pm$ 3.04 and 9.40 $\pm$ 5.51% in control and GDNF treated cultures, respectively) (Figure 7). Sertoli cell proliferation seemed to be related to the degree of confluency (Figure 7) Occasionally, Sertoli cells organized themselves surrounding empty areas, which resemble seminiferous

tubules cross-sections. (Figure 6(B)). In some cultures, elongated cells resembling peritubular cells appear in some areas, usually near colonies. (Figure 6(C)). Another cell type occasionally appeared in the monolayer in some cultures, showing a low nucleus/cytoplasm ratio, a globular cytoplasm and irregular and basophilic nuclei. These appeared alone or in connection to each other to form elongated structures, were DBA negative (Figure 6(D)) and actively engaged in proliferation (BrdU positive) in some areas.

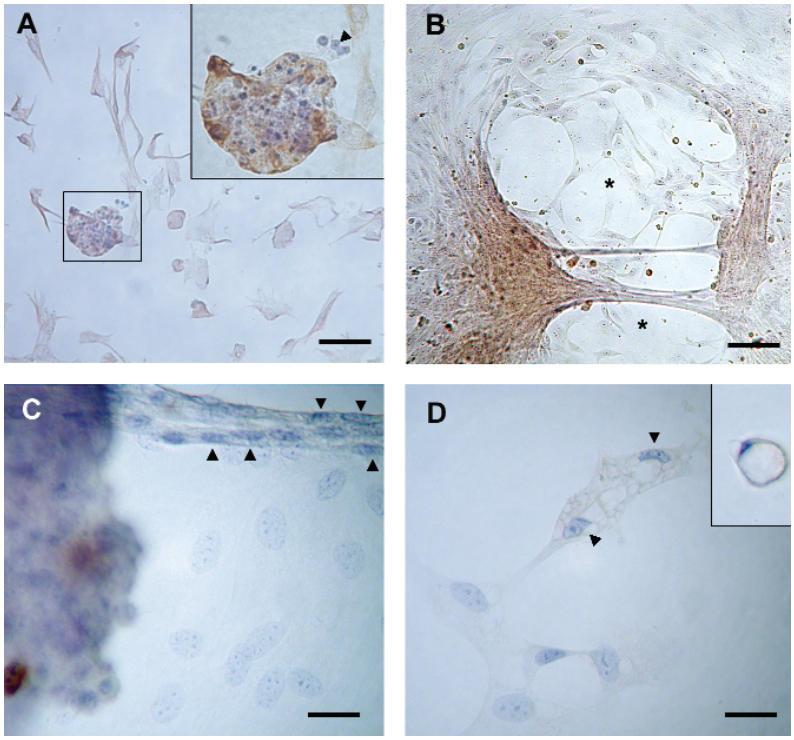

Fig. 6. Bovine Sertoli cell monolayer. (A) Vimentin immunostaining. Most cells in the monolayer and some within colonies are positive. Inset shows an enlargement of the boxed area. Germ cells are vimentin negative (arrowhead). (B) Round empty spaces in the monolayer of Sertoli cells resembling flat cross-sections of seminiferous tubules (asterisks). (C and D): Contaminating cells; (C) elongated cells, probably peritubular, adjacent to a colony (arrowheads). The colony (left) shows DBA positive cells (spermatogonia); (D) polygonal cells with large cytoplasm (arrowheads). Insert: these cells are single in some cultures and show a globular shape. Bar (A, B) = 80 μm; (C, D) = 20 μm.

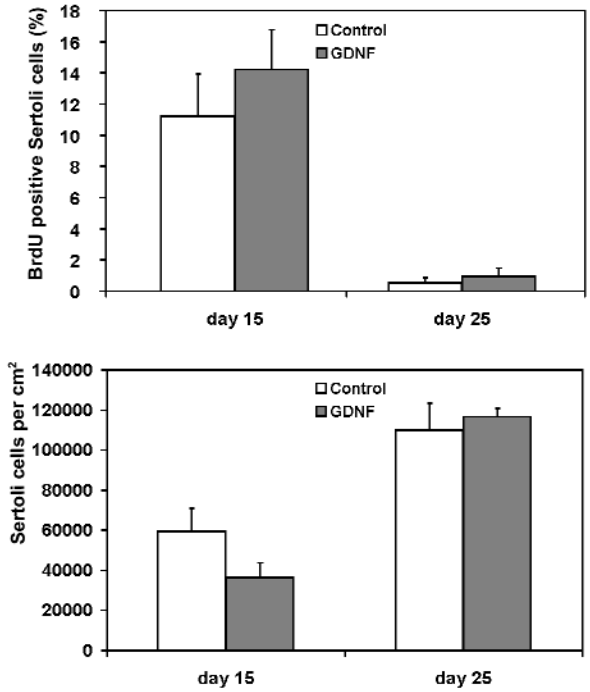

Fig. 7. Labeling index (A) and density (B) of Sertoli cells in control and GDNF treated cultures at Days 15 and 25.

Discussion

The present results provide a detailed description of the development of cultures of prepubertal bovine A spermatogonia on a monolayer formed by their accompanying premature Sertoli cells. Proliferation of type A spermatogonia and clone formation was followed and colonies of spermatogonia were found to appear from Day 6 onwards. In addition, the effect of GDNF on the cultures was studied and this growth factor was found to enhance spermatogonial numbers and the relative number of stem cells compared to controls. Our cultures started with a suspension of single cells consisting mainly of A spermatogonia and Sertoli cells. During the first days spermatogonia started to proliferate and formed pairs and chains of A spermatogonia. Also, many spermatogonia showed morphological signs of apoptosis and because of this there was an initial decrease in spermatogonial numbers. After Day 7, the decline in spermatogonial numbers slowed down. At about that time colonies started to appear in the form of mixed aggregates of loosely connected spermatogonia and Sertoli cells. These colonies continued to grow and condense. This process appeared to be the result of a balance between proliferation and cell death, since while some of the spermatogonia in the colonies incorporated BrdU, others showed morphological signs of apoptosis. The majority of the colonies continued to increase in size and became tightly packed.

Spermatogonia of various morphology coexisted within the cores of these colonies, suggesting that spermatogonial differentiation was taking place. Colony numbers declined during culture, very likely because some of the initial colonies disintegrated, as the percentage of small colonies decreased from Days 15 to 25. Therefore, we speculate this may be the result of apoptosis or detachment of differentating germ cells. However, the remaining surviving colonies increased in size. The Sertoli cell monolayer continuously developed from the start of the cultures both by an increase in cell numbers and by elongation of cytoplasmic extensions. The prepubertal origin of Sertoli cells, taken before their terminal differentiation into non-dividing cells, probably contributed to the success of the monolayer formation and its ability to support germ cells [24,25].

At the end of the culture (Day 25) this capacity seemed to diminish, since the number of spermatogonial clones on top of the monolayer clearly decreased. Furthermore, some Sertoli cells began to show vacuoles in the cytoplasm at this time. This has been reported as a sign of senes-

cence in 20–24 days cultured murine Sertoli cells of prepubertal origin [26]. In the human testis, vacuolization was also associated with aging [27]. We previously also found an initial heavy decline in spermatogonial numbers, but these increased again afterwards. [28]. In the present cultures spermatogonial numbers kept slowly decreasing after Day 7. This discrepancy is probably related to differences in seeding densities and/or in well size or shape, which were different in the present cultures. Nevertheless, we found comparable numbers of spermatogonia at Day 15, 1189 versus 1500 [28] spermatogonia per 0.28 cm^2 well. Bovine SSC were found to proliferate on an embryonic fibroblast feeder layer (BEF) during the first week of culture, as demonstrated by transplantation of the cultured bovine spermatogonia into nude mice [29]. Although a significantly higher number of SSC could be demonstrated at Day 7, their number dropped during the second week to values equal or lower than the starting condition of freshly thawed germ cells [29]. The addition of 100 ng/ mL GDNF to the culture did not further increase the number of SSC. Since the BEF monolayer was shown to produce GDNF, possibly the sum of exogenous and endogenous GDNF may have exerted a negative effect on SSC in this case. Furthermore, the increase of SSC activity was 2-fold when only the BEF feeder layer was used, and 1.35-fold after adding 100 ng/mL GDNF. A two times increase in SSC numbers, as we saw under our experimental conditions, seems to be more physiological, since SSC probably have the chance to divide only once during the first week. In our culture system probably the basic production of GDNF by the cultured Sertoli cells is very low as it was found to be FSH dependent [10] and FSH was not added to the cultures. In another study, pieces of 1–2- month-old calf testes were cultured and a 2.7-fold increase in germ cell numbers was found during the first 7 days and thereafter cell numbers remained stable up to Day 15 [9]. However, this will have mainly concerned gonocytes as at this age bovine spermatogenesis did not yet start [14,30]. Taken together, the data suggest that in general in bovine spermatogonial cultures many differentiating spermatogonia die during the first few days of culture and that many of the remaining cells at Day 7 are stem cells with the capability to generate the spermatogonial colonies. SSC are believed to be resistant to various insults for example irradiation [31–33] and high temperatures [34] and may preferentially survive over their differentiating cell progeny during the first week of culture. The great majority of the colonies in our bovine culture system, which started to appear from Day 7 onwards morphologically resembled the radial colonies described by Izadyar et al., [28]. The rare round colonies we found were very akin to the ones described by Izadyar et al., with respect to morphology, numbers

and size ranges. However, round colonies were rare and did not appear in every culture. Experiments have also been carried out with testis cells from 3-week-old pigs, also using a co-culture with Sertoli cells but with a 40% lower seeding density than that in our experiments. A continuous decline in spermatogonial numbers was observed during 9 days of culture (2.5-fold) [35]. Likewise, in a similar system, a decrease of the viability of mouse spermatogonia during culture has been reported [25]. The formation of colonies was not reported in either of these culture systems [25,35]. Nevertheless, colonies have been described as vesicular structures in primary cultures of rat testicular germ cells [36] and more recently for cultures of mouse germ cells [20,21,37]. In one of the latter reports, mouse colonies have also been described as compact masses of cells [37]. Interestingly, these authors also used serum in their culture medium but did not observe colony formation without the addition of growth factors. In our studies, colonies appeared in control cultures to which no growth factors had been added, suggesting species differences in factors needed to support colony formation. As demonstrated by the WST-1 assay, cell numbers increased after addition of GDNF to our cultures, in a dose-dependent manner. Since we could not find a stimulation of the proliferative activity of both spermatogonia and Sertoli cells by GDNF during long-term culture, it seems more likely that GDNF served as a survival factor for spermatogonia. Indeed there was a significantly higher number of small clones in GDNF treated cultures. Alternatively, GDNF preferentially stimulated proliferation within the colonies where we could not determine the numbers of BrdU positive cells. Interesting numerical relationships were observed with respect to spermatogonial clonal sizes. Significantly higher ratios between A_s and A_{al} were found in GDNF treated cultures during the first 2 weeks, suggesting a preference for self-renewal of the stem cells as an effect of GDNF. These results are in line with those showing that mouse spermatogonial suspensions coming from GDNF exposed cultures produced a higher colonization after transplantation into nude testes, than those coming from untreated cultures [7,8,20]. Colonies probably start to develop when spermatogonia and Sertoli cells make contact, apparently creating a microenvironment that favors their development. SSC might need specific microcontacts with niche-offering Sertoli cells during culture. The number of these special microenvironments is probably limited during culture, especially at the beginning, when Sertoli cells are still immature due to their prepubertal origin. SSC not finding their niche would probably commit to differentiation or become arrested in quiescence. Likely, each colony was founded by one SSC. In our cultures, the initial seeding density (4×10^4 spermatogonia/cm^2) represents 1

cell/5000 μm^2 and therefore in a theoretical even distribution there would be a distance of about 70 μm between cells. This would be enough to allow individual cells to autonomously start a colony. As GDNF did not enhance the number of colonies formed, the daughter cells of a stem cell carrying out a selfrenewing division apparently did not migrate away from each other to form separate colonies, but remained inside the colonies. Many of the colonies that appear when GDNF is added to the cultures, showed a high number of large, round shaped spermatogonia, typical of the immature, undifferentiated state [17,28,38]. There were around two times more of these cells in the outskirts of the colonies exposed to GDNF than in the controls. This, in addition to the increased proportion of A_s cells, also indicates an anti-differentiation activity of GDNF. Colonies with a compact appearance were found more frequently in control cultures. Their composition of very densely arranged smaller germ cells suggests that these cells were on the differentiation pathway. Since the c-kit receptor, a known marker for differentiating germ cells [39], has been found in mouse [21], and bovine [28] germ cell colonies, these small tightly packed cells are probably differentiating germ cells intermingled with their progenitor stem cells. The round spermatogonia frequently found at the periphery of our colonies in GDNF treated cultures morphologically resemble those in spermatogonial colonies of mouse origin that were found to appear only after addition of GDNF [37]. The latter cells were shown to be positive for α6- and β1-integrin, that in the testis are markers for SSC [37,40]. In our transplantation experiments there were about two times more stem cells in cultures to which GDNF was added than in control cultures. This suggests that these cells could be better maintained under the influence of GDNF during short-term culture. Since we found a higher single/spermatogonial clones ratio in the GDNF treated cultures at Days 7 and 15, suggesting a blockage of differentiation/enhanced selfrenewal, likely the transplanted cell suspensions previously cultured with GDNF had more SSC, the only cells which can relocate to the basal membrane in the seminiferous tubule. A better survival rate of SSC under the influence of GDNF during culture cannot be disregarded. In conclusion, the present study rendered a detailed description of the development of bovine spermatogonial colonies in a basic culture. This will provide a basis for further studies on the specific effects of growth factors. One of these growth factors, GDNF, was found to clearly favor self-renewal of SSC in cultures of bovine spermatogonia. In cultures with GDNF there were more As spermatogonia and within the colonies, where separate clones could not be discerned, many more primitive looking cells were present. In contrast, in control cultures, spermatogonial morphology changed as cells became smaller and more densely packed probably as a consequence of the start of differentiation of these cells.

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Propagation of bovine spermatogonial stem cells in vitro

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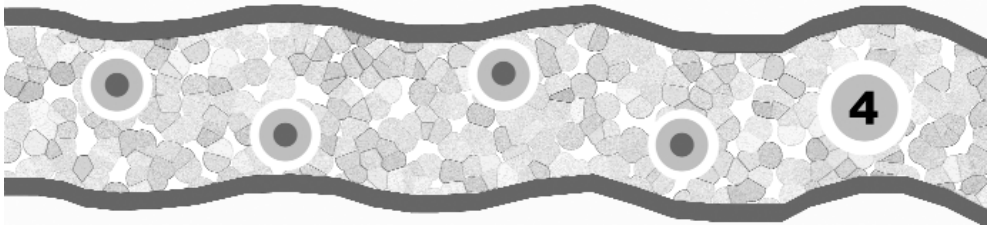
Chapter



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Abstract

The access to sufficient numbers of spermatogonial stem cells (SSCs) is a prerequisite for the study of their regulation and further biomanipulation. A specialized medium and several growth factors were tested to study the in vitro behavior of bovine type A spermatogonia, a cell population that includes the SSCs and can be specifically stained for the lectin *Dolichos biflorus* agglutinin. During short-term culture (2 weeks), colonies appeared, the morphology of which varied with the specific growth factor(s) added. Whenever the stem cell medium was used, round structures reminiscent of sectioned seminiferous tubules appeared in the core of the colonies. Remarkably, these round structures always contained type A spermatogonia. When leukemia inhibitory factor (LIF), epidermal growth factor (EGF), or fibroblast growth factor 2 (FGF2) were added, specific effects on the numbers and arrangement of somatic cells were observed. However, the number of type A spermatogonia was significantly higher in cultures to which glial cell line-derived neurotrophic factor (GDNF) was added and highest when GDNF, LIF, EGF, and FGF2 were all present. The latter suggests that a proper stimulation of the somatic cells is necessary for optimal stimulation of the germ cells in culture. Somatic cells present in the colonies included Sertoli cells, peritubular myoid cells, and a few Leydig cells. A transplantation experiment, using nude mice, showed the presence of SSCs among the cultured cells and in addition strongly suggested amore than 10 000-fold increase in the number of SSCs after 30 days of culture. These results demonstrate that bovine SSC self-renew in our specialized bovine culture system and that this system can be used for the propagation of these cells.

Introduction

Spermatogonial stem cells (SSCs) have the potential to selfrenew and at the same time generate the cascade of differentiating germ cells that will eventually lead to the formation of sperm. The regulation of the SSC self-renewal process is not yet completely understood. To study the characteristics of SSCs, large enough populations of pure SSCs must be isolated. One way to reach this goal is to propagate these cells in vitro and various attempts to achieve this have been carried out [1]. Interestingly, using a combination of growth factors and a special stem cell medium, Kanatsu-Shinohara et al., (2003b), by way of continuous subculturing during 4–5 months, could achieve an expansion of mouse SSCs in the order of 10^{12}-fold [2]. Kubota et al., (2004b) also induced

SSC renewal in a serum-free culture system based on a mitotically inactivated feeder layer and showed that the combination of glial cell line-derived neurotrophic factor (GDNF), soluble receptor for GDNF (GFRA1), and fibroblast growth factor 2 (FGF2) was best to induce self-renewal [3]. In both these systems, SSCs were found to form colonies. When the cells of these colonies were enzymatically dispersed and replated, their constituent SSCs could start new colonies. Colonies also appeared when bovine SSCs were grown on top of a Sertoli cell monolayer [4, 5]. In this system, the colonies appeared to be the result of complex interactions between SSCs and Sertoli cells [4, 5]. These colonies were also able to regenerate new colonies upon subculturing [6]. When adding GDNF, a member of the transforming growth factor-β superfamily, to these cultures, type A spermatogonia in the culture increased in number [5]. Moreover, SSC numbers increased twofold when compared with controls after 1 week of culture under the influence of GDNF [4, 5]. After transplantation of a germ cell suspension into the seminiferous tubules of a recipient mouse testis, the SSCs among the transplanted germ cells migrate to the basal membrane of the seminiferous tubules and subsequently start to form a repopulating colony of donor spermatogenesis. In this colony at first, stem cell renewal will prevail [7] but then also differentiating spermatogonia and subsequent germ cell types will be formed [8, 9]. However, because of the large phylogenetic distance between mouse and bovine, only type A spermatogonia are formed in the colonies originating from the bovine SSCs in the recipient mouse testis [6]. With time, the colonies grow along the length of the seminiferous tubules. One SSC will only give rise to one repopulating colony and when, as in this study, relatively few SSCs are transplanted very few if any donor SSCs will by chance seed in the same area and produce overlapping colonies. The more SSCs are transplanted the more colonies will be formed. With time, in each of these colonies the founding SSCs will give rise to more and more progeny, in the present experiment consisting of new SSCs (which are also type A spermatogonia) and type A spermatogonia that are at the beginning of the differentiation pathway. Therefore, in each transplanted mouse testis, the total number of bovine type A spermatogonia will be directly related to the number of originally transplanted bovine SSCs. In the present study, we tested a specific stem cell culture medium and various growth factors for their effects on qualitative and quantitative aspects of bovine SSC behavior in vitro. In particular, we studied the cellular composition of the colonies formed in culture and estimated changes in SSC numbers using the transplantation assay. The results indicate that bovine SSCs can be successfully propagated in culture, a crucial step in the stem cell technology field that will most likely impact the cattle industry.

Materials and Methods

Cell isolation and purification

Testes were collected from calves between 4 and 6 months of age at a commercial slaughterhouse. Isolation was performed as described previously [5, 6, 10]. Briefly, about 20 g testis material was minced into small pieces and suspended in MEM (Gibco, Invitrogen Corporation) supplemented with 0.1275% w/v $NaHCO_3$ (Sigma), 4 mM L-glutamine, 0.1 mM single-strength nonessential amino acids, 100 IU/mL–100 mg/mL penicillin– streptomycin, 40 μg/mL gentamicin, and 15 mM HEPES (all from Gibco), subsequently referred to as supplemented MEM. The procedure included two enzymatic digestions, filtration, overnight differential plating, and discontinuous Percoll density gradient centrifugation [10]. Fractions were evaluated under a Nomarski interference microscope and an Axioskop (Zeiss, Göttingen, Germany) for estimation of the percentage of type A spermatogonia. The fractions consisting of between 50 and 80% type A spermatogonia were used. The viability of the primary isolated cells was evaluated using LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR, USA) and the total number of type A spermatogonia in the suspension was estimated with a hemocytometer.

Short-term culture

Cells were seeded at a concentration of 100 cells/mL under seven different culture conditions (Table 1). The defined medium used for conditions 2 through 7 was originally used by Kanatsu-Shinohara et al., (2003b) [2]. Briefly, the medium consisted of StemPro-34 serum free medium (SFM) (Invitrogen) supplemented with StemPro supplement (Invitrogen), 25 μg/mL insulin, 100 μg/mL transferrin, 60 μM putrescine, 30 nM sodium selenite, 6 mg/mL D-(1)-glucose, 30 μg/mL pyruvic acid, 1 μL/mL DL-lactic acid (Sigma), 5 mg/mL bovine albumin ImmunO (MP Biochemicals LLC, Solon, OH, USA), 2 mM L-glutamine, 5 x 10^{-5} M 2-mercaptoethanol, minimal essential medium (MEM) vitamin solution (Invitrogen), MEM nonessential amino acid solution (Invitrogen), 10^{-4} M ascorbic acid, 10 mg/mL D-biotin, 30 ng/mL b-estradiol, and 60 ng/mL progesterone (Sigma). When present, the growth factors were added at the following concentrations: EGF 20 ng/mL, FGF2 10 ng/mL, LIF 100 ng/mL, and GDNF 40 ng/mL (all human recombinant from Sigma). Fetal calf serum (FCS; Invitrogen) was used at a concentration of 2.5%

v/v for condition 1, as in our previous work involving supplemented MEM [5, 6]. For the other conditions, the concentration was 1% v/v [2]. Cells were cultured in 4-well chamber slides of 2 cm2/well (Lab-Tek, Nalge Nunc Inc., Naperville, IL, USA) at 37 °C, a humidified atmosphere with 5% CO_2, and refreshed twice a week. The colonies were collected at day 15 by pipetting with a manually bended pipette tip under a stereomicroscope (Nikon SMZ800, Tokyo, Japan) and kept on PBS at 4 °C until fixation.

Table 1 Summary of experimental culture conditions.

Condition	Medium	Fetal calf serum (%)	Growth factor (s)
1	Supplemented MEM	2.5	---
2	Stem cell medium	1	---
3			LIF, EGF, FGF2, GDNF
4			LIF
5			EGF
6			FGF2
7			GDNF

Histology

The colonies were fixed in Bouins fluid and kept in ethanol 70%. To avoid loss during histological processing, the colonies were stained with Mayer's hematoxylin (Sigma) for 30 s, transferred to a drop of 0.5% w/v Pronarose LEEO agar (SphaeroQ, Gorinchen, The Netherlands) at 41°C over a hot plate and let cool at room temperature before placing the specimen in biopsy embedding cassettes (Adamas Instruments, Leersum, The Netherlands) to be further histologically processed. The samples were embedded in paraffin and the colonies were serially sectioned.

Immunohistochemistry

Type A spermatogonia were identified through DBA immunohistochemistry, as described by Ertl & Wrobel (1992) and Izadyar et al., (2002a) [6, 11]. Briefly, the sections were rehydrated, treated with 3% v/v H_2O_2 (Sigma) for 10 min in the dark to inhibit endogenous peroxidase, and rinsed in PBS. Unspecific site blocking was done with 5% w/v BSA in PBS for 15 min and then sections were washed with HEPES buffer (1% v/v HEPES buffer, Invitrogen, 0.1 M NaCl in

distilled water). Subsequently, the samples were incubated with biotinylated DBA (Vector Laboratories, Inc., Burlingame, CA, USA) at 1:500 in HEPES for 1 h at 37 °C in a moist chamber. After incubation with DBA, the samples were rinsed with HEPES and washed three times in PBS. The horseradish peroxidase–biotin complex reaction was performed using an ABC Kit (Vector Laboratories), 1:500 each in PBS. Lectin binding was visualized using 25 mg of 3,3'- diaminobenzidine tetrahydrochloride (DAB; Sigma), as chromogen substrate, and 17 μL of 35% v/v H_2O_2 per 50 mL in PBS for 1 min. The samples were rinsed thoroughly in distilled water, counterstained with hematoxylin (Sigma), dehydrated in graded alcohols, cleared in xylol, and mounted in Pertex (Histolab, Göteborg, Sweden). Negative control sections were incubated in 1% w/v BSA in PBS without lectin. Vimentin immunostaining was carried out in order to identify Sertoli cells. After deparaffination and rehydration, endogenous peroxidase was inhibited, samples washed with PBS, and treated with Triton X-100 (Sigma–Aldrich) at 0.5% v/v for 30 min. Unspecific-site blocking was done with Normal Goat Serum (Vector Laboratories). The first antibody was mouse antivimentin and non-hematopoietic (Biogenex, San Ramon, CA, USA) at concentration 1:50. The second antibody was immunoglobulin M (IgM) anti-mouse (Vector Laboratories). ABC and DAB steps were done as described for the DBA immunostaining. All inter-step PBS washes, except those after ABC treatment, were followed by a simple wash with 0.1% Tween (Merck- Schuchard) in PBS as well as the blocking and antibody steps. Similarly, α-SMA immunostaining was performed to identify myoid peritubular cells. A pretreatment was done with Triton X-100 0.4% for 10 min (Sigma). Blockage was with Normal Horse Serum 5% v/v (Vector Laboratories) for 1 h at room temperature. The first antibody was anti-α-SMA Mu-128 (Biogenex) 1:500, in 1% w/v BSA in PBS, at 4 °C, overnight. The second antibody was IgM anti-mouse (Vector Laboratories), 1:200, in 1% BSA in PBS at room temperature. The rest of the procedure was as in DBA and vimentin immunostaining. Staining 3 β -HSD type 1 was performed as described by Teerds et al., (1999) [12]. Briefly, the sections were incubated overnight at 4 °C with a polyclonal anti-3β-HSD type 1 antibody raised in goat (Santa Cruz, (C-18, SC-30821), Santa Cruz, CA, USA) diluted 1:300 in TBS+BSA-c (Aurion, Wageningen, The Netherlands). This was followed by five washes with TBS and incubation with a biotinylated secondary rabbit-anti-goat antibody (Vector Laboratories) diluted 1:200 in TBS+BSA-c for 1 h at room temperature. Slides were again washed with TBS and incubated for 60 min with the components avidin (A) and biotin (B) of the ABC staining kit elite (Vector Laboratories) diluted 1:1500 in TBS+BSA-c. Bound antibody was visualized using 0.6 mg/mL solution of 3,3'- diaminobenzidin

in TBS to which 0.03% H_2O_2 was added. The slides were counterstained with Mayer's hematoxylin. In the control experiments, the first antibody was replaced by normal goat serum. No background staining was observed (not shown).

Stereology

Volume density (or cells per volume unit), and absolute numbers of DBA-positive cells (spermatogonia) in both colonies and transplanted testes were estimated through unbiased stereological methods. For this purpose, one in every tenth 5 μm thick sections was used (with a random sectioning start). Colony volumes were estimated by the Cavalieri method [13], by adding the several sub-volumes constituting the colony. Each sub-volume was defined as the product of the area of the colony profile in the section by the distance to the next section analyzed, that is the area of the profile of the colony x 5 μm, which is the section thickness x 10, or the number of sections in between samples (in summary, profile area x 50 μm). The areas of the profiles of the colonies were measured using the ImageJ 1.34S program, developed by Dr. W Rasband at the National Institute of Health, Bethesda, USA, and downloaded from the Internet at http://rsb.info.nih.gov/ij/, previously applying the appropriate calibration. Spermatogonial density (spermatogonia per volume unit) is a required input to calculate the absolute numbers of these cells. It was estimated using a stereological method: the physical disector. For this, colony images from serial consecutive pairs of sections (a consecutive pair taken every tenth section with a random start in the series) were digitalized. The image pairs were exposed simultaneously side by side on a wide computer screen and square-shaped counting frames were randomly generated and positioned in the same corresponding area in both images with the help of software developed by Dr M Terlou, Department of Biology, Utrecht University, The Netherlands. DBA-positive cells that appeared in the first image (called reference section) but had disappeared in the same area in the second section (look-up section) were counted. Whenever a DBA-positive cell appeared in both sections, it was not included in the counting [13]. The area of the counting frame was recorded and used to calculate the sample volume to refer to the counting. This volume was equal to the counting frame area times the length of the space between the consecutive pair of sections (5 μm). The division of the reference volume by the counted DBA-positive cells yielded figures of density, i.e. number of spermatogonia per volume unit (spermatogonia/μm^3). Absolute numbers of spermatogonia were estimated by the product of the volume of the colony

(estimated through the Cavalieri method, as previously described) times the spermatogonial density, that is, colony volume (μm^3) x density (spermatogonia/μm^3). Absolute numbers of spermatogonia per colony were multiplied by the number of colonies in each well to obtain the number of spermatogonia per well.

Long-term culture

The cells were seeded at 50 cells/μL on 6-well dishes (Lab-Tek, Nalge Nunc Int.) with stem cell medium, 1% v/v FCS, and growth factors (EGF, FGF2, LIF, and GDNF at concentrations specified elsewhere) during 26–30 days, during which the cells were subcultured six to seven times. The cells (germ and somatic cells) were separated through enzymatic digestion and gentle pipetting. The cells were cultured in culture dishes in which the medium was bathing the cells from above. The cultures were refreshed twice a week with the corresponding growth factors, and passaged 1:2 to 1:4 every 3–5 days, depending on the rate of somatic cell proliferation. The cells had to be passaged just before 100% confluency to avoid contact inhibition and subsequent detachment of the monolayer from the culture plate. Whenever detected incidental foldings or pealing off the monolayer, mainly during early pilot experiments, we aborted the cultures and started all over again.

Type A spermatogonia quantification during 26-day culture

The number of type A spermatogonia at different time points during culture could be estimated through the formula: Type A number = Total number of cells x Type A purity. At every passage, the total number of cells was estimated with the aid of a hemocytometer and the purity of type A spermatogonia (%) was determined in smears of the cells that previously underwent DBA staining. This culture was started with freshly isolated cells.

SSC transplantation

The cells were frozen at day 0 (directly from a primary cell isolation) and day 30 (end of serial subculture) to be thawed on the day of transplantation. Freezing and thawing protocols for bovine spermatogonia have been described previously [14]. The cells from each of the two experimental groups (n=3) were transplanted into irradiated NMRI nude mouse (NU/NU, Harlan, Horst, The

Netherlands) testes (5000 cells/μL or 10^5 cells in 20 μL cell suspension per transplanted testis) for SSC functional testing as described previously [6, 15]. The mice were killed after 2 months and the testes collected for histological processing. DBA staining was carried out to evaluate the presence of bovine type A spermatogonia in the recipient mouse testes. DBA-positive cells were counted in an unbiased way in serial sections using the disector method [13]. The experimental protocol of this experiment followed the Guidelines for the Care and Use of Laboratory Animals and was approved by the Animal Care and Use Committee of the Utrecht University.

Estimation of SSC propagation

Using DBA immunohistochemistry, the numbers of type A spermatogonia in the starting germ cell population and in the suspensions of germ cells after culture were estimated and about similar numbers of cells (100 000) with known contents of type A spermatogonia were transplanted into each recipient mouse testis. Two months after transplantation, the numbers of bovine type A spermatogonia in the recipient mouse testes were estimated using unbiased stereological methods (see section Stereology). Subsequently, the dilution factor of the cultured type A spermatogonia was calculated as these cells went through multiple passages in which they were diluted. Knowing these data, the fold increase in SSC numbers after culture could be calculated, as described.

Statistical analysis

General statistical analysis was performed using the independent samples Student's t-test or one-way ANOVA. Multiple comparisons were performed by means of Bonferroni post hoc test. Results are presented as the mean ± S.E.M. All calculations were performed using the software SPSS for Windows v11.5 (Chicago, Illinois, USA).

Results

Colony formation

All cultures started with a mixed population of cells including bovine type A spermatogonia and somatic cells. The percentage of contamination with somatic cells was about 60% after differential plating, and then dropping to 30% after Percoll discontinuous gradient centrifugation. Colonies formed locally in some areas of the culture plate. The colony-forming cells in the initial suspension, including both somatic and germ cells, approached each other and became tightly packed as the colonies continued to grow during their development (Figure 1A–D). This behavior was regardless of the treatment. Between days 7 and 15 of culture, the colonies grew about sevenfold in volume independently of the treatment. Colonies appeared de novo and not as a result of cells detaching or the monolayer folding upon confluency. Some somatic cells formed the monolayer while some others committed to colony formation. Cytoplasmic extensions from peripherally located somatic cells in the colonies connected with nearby germ cells at the time colonies could be observed for the first time (Figure 1E). Type A spermatogonia also showed contacting cytoplasmic extensions (Figure 1F).

The growth of the colonies seemed to occur preferentially at the outskirts of the colonies, since in these areas many somatic cells showed mitotic figures (Figure 2E). As development progressed, the colonies remained attached to the underlying monolayer by a stalk. Thus, once formed, colonies maintain a short physical connection with the monolayer. This became evident pulling out the colonies for histological processing. Additionally, in some sectioned colonies, part of the broken stalk could be seen to be made up of cells similar to those in the monolayer. Comparison of cultures in supplemented minimum essential medium (MEM) and stem cell medium A specialized stem cell medium was used, based on a hematopoietic stem cell proprietary medium with several enrichments [2]. The new stem cell medium outperformed supplemented MEM in terms of number of colonies appearing in culture ($P < 0.01$) and number of *Dolichos biflorus* agglutinin (DBA)-positive cells, i.e., type A spermatogonia ($P < 0.01$; Figure 3). Consequently, in further experimentation, only the stem cell medium was used. Colonies appearing in cultures in which supplemented MEM was used were smaller than those with the stem cell medium (0.003 ± 0.002 mm^3 *vs* 0.024 ± 0.008 mm^3 respectively; $P < 0.05$). Supplemented MEM

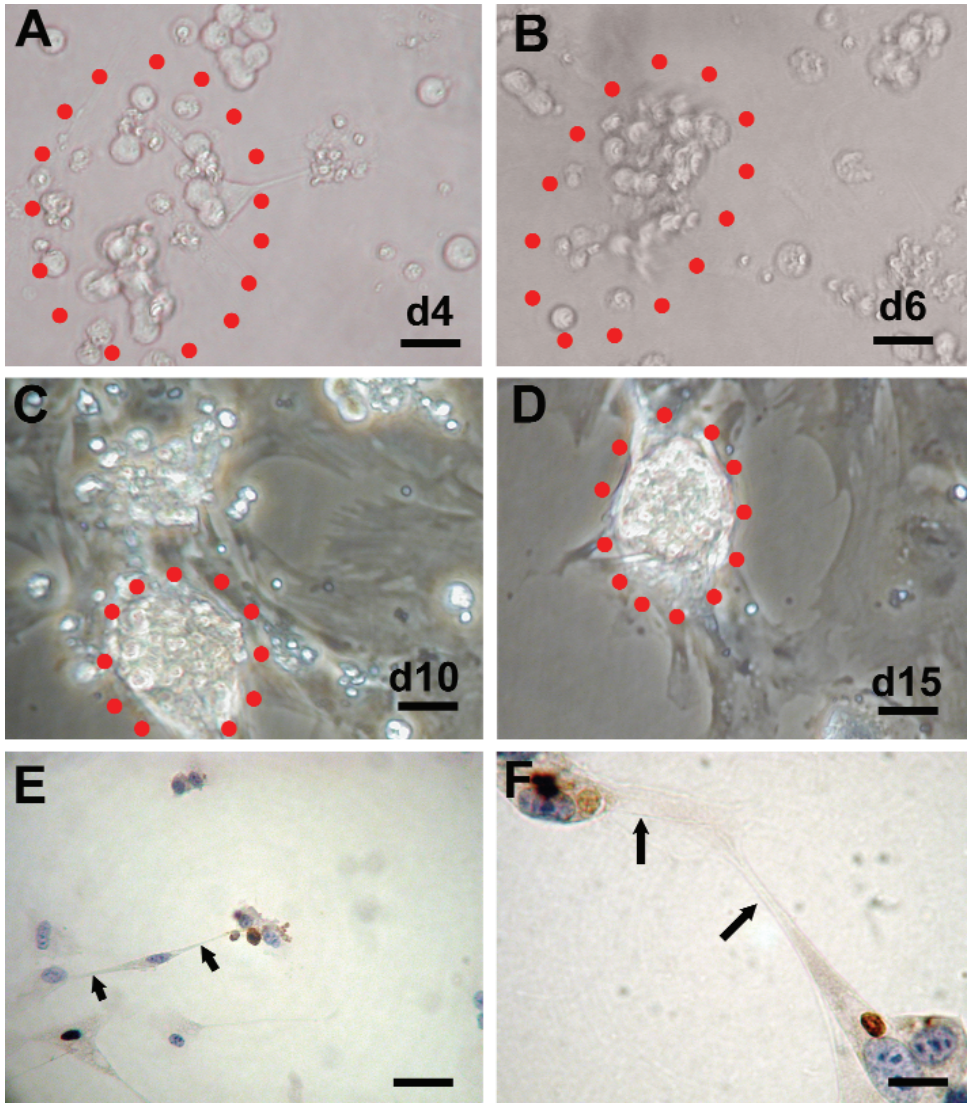

Fig. 1. Typical development of colonies during short-term culture in supplemented MEM medium without growth factors. (A–D) Serial microphotographs of one area of the culture plate showing the development of one bovine germ cell–somatic cell colony during short-term culture; d4, d6, d10, and d15 are days 4, 6, 10, and 15 respectively. Red dots encircle the cells involved in the formation of the colony shown. Nomarski optics. Bar=20 μm. (E) Cytoplasmic extensions (arrows) of one somatic cell reaching a small group of colony-forming typeA spermatogonia. Bar=25 μm. (F) Cytoplasmic extensions (arrows) of type A spermatogonia contacting each other within a forming colony. Bar=10 μm. (E) and (F) were obtained from 4-day culture plates immunostained for DBA (brown) and counterstained with Mayer's hematoxylin (blue). DBA (brown) joins carbohydrates at the Golgi system of type A spermatogonia [11]. The process of colony formation that took place using MEM medium was similar to other treatments involving the use of stem cell medium.

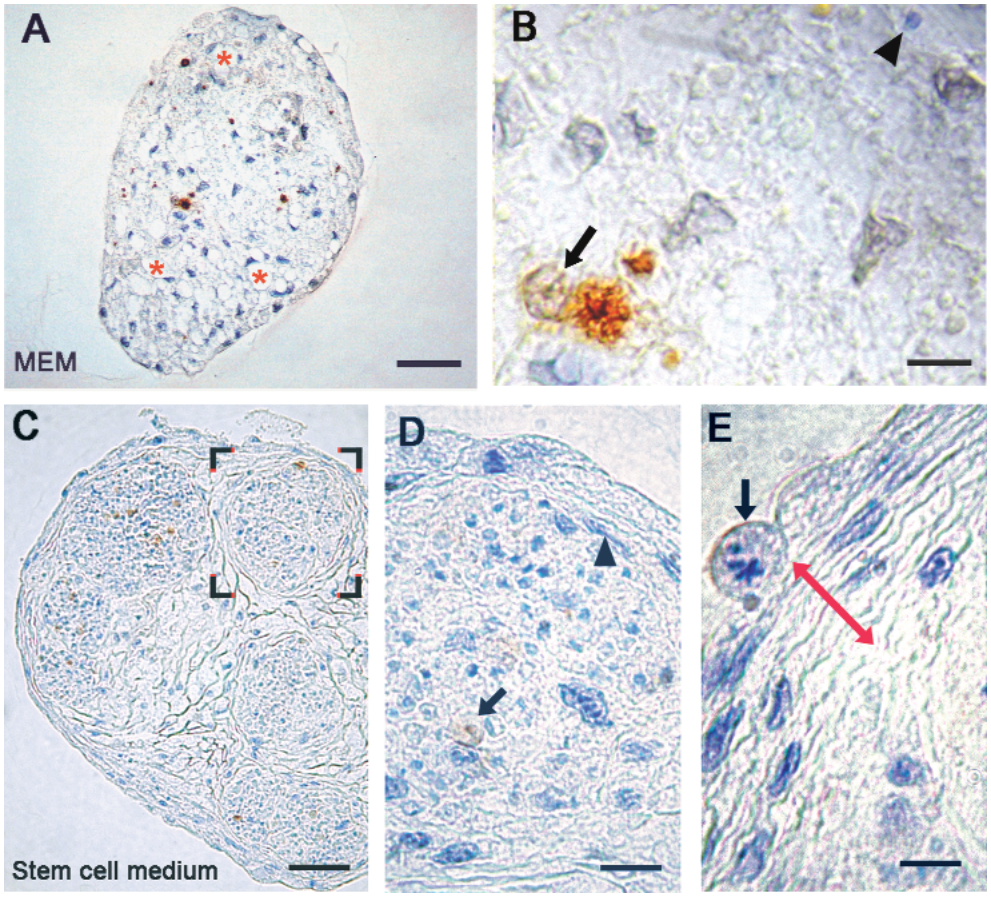

Fig. 2. Comparison of two different culture media (MEM versus stem cell medium) on the morphology of bovine spermatogonial cultures after 15 days. All images originate from histological sections of colonies immunostained for DBA (brown) and counterstained with Mayer's hematoxylin (blue). (A and B) MEM (MEM refers to supplemented MEM, see Materials and Methods). (C–E) Stem cell medium. (A) MEM colonies were smaller in size when compared with colonies appearing with stem cell medium and showed wide intercellular spaces and even lacunae in the core of the colonies (*). Bar = 85 μm. (B) MEM colonies showed numerous pycnotic nuclei (arrowheads) among normal looking type A spermatogonia (big round nucleus with one large central nucleolus) (arrows). Bar = 15 μm. (C) With the stem cell specialized medium, areas resembling round crosssections of seminiferous tubules are formed inside the colonies (one example in framed area). Notably, no such structures were seen in colonies arising using supplemented MEM. Bar = 200 μm. (D) Type A spermatogonia were always located inside the round areas (arrow). These areas had surrounding layers of somatic flattened cells (arrowhead). Bar = 20 μm. (E) The colonies had an outer capsule made up of somatic flattened cell layers (red double head arrow) which showed evidence of proliferation as mitotic Fig.s were seen (arrow). Bar = 15 μm.

colonies showed that many empty intercellular spaces surrounding irregular areas of dead cells and debris where spermatogonia could be seen in scattered patterns (Figures 2A and B). By contrast, the sections of stem cell medium colonies contained round structures within the core of the colonies (Figure 2C and D). A three-dimensional reconstruction of these round structures would most likely render a cyst. Cross-sections of these structures were round with clear sharp boundaries organized by flat somatic cells, morphologically similar to peritubular myoid cells. Interestingly, the DBA-positive type A spermatogonia were always within the boundaries of these structures. Most other somatic cells remained outside of the round structures, forming the bulk of the colonies (Figure 2C). Overall, in the stem cell medium, the somatic cell areas had a higher cell density than in the supplemented MEM colonies. To learn more about the nature of the somatic cells in culture, immunohistochemistry was carried out using a-smooth muscle actin (α-SMA) as a marker for peritubular myoid cells, vimentin as a marker for Sertoli cells, and 3β-hydroxysteroid dehydrogenase (3β-HSD) type 1 to detect Leydig cells. Indeed in sections of

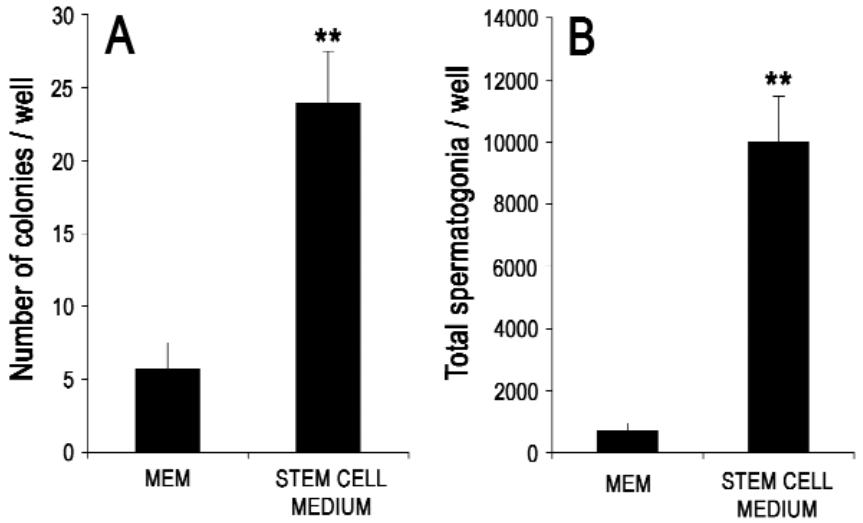

Fig. 3. Comparison of the effect of two different culture media (supplemented MEM versus stem cell medium) on the formation of colonies in bovine spermatogonial cultures. (A) Number of colonies per 2 cm^2 well after 15 days of culture (**$P < 0.01$). (B) Absolute number of DBA (*Dolichos biflorus* agglutinin)-positive bovine type A spermatogonia in colonies as estimated by stereological methods. MEM refers to supplemented MEM (see Materials and Methods). The results are presented as the mean ± S.E.M. of three independent experiments (**$P < 0.01$)

bovine testes, the antibodies against α -SMA, vimentin, and 3β -HSD detected peritubular myoid cells, Sertoli cells, and Leydig cells respectively (Fig. 4E–G). In cultures, immunohistochemistry for α-SMA showed reaction primarily at the outskirts of the colonies with some positive cells scattered through the colonies, but only occasionally within the round structures (Fig. 4A and C). Vimentin-positive cells were located also in the outskirts but were more widely spread within the colonies than α-SMA-positive cells, especially within the round structures (Fig. 4B and D). Some flat cells circumscribing the round structures were vimentin positive, while a smaller number stained positive for α-SMA (Fig. 4C and D). Immunohistochemical staining for 3β –HSD type 1, revealed a few rare Leydig cells in the culture (Fig. 4H), their number being less than 1 per 200 cells..

Effect of adding growth factors

Leukemia inhibitory factor (LIF)

When LIF was added, the interior of the colonies was similar to that in colonies arising in cultures in stem cell medium only, but the border of the round structures was less defined and the spermatogonia within these areas were lying in a non-characteristic pattern together with many cells with pycnotic nuclei. The round areas were surrounded by several compact, concentric layers of somatic cells with few intercellular empty spaces (Fig. 5A, D and G).

Epidermal growth factor (EGF)

Under the influence of EGF, the colonies appeared as spherical complexes joined by bridges (Fig. 5B, E and H). These bridges were composed of somatic cells that merged with those present within the spherical units of the colonies. The interior of the colonies was rather spongiform with scattered, ill-defined round structures where spermatogonia could be seen inside. Occasionally, medium to large empty, cyst-like areas could be observed.

Fibroblast growth factor 2 (FGF2)

Unlike the other growth factors, FGF2 induced the formation of rather large elongated worm-shaped structures, three-dimensional in shape as could be observed through the stereomicroscope. Occasionally, they appeared as the usual spherical colonies. Inside of the colonies, there were

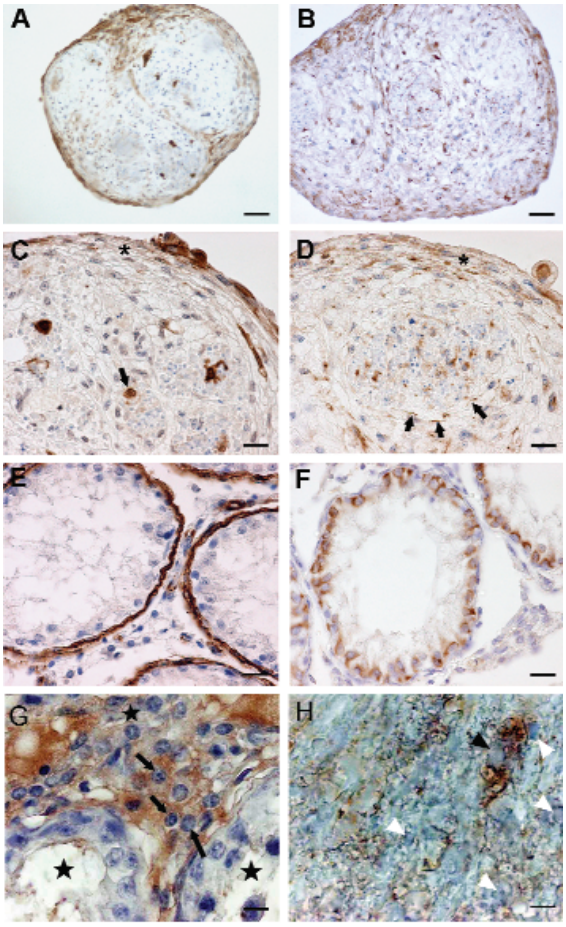

Fig. 4. Identification of somatic cells in bovine spermatogonial cultures. (A and B) General pattern of immunostaining (brown) of α-smooth muscle actin (α -SMA) (A) and vimentin (B) in histological sections of spermatogonial colonies from 15-day old cultures and treated with four growth factors (EGF FGF2, LIF, and GDNF). Bar = 80 μm. (C and D) Details of immunostaining (brown) of α -SMA (C) and vimentin (D) of histological sections of the described colonies. Many vimentin and α-SMA positive cells are located at the colony outer somatic cell concentric layers (*). Rarely, α-SMApositive cells were surrounding round areas resembling cross-sectioned seminiferous tubules (arrow, C) while some surrounding flat cells were vimentin positive (arrows, D). Bar = 40 μm. (E) α -SMA and (F) vimentin immunostainings of bovine seminiferous tubules showing α -SMA is a specific myoid peritubular cell marker. Some endothelial cells of the capillaries in the intersticium stain positive as well; this has been shown to be a specific pattern of staining for bovine species [16] (E, brown), while vimentin specifically marks Sertoli cells within the seminiferous tubules (F, brown). In both panels, (E) and (F), cross-sections of seminiferous tubules correspond to prepubertal bulls closely after the start of spermatogenesis. Thus, the only germ cells present are a few type A spermatogonia (not stained). Bar = 40 μm. Immunohistochemical staining for the presence of 3β -HSD type 1, a marker for Leydig cells. (G) Pre-pubertal bull testis, 3β-HSD-positive Leydig cells (arrows) are located in the interstitium surrounded by seminiferous tubules (asterisk). Bar = 13 μm. (H) Stem cell culture, a contaminating Leydig cell is indicated by a black arrowhead. The surrounding cells are indicated by white arrowheads. Contaminating Leydig cells were rarely observed in the cultures (< 0.5%). Bar = 6 μm. All stainings were counterstained with Mayer's hematoxylin (blue).

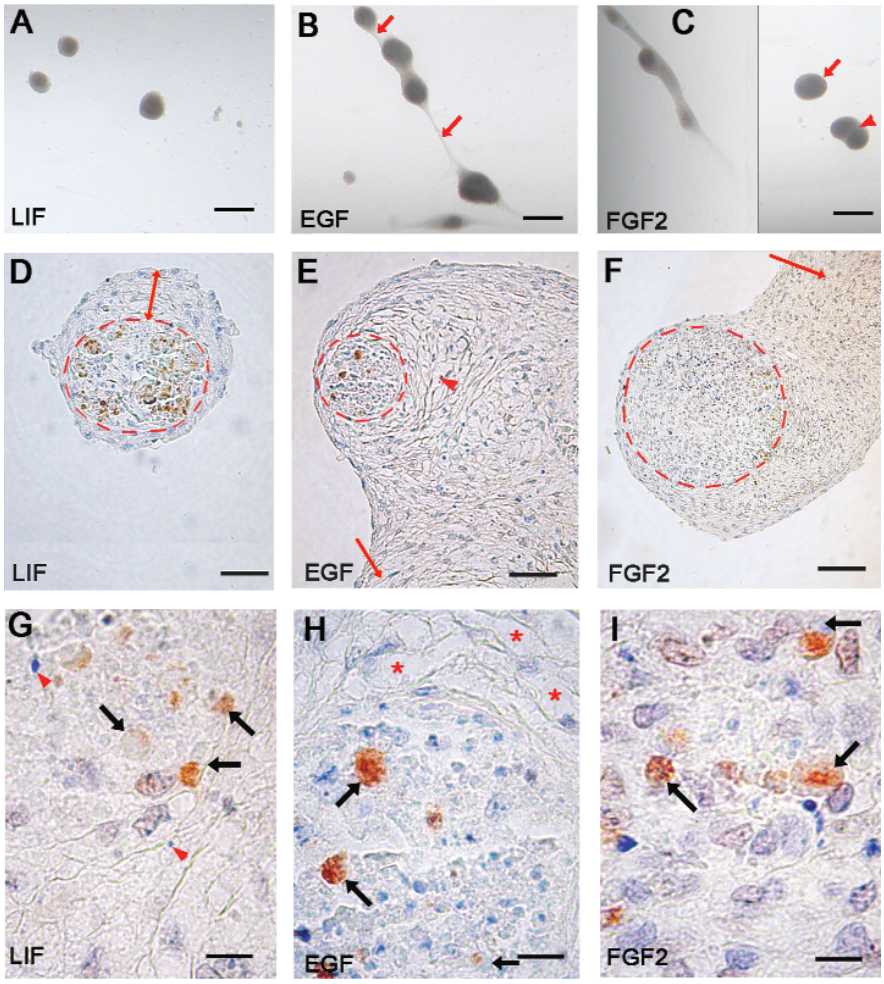

Fig. 5. Morphology of colonies appearing under the effect of the growth factors LIF, EGF, or FGF2 (short-term culture, 15 days). (A–C) The general morphology of the colonies. LIF colonies (A) were spherical, while EGF colonies (B) were made up of spheres connected by bridges (arrows) and FGF2 (C) colonies were elongated structures with sometimes single (arrow) or double spheres (arrowhead). (A–C) Bar = 0.5 mm. Sections of colonies were immunostained for DBA (brown) to detect bovine type A spermatogonia. Note that these cells concentrate in round areas resembling round cross-sections of seminiferous tubules in all cases. Counterstaining with Mayer's hematoxylin stained the nuclei of all cells (blue); LIF (D and G), EGF (E and H), FGF2 (F and I). (D–F) Depict the round areas (red outline) in colonies appearing in the presence of LIF, EGF, and FGF2 respectively. LIF colonies (D) had a not well-defined border of the round areas, surrounded by thick layers of concentric somatic cells (red double head arrow). EGF colonies (E) showed empty cystic spaces (arrowhead) and bridge areas were composed solely of somatic cells (red arrow). The elongated parts of FGF2 colonies (F) had no round structures and consisted of somatic cells (red arrow). (D and E) Bar = 50 μm and (F) 100 μm. (G–I) High magnification images that show round areas with type A spermatogonia inside (arrows). With LIF (G), round areas have irregular boundaries and pycnotic nuclei (arrow heads). EGF colonies (H) show lacunae, scattered in the intercellular space in somatic areas (*). The somatic areas of FGF2 colonies (I) appear very tightly packed with scarce intercellular space. (G–I) Bar = 10 μm.

also round structures with unclear borders, inside which type A spermatogonia were confined. The surrounding somatic cells showed a very compact arrangement with scarce empty intercellular spaces (Fig. 5C, F and I).

Glial cell line-derived neurotrophic factor (GDNF)

In cultures to which GDNF was added, the spherical colonies were formed with large internal round areas with many bovine spermatogonia (DBA positive) with the typical morphology (big round nucleus with one large central nucleolus) inside. In some colony sections, the round areas represented as much as about 50% of the colony area and were usually located eccentrically (toward the outer limit of the colonies) with scarce surrounding somatic cells (Fig. 6A–C).

LIF, EGF, FGF2, and GDNF

When cultures were carried out using a combination of the four growth factors, colonies appeared as very large ovoid structures and showed a mixture of characteristics seen when individual growth factors were added (Fig. 6D–H). For instance, large internal round areas commonly placed toward the periphery with many morphologically normal spermatogonia inside with a big round nucleus and single large nucleolus, as seen with GDNF, but embedded in compact somatic cells with scarce empty intercellular spaces which is typical for cultures to which only FGF2 was added (Fig. 5). The stereological variable volume density of the round areas (percentage of the total colony volume occupied by round areas) was significantly smaller in the EGF treated colonies ($17.8 \pm 0.3\%$), ($P < 0.05$), than with the rest of the growth factor treatments (range 27.8 ± 0.7 to $48.8 \pm 0.7\%$). In general, somatic cell numbers increased during culture. The germ cell:somatic cell ratio changed during culture with the four growth factors from 2.33 (starting cell suspension) to 0.146.

The effects of growth factors on spermatogonia

More colonies tended to appear in cultures to which all four growth factors were added, but no statistical significance was found with the different growth factor treatments (Fig. 7A). When the data were pooled, there were 21.0 ± 1.6 colonies/well. Nevertheless, there were significant

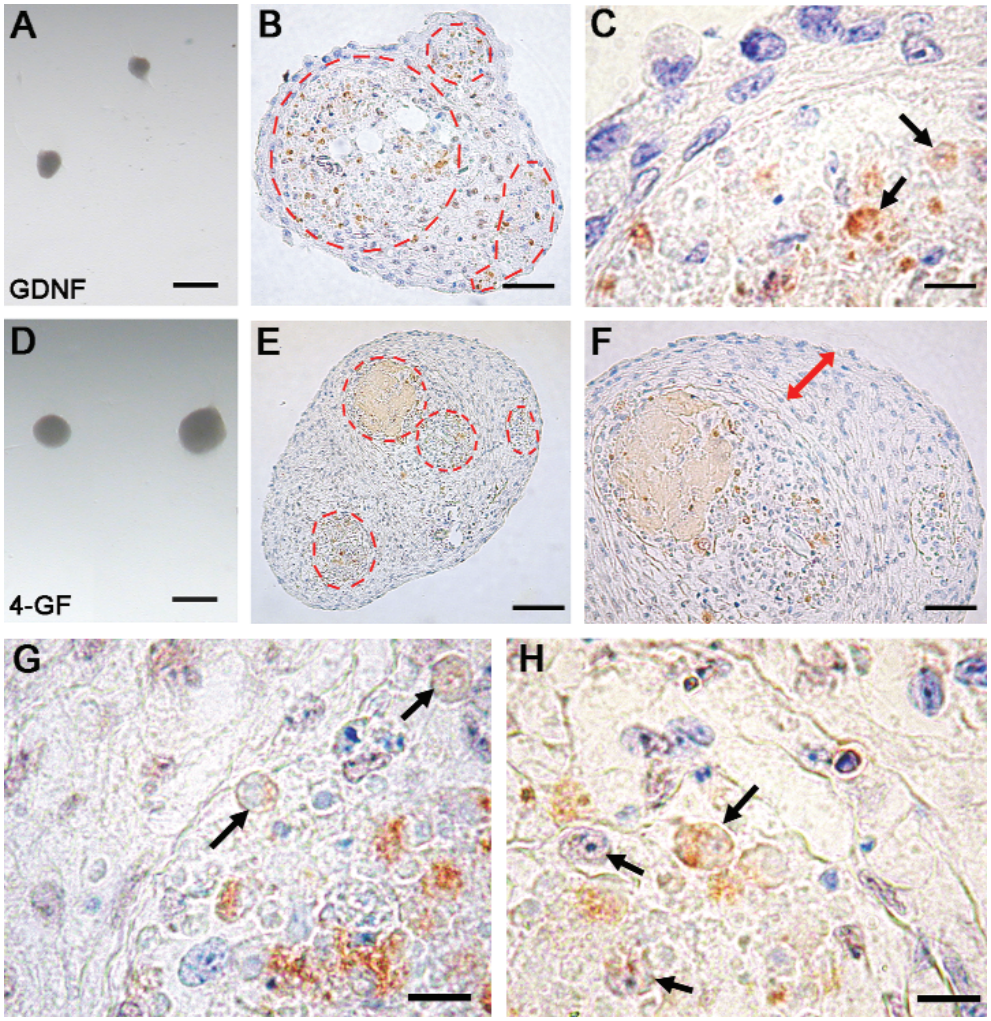

Fig. 6. Morphology of colonies appearing under the effect of the growth factors GDNF or a combination of LIF, EGF, FGF2, and GDNF (short-term culture, 15 days). (A and D) The general morphology of the colonies. GDNF colonies (A) were always spherical, while all four growth factors colonies (D) were from spherical to ovoid and were bigger. (A and D) Bar = 0.5 mm. Sections of colonies were immunostained for DBA. DBA positive cells (brown) are type A spermatogonia. They were present in round areas resembling round cross-sections of seminiferous tubules in all cases. Counterstaining with Mayer's hematoxylin stained the nuclei of all cells (blue); GDNF (B and C), all four growth factors (E–H). In GDNF colonies (B), some of the germ cell centers were peripherally located in colonies and represented a very high percentage of the colony bulk (round areas, red outlined). (B) Bar = 50 μm. (C) A very narrow layer of somatic cells (arrowheads) separated the round areas from the external milieu. Type A spermatogonia are present inside the round areas (arrows). Bar = 10 μm. (E) Round areas in all four growth factors colonies (red outlined). Note the density of somatic cells, as in FGF2. Bar = 100 μm. (F) In some areas, there is a thick layer of concentric rings of somatic cells as in LIF (red double head arrow). Also irregular boundaries around round areas can be seen. Bar = 50 μm. (G and H). High magnification images of round areas showing the high density of healthy type A spermatogonia (arrows). Bar = 10 μm.

differences between treatments with respect to the absolute numbers of type A spermatogonia that were found in culture (Fig. 7B). The colonies grown in the presence of all four growth factors contained the highest numbers of spermatogonia after 2 weeks of culture (15 968 ± 2160 spermatogonia/well), closely followed by GDNF alone (12 422 ± 2172 spermatogonia/ well). The groups with only LIF, EGF, or FGF2 had the lowest numbers (range 5268 ± 1036 to 7158 ± 1256 spermatogonia/well). Normally, bovine type A spermatogonia have a round- to oval-shaped nucleus with one to three distinct nucleoli. This typical morphology could be observed more often in the colonies grown with a combination of the four growth factors and with GDNF alone.

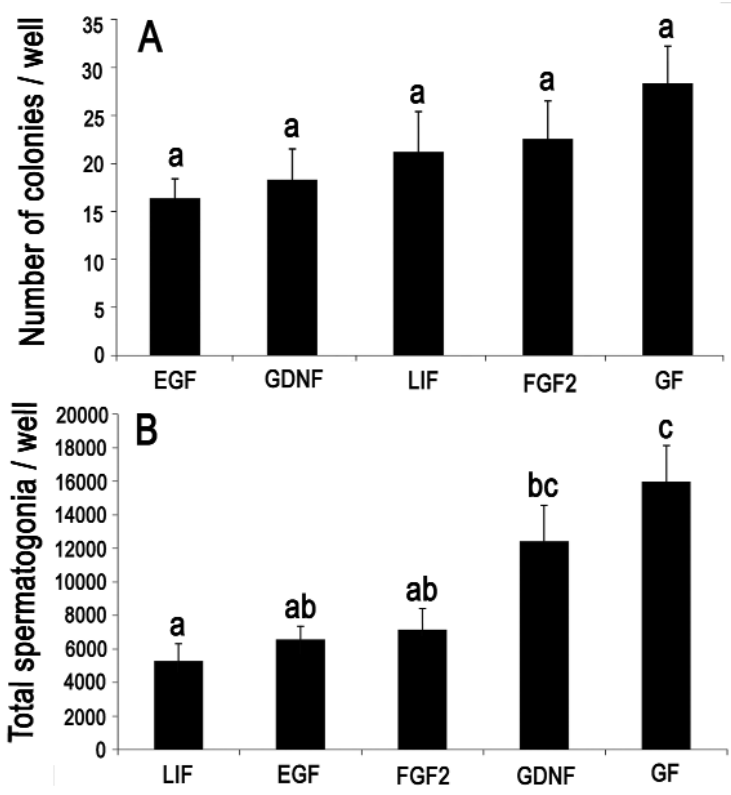

Fig. 7. Comparison of the effect of several growth factors (GDNF, LIF, EGF, FGF2 and their combination) on the formation of colonies in bovine spermatogonial cultures. (A) Number of colonies per 2 cm^2 well after 15 days of culture. (B) Absolute number of DBA positive, bovine type A spermatogonia, in colonies as estimated by stereological methods. Different superscripts represent significant differences between culture conditions. The results are presented as the mean ± S.E.M. of three independent experiments.

Bovine type A spermatogonial kinetics during long-term culture

In order to perform long-term culture experiments, the stem cell medium with four growth factors (LIF, EGF, FGF2, and GDNF) was used because it provided the best maintenance conditions during short-term culture. Besides, the cells were serially subcultured during long-term experiments. The colonies did not form during long-term culture, probably due to the short passaging intervals and ongoing variations of somatic to germ cells ratios. Type A spermatogonial numbers, as determined with the help of the marker DBA, strongly increased during long-term subculturing. In one experiment of 26 days in culture, about 12 x 10^6 type A spermatogonia were formed from a total starting population of 18 x 10^4 cells. This growth represented an increment of 365-fold resulting in what closely resembled an exponential increase with time (Fig. 8A). In correlation with this finding, the cells with the typical morphology of type A spermatogonia could be seen scattered on top of the monolayer up to at least 26 days of culture (Fig. 8B).

Bovine SSC kinetics during long-term culture

In order to determine whether the SSCs among the type A spermatogonia also increased in the cultures, SSC transplantation was carried out. In the recipient mouse testes, SSCs can home to the basal membrane, proliferate, and start to form a repopulating colony. However, the phylogenetical distance between mice and bovine being too large, no full bovine spermatogenesis was established and only type A spermatogonia were formed [6, 17]. The numbers of type A spermatogonia present 2 months after transplantation (DBA-positive cells) were counted and were taken as a measure of the numbers of stem cells transplanted. Mouse type A spermatogonia do not stain for DBA as could be seen in the few areas with endogenous mouse spermatogenesis in irradiated nontransplanted mouse testes nor in normal mouse testes. Spermatogonial transplantation was successfully performed in six animals but unfortunately one died before the end of the experiment (Fig. 8C). In this specific culture experiment, from the starting cell suspension, 100 000 cells were transplanted to each mouse testis, 70 000 of which were type A spermatogonia. After 2 months, about 253 bovine type A spermatogonia were found per transplanted testis. This means that the SSCs present in the transplanted cell suspension had given rise to a number of colonies that in total had formed on the average 253 type A spermatogonia per transplanted

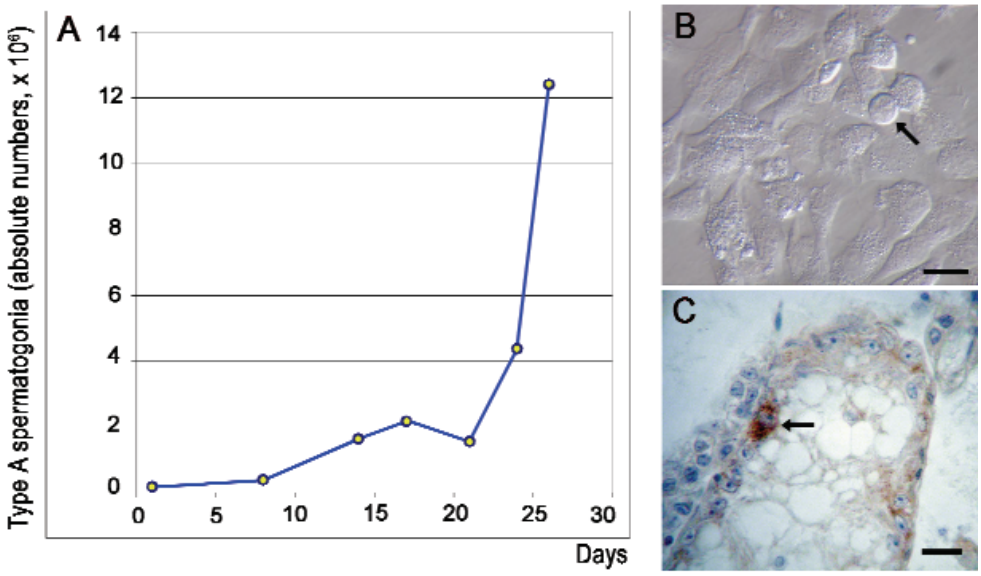

Fig. 8. Expansion of the type A spermatogonia population on long-term culture and identification of SSCs. (A) Expansion of the population of type A spermatogonia during a first experiment of 26-day culture with stem cell medium and the combination of four growth factors (GDNF, LIF, EGF, and FGF2). At each time point (passage), the total number of cells was estimated with a hemocytometer and corrected for viability and DBA-positive cells percentage (smears were prepared that were stained for DBA to determine the purity of the type A spermatogonia), see Materials and Methods. A total expansion of type A in this experiment was 365-fold. (B) Cell with the typical morphology of type A spermatogonia (arrow) laying on top of a somatic cell monolayer after the last of several passages during a culture experiment of 30 days, established to test the expansion of SSCs. A total expansion of the type A spermatogonial population in this experiment was 1780-fold. These cells were marked with DBA in sample smears to estimate their numbers at different time points (staining not shown, see Materials and Methods). Nomarski optics. Bar= 10 μm. (C) Type A spermatogonium (arrow) present in the basal compartment of a seminiferous tubule of a nude mouse 2 months after transplantation and originally obtained from a 30-day serial subculture. In all cases, the bovine type A spermatogonial marker was DBA (brown). Counterstaining with Mayer's hematoxylin stained the nuclei of the cells (blue). Bar = 10 μm.

testis. At the end of the culture period (day 30), a cell suspension was obtained of which also 100 000 cells were transplanted into each mouse testis. In this case, the purity of the type A spermatogonia was 12.74% purity (% of DBA-positive cells) and hence 12 740 type A spermatogonia were transplanted per recipient mouse testis. Two months after transplantation, per recipient mouse testis, 366 type A spermatogonia were counted. These data indicated that there was a higher proportion of SSCs among the cultured type A spermatogonia than among the type A spermatogonia of the starting cell population. The difference could be calculated to be 366/12740 divided by 253/70 000 = 8-fold. In addition, we had to take into account that at each passage of the cultures the cells were considerably diluted. At each passage, a small aliquot of cells was re-plated and the rest taken out of the experiment. If the discarded cells had also been cultured, the total number of SSCs at the end of culture would be higher, in proportion to the total dilution factor. At the start of the experiments, 36 500 type A spermatogonia were put in the culture; and at the end of the culture, 680 000 type A spermatogonia were harvested. Taking into account the dilution (2 x 2 x 4 x 2 x 2 x 1.5 = 96), the culture during 30 days rendered a 1780-fold increase in type A spermatogonial numbers. The fold increase of SSCs during culture was then calculated as the increase in the purity of SSCs (eightfold) multiplied by the increase in type A spermatogonial numbers (1780-fold) is 14 240-fold.

Discussion

Transgenesis technologies were first developed in laboratory animals. Recently, interest arose in the possibilities to use the male germline to create transgenic animals. One key point to advance in this important aspect of stem cell research is the acquisition of large enough quantities of SSCs. The possibility to propagate these cells in vitro would be very helpful in this respect, especially considering the low numbers of SSCs presents in the testis, about 0.03% of all germ cells in the mouse [18], and the small testicular samples that will most likely become available in genetically valuable large animals. In the present study, we successfully propagated bovine SSCs in the culture using a stem cell specific medium and tested the effects of several growth factors. We used a highly enriched medium, originally used by Kanatsu-Shinohara et al., (2003b) [2] to stimulate proliferation of SSCs in cultures of mouse spermatogonia. Comparing this medium to our routinely used supplemented MEM cell culture medium, we found that the stem cell medium outperformed supplemented MEM in terms of number of colonies arising during culture and total numbers of bovine spermatogonia within the colonies. In addition, the morphology of the colonies was very much different in that a complex arrangement of somatic and germ cells was observed. In a previous paper, the colonies were defined as clusters of type A spermatogonia of at least 64 cells [5]. Interestingly, with the present culture conditions, the colonies evolved to a higher level of organization and consisted of a dense core of cells including spermatogonia, intermingled with somatic cells. The colonies at this degree of development were difficult to characterize because of the tight arrangement of the constituent cells. Therefore, we sectioned the colonies to get a clearer picture of their internal structure. The morphology of the colonies that were formed in the stem cell medium included densely packed somatic cell areas surrounding round distinct, flat somatic cells lined, less dense areas, inside which spermatogonia were always located. Apparently, the growth factors and other components of the stem cell medium induced the formation of organized structures in vitro. The fact that no round areas were found, which did not contain spermatogonia with or without any of the growth factors, indicates that the formation of these structures depends on the presence of germ cells. The spherical colonies do not seem to originate from aggregation of detached cells during monolayer formation but likely self-generate during early monolayer formation. Apparently, spermatogonia interact with some somatic cells that do not join the ongoing monolayer formation, in such a way that the somatic

cells contact, intermingle, and surround them resembling what happens during testis chord formation during fetal development. One part of the mechanism of colony formation may involve the cytoplasmic extensions of somatic cells that contact germ cells during the early stages of colony formation. These contacts may trigger the formation of a conglomerate that will become a spherical colony, while leaving a connection or stalk with the monolayer made up of a narrow bundle of somatic cells. Once inside the colonies, the germ cells seem to be compartmentalized by surrounding flat somatic cells. In parallel, the somatic cells continue to proliferate, increasing the size of the colonies by depositing layers of peritubular myoid and some Sertoli cells toward the outskirts, also showing proliferative activity as evidenced by mitotic figures. At first glance, many of the cells inside the colonies morphologically resemble somatic cells of testicular origin. Interestingly, the cells surrounding the round areas inside the colonies were very similar to the peritubular myoid cells seen in vivo. As Sertoli cells are present as contaminants in bovine type A spermatogonia suspensions in a range of 20–50% [5, 6], these cells also contribute to the somatic components of the colonies. Immunohistochemistry using a Sertoli cell marker (vimentin) and a peritubular myoid cell marker (α-SMA) revealed that peritubular myoid cells together with Sertoli cells participated in the formation of a 'capsule' in the periphery of the colony. Furthermore, both cell types appear scattered in the core of the colony, not showing a preferred location with respect to the round areas containing germ cells. However, Sertoli cells are more abundant. Surprisingly, the flat cells surrounding the round areas are mainly Sertoli cells and not peritubular myoid cells. The change of the morphology of some Sertoli cells to a flattened phenotype is probably due to unknown responses to components in the stem cell medium. Interestingly, spermatogonia inside the round areas of the colonies are in close vicinity to Sertoli cells but the cytoarchitecture is not the typical one of a seminiferous tubule, as germ and Sertoli cells appear randomly arranged in the colonies. Apparently and despite the richness of the culture medium used, it was not possible to trigger the formation of true seminiferous tubules in the same manner as was achieved when transplanting a testicular cell suspension of pig origin into the mouse subcutaneous tissue [19]. However imperfect, the patterns observed in our study still show an interesting morphogenic rearrangement of somatic and germ cells in vitro to form a well-defined and organized structure. Future work will hopefully lead to conditions allowing the formation of de novo seminiferous tubules in vitro. The contamination of the stem cell cultures with Leydig cells was very low. The expression of the marker enzyme 3β-HSD type 1 in Leydig cells is not de-

pendent on the presence of luteinizing hormone (LH). Even in the continuous absence of LH signaling, in LH receptor knockout mice, it is possible up to adulthood to detect 3β- HSD type 1 immunoreactivity in Leydig cells [20]. Although Leydig cell progenitors and immature Leydig cells can survive in culture for several weeks up to months, even in the absence of LH (Teerds, de Boer and Rommerts unpublished observations), the proliferative activity of these cells is negligible [21], nor do these Leydig cells produce measurable amounts of testosterone. Thus, even though low numbers of Leydig cells may be present in the stem cell cultures, it is highly unlikely that these cells will somehow affect the stem cells under the present conditions. Other cell types were not characterized, but might include endothelial cells and fibroblasts. As α-SMA seems to stain some endothelial cells, the number of myoid peritubular cells in the cultures might appear lower than expected. This has been shown to be a specific pattern of staining for bovine species [16]. The growth factors used in our experiments are quite generally used in male germline stem cell cultures, including SSCs. In the present study, we have attempted to learn more about their individual effects. Histological sections of the colonies arising in the stem cell medium to which LIF, GDNF, FGF2, EGF, or a combination of the four growth factors was added, in principle all showed the described morphology. Often, the dead cells with pycnotic nuclei were intermingled with healthy spermatogonia inside the round areas, especially in LIF colonies. Probably, with this growth factor, the surrounding somatic cell layers become a barrier for some nutrients. Interestingly, GDNF alone or in combination with the three other growth factors induced the formation of colonies in which the round areas were preferentially located at the outskirts, with only a very thin somatic cell layer separating them from the culture medium. This is in accordance with our previous report showing that GDNF induced the presence of a higher number of type A spermatogonia at the periphery of intact (not-sectioned) bovine colonies in culture [5]. Davis & Schuetz (1977) and Davis (1978) cultured cells dissociated from the testes of 15-day-old rats and obtained monolayers supposedly consisting of Sertoli cells, in which vesicle-like structures were found as well as tubes that arose by rolling of the cells at the edges of the monolayer [22, 23]. These structures are not comparable with those we found in our cultures in which germ cells did stay present. In another study on mouse SSCs, a different set of growth factors was used (LIF, FGF2, kit ligand, platelet derived growth factor, and insulin-like growth factor-1) than in the present study but an increase in the rate of proliferation inside the colonies was found [24]. In both systems (bovine and mouse), the colonies appeared with a similar timing, that is, around 7

days of culture. Noteworthy, the combination of growth factors used in the mouse cultures did not include GDNF. Differences in the stimulatory effect of different groups of growth factors might be related to the nature of the starting cell population (i.e., age, species, cell density, and somatic cell contamination). In general, colonies tended to be spherical in shape. However, the use of additional growth factors (LIF, GDNF, FGF2, EGF, or a combination of all four) induced differences in the morphology of the colonies. For instance, under the influence of EGF and FGF2, big odd-shaped colonies appeared. Stereological analysis of the colonies indicated that although EGF has been described as a spermatogonial growth factor [25, 26], the cultures with this growth factor showed the largest volume of somatic cells. In EGF-treated cultures, spherical colonies arose interconnected by bridges. Whether separate spherical colonies merge with one another through somatic cell proliferation or start as one colony and sprout to make new spherical daughter colonies in rows connected by somatic cell bridges remains to be studied. The origin of these bridges may lie in the cytoplasmic extensions of peripherically located somatic cells in one colony connecting with other germ cells that are in the process of forming a separate colony nearby. While this second spherical colony forms, it probably remains connected to the first one through what will be a 'bridge' later in the process. We have seen these very long cytoplasmic processes trying to connect with germ cells in early stages of the cultures, but cannot definitely relate this complex cell to cell interaction with the process of bridge formation between colonies. The effect of EGF may be direct on somatic cells (Leydig cells, Sertoli cells, and peritubular myoid cells) as well as spermatogonia as both express EGF receptors in rats [26]. In bulls, in the seminiferous epithelium, the EGF receptor is restricted to germ cells, but it is also present in the somatic cells of the straight tubules and rete testis [27]. In our bovine culture system, FGF2 produced large elongated colonies with an abundance of somatic cells with the morphology of Sertoli cells. In rats, the FGF2 receptor has been traced to several somatic cell types (Sertoli cells, Leydig cells, and myoid peritubular cells; [28, 29]). Indeed, FGF2 has been associated with promoting both the proliferation and survival of somatic cells in vitro [30-32]. Interestingly, Van Dissel- Emiliani et al., (1996) also found an effect of FGF2 on culture morphology as they observed the formation of groups of tightly packed Sertoli cells in cocultures of rat Sertoli cells and gonocytes [31]. The relatively small size of LIF colonies in our experiments might be related to the fact that at least in rats, the receptor is limited to the spermatogonial population [33]. When a combination of all growth factors was used, big colonies of mixed characteristics appeared.

Since DBA-positive cells were very abundant inside broad round areas, these complex colonies seem to support spermatogonia most effectively. Immunohistochemical staining for DBA allowed us to identify type A spermatogonia in the colonies [10, 11]. Our finding that the medium including all four growth factors was superior with respect to the numbers of spermatogonia formed is consistent with previous results indicating that a combination of growth factors can induce self-renewal of SSC in vitro [2, 3, 34]. GDNF-only treatment came second with respect to spermatogonial numbers, which provided further evidence that, as in the mouse [2, 3, 35, 36]. GDNF has an important role in bovine SSC self-renewal physiology. Remarkably, spermatogonia had a healthy appearance (i.e., normal size of about 10–12 μm diameter, round nucleus with usually one large nucleolus and no morphological signs of apoptosis) when cultured with GDNF as well as the combination of the four growth factors (including GDNF). Germ cells were very rarely seen outside of the colonies; hence spermatogonia apparently do not survive outside of the colonies for periods as long as 15 days. Furthermore, the highest numbers of spermatogonia were found with conditions allowing the formation of the most well developed colonies. In view of the steep increase in spermatogonial numbers with culture time, the increase in spermatogonial numbers is very likely associated with increased proliferation. This has also been shown in SSC colonies in the mouse [24]. Apparently, the initial SSCs in our culture were surrounded by somatic cells to form a colony and were able to self-renew under the influence of the combination of growth factors. At a comparable time point (day 14) in two different type of experiments (short versus long term), there were around seven times more type A spermatogonia in cultures that involved passaging than in those left undisturbed for 15 days. Without subculturing some expansion of the type A spermatogonial population did occur, but the increase was definitely more important in the long-term approach which included frequent passaging. Enzymatic dissociation of the cultured cells at every passage probably opens new niche sites in the cultures inducing SSCs to self-renew. The type A spermatogonial population grew during long-term culture experiments of 26 and 30 days of duration. The growth was superior in the 30-day culture (1780-fold) than in the 26-day culture (365-fold) probably because in the first case the cells, besides having more time to proliferate, had been frozen, in contrast with the second case, in which freshly isolated cells were used. In this respect, Kanatsu-Shinohara et al., (2003a) suggested that a possible mechanism of positive selection of SSCs related to the freezing procedure [37]. In parallel, the growth factors used also affected the somatic cell population which increased in size as observed

in the shift of the germ cell:somatic cell ratio during culture. The detailed nature of these effects will require further investigations. Upon transplantation of the cultured cells, colonization of the recipient mouse testes was found, implying that at least some of the cultured type A spermatogonia were SSCs that were able to migrate to the basal membrane of the tubules and did retain their stem cell properties. Although transplanted bovine SSCs did not give rise to colonies with full spermatogenesis in mouse seminiferous tubules, these cells did proliferate and colonized the recipient mouse testis with type A spermatogonia, as described previously [6]. The numbers of type A spermatogonia present 2 months after transplantation (DBA-positive cells) were taken as a measure of the numbers of stem cells transplanted. In our experiments, almost all bovine DBA-positive cells sitting on the basal membrane of the mouse seminiferous tubules 2 months after transplantation were single cells. This has also been observed by others [17, 38]. The duration of spermatogenesis in the bull being 61 days, after 2 months any non-stem germ cell 'contaminants' of the original transplanted cell suspension will have had enough time to undergo the whole spermatogenic process, if capable of this, and will have been shed as sperm. Therefore, the bovine type A spermatogonia, as detected by DBA staining, in the recipient mouse testes are daughter cells of transplanted stem cells and, as already discussed, their numbers can be expected to be proportional to the number of stem cells transplanted. The results of our transplantation experiment strongly suggest that our culture conditions allowed the in vitro propagation of SSCs. A more than 14 000-fold increase in SSC number was found after 30 days of culture, indicating that our culture system has the capacity to expand the SSC population. It would be interesting to study the ability of bovine SSCs to repopulate a recipient bovine testis and to give rise to full spermatogenesis. Important advances in this respect have been achieved in ruminants. In goats, full spermatogenesis and derived offspring has been obtained from transplanted germline cells [39, 40]. However, in bulls, colonization and full spermatogenesis has been achieved only after autologous transplantation of SSCs [41]. After homologous and heterologous transplantations, full spermatogenesis has not been shown [41, 42], probably because stem cells might not survive to an immunological reaction against them, so no or few daughter cells are formed. Our cell suspensions, transplanted into nude mouse testes, contained somatic cells besides type A spermatogonia. The somatic cell component of the cell suspensions will require further investigation as these cells possess receptors for the growth factors used [26-29, 33]. It will be interesting to study the prevalence of the different somatic cell types under the action of the different growth factors

and the effect of specific somatic cell types on the homing process of bovine SSCs in the mouse seminiferous tubule environment. In this respect, it has recently been shown that bovine DBA-positive cells can colonize mouse seminiferous tubules and survive for 2.5 months supported by cotransplanted bovine Sertoli cells [38]. In conclusion, it is possible to long-term culture SSCs from bovine calf testes. In culture, these SSCs were found to quickly increase in number while retaining their stem cell properties. While our previous results also pointed in this direction, the use of a specific stem cell medium greatly improved the efficiency of the system. It will now be possible to efficiently acquire large numbers of bovine SSCs in a way comparable with that for mouse SSCs and probably to establish bovine cell lines comparable with the germline stem cells available for the mouse [34, 43]. This will open the way to carrying out detailed research on bovine SSCs, possibly leading to applications important for the cattle industry like the production of transgenic animals by transfection of SSCs and transplantation to recipient bull calves.

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Development of bovine spermatogonial colonies during culture in agar medium

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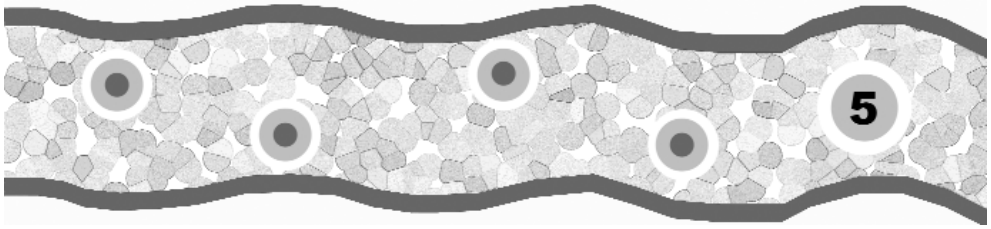
Chapter



Submitted for publication

Abstract

Generally, cultures of mammalian testicular cells (SSCs) have been carried out using liquid media. We now have compared cultures in a liquid medium with those in a semisolid medium of agar. Germ cells isolated from prepubertal bulls were cocultured with contaminating somatic cells in either a liquid medium or in agar. Germ cells in agar cultures developed in a similar way as those in liquid medium. Somatic cells grew as a monolayer on top of the bottom agar layer and germ cells nestled on top of the monolayer and formed colonies. Colonies appeared around day 7 and subsequently grew regardless of the culture medium used. However, a 4-fold higher number of colonies was observed in agar cultures than in liquid medium (17.3 ± 1.4 and 4.8 ± 1.9; $P < 0.01$) and colonies formed in agar cultures were about 3-times smaller (23857 ± 2440 and 71800 ± 10497 μm^2; $P < 0.01$) at day 15, end of the culture ($P < 0.01$). The number of cells with apoptotic morphology was much higher in agar cultures (530 ± 64 cells / mm^2) than in liquid medium (MEM, 154 ± 29), ($P < 0.01$). The differences in colony numbers and sizes between treatments are likely caused by cell migration of SSCs, merging of colonies and cell loss during liquid medium refreshment which does not happen with agar cultures, lowering the numbers of colonies. In the agar cultures all cells originally seeded remain in the system, even the dead cells which are prevented from detaching. Single germ cells may start an individual colony. We conclude that for the purpose of assaying SSC numbers by determination of colony numbers in culture, cultures using agar will be essential in order to obtain realistic SSC numbers.

Introduction

Spermatogonial stem cells (SSCs) represent the male germ stem cells in the testis. Stem cells are present in most tissues and they are able to divide and generate daughter cells that are either committed to differentiate or to become new stem cells (self-renewal). SSCs are scarce cells located in tissue areas where supporting cells produce a specialized microenvironment which provides nutrition and protection, called the niche. In the testis, the SSC niche has been shown to be related to the presence of the vasculature in the interstitial tissue [1-3].

SSCs constitute a small percentage of the type A spermatogonia, that for the rest are descendants of SSCs committed to the differentiation pathway [4]. SSCs represent a unique opportunity for biotechnological manipulation, since they are the only stem cell in the body with the capability

of delivering genetic information to next generations. These cells are at the base of the complex spermatogenenic process, specialized in producing spermatozoa. The self-renewal and differentiation potential of SSCs ensures the long term capacity of the testis to produce sperm and even to recuperate from damage inflicted to germ cells.

In recent years great progress has been made in the development of culture systems for SSCs [5-13]. The protocols for the culture of SSCs have been customarily based on the use of liquid media. These systems offer a simple microenvironment in which the conditions for studied factors are relatively easy to control and therefore easier to interpret. Compared to liquid media, a semisolid medium for SSC culture would offer potential advantages over the use of liquid media as a tool for the study of SSC, for example it could enable the development of an assay based on the clonogenic properties of these cells. When SSCs are isolated and dispersed in a culture system, they will self-renew if properly stimulated and each stem cell will produce a colony of daughter SSCs and possibly differentiating cells. This is also the rational for the in vivo SSC assay in which spermatogonial cell suspensions are transplanted to nude mouse testes with little or no endogenous spermatogenesis. The colonizing ability of SSCs allows them to settle in the basal compartment of the seminiferous tubules and to start spermatogenetic colonies whose numbers will correlate with the initial number of SSCs that was seeded [13-20]. An ideal model for the study of SCC in vitro, would be one in which the germ cells become evenly dispersed and settled in the culture well area and where each stem cell would remain in an unique spot, start dividing and form a colony. The number of colonies appearing in vitro would then be similar to the initial number of SSC, under the assumption that one SSC produces one colony. Unfortunately, in a liquid medium there is always doubt whether some of the colonies are formed by more than one stem cell as SSC are able to actively migrate during culture in a liquid medium [21]. Furthermore, SSCs might passively move and randomly seed close to one or more other SSCs, and together form a heterogeneous colony. Other approaches to study the clonogenicity of SSCs are very laborious and consist of micromanipulation of single SSCs to start colonies in individual culture wells [22] and do not consider the importance of the presence of Sertoli cells as niche providers [5, 6, 23].

An important advantage of a semisolid medium is that movement of the cells in culture is not possible while molecules can freely diffuse through the culture medium. A medium which

offers these conditions is agar [24]. Indeed, a pore in an agar gel is 100 to 600 nm wide [25], a dimension that allows the passage of many kinds of molecules. Moreover, agarose, the main constituent of agar, has a neutral pH [24], which contributes to the biocompatibility of the system. Furthermore, most cells will not take up agar or degrade it in their metabolism. Accordingly, agar has been used for decades to culture prokaryotic cells in research and diagnostic applications [24] and eukaryotic cells, for example malignant human tumor cells in research for cytotoxic drug development and cancer cell biology [26-28]. A recent study using agar as a culture medium concentrated on the process of germ cell differentiation in mice [29]. In this study, cells kept in agar formed colonies in which some germ cells were positive for meiotic markers. Interestingly, a better survival rate was achieved when using monolayers made of contaminant somatic cells.

Given the importance of ruminant species in the food production chains, it is important to develop in vitro procedures to manipulate testicular cells in order to improve the reproductive techniques available. The objective of the present work was to study the development of bovine germ cell colonies in co-culture with somatic cells in a semisolid medium of agar in comparison to the well documented behavior in a liquid medium, as shown previously [5, 10]. Furthermore we wanted to compare colony formation in terms of their number and size in liquid and in semisolid medium as a basis for future characterization purposes.

Material and methods

Cell isolation and liquid culture system

Testes were collected from *Bos taurus* calves between 4 and 6 months of age at a commercial abattoir (Apeldoorn, The Netherlands). Isolation was performed as described previously [5, 9, 10]. Briefly, about 20 g of testis material was minced into small pieces and suspended in minimum essential medium (MEM; Gibco, Invitrogen corporation, Paisley, UK) supplemented with 0.1275 % w/v $NaHCO_3$ (Sigma, St Louis, MO, USA), 4 mM L-glutamine, 0.1 mM single-strength non-essential amino acids, 100 iu/ml–100 μg/ml penicillin–streptomycin, 40 μg/ml gentamicin and 15 mM Hepes (all from Gibco), subsequently referred to as supplemented MEM. The procedure included two enzymatic digestions, overnight differential plating and discontinuous Percoll density gradient centrifugation [9]. Fractions were evaluated under a Nomarski interference microscope and an Axioskop (Zeiss, Göttingen, Germany) for estimation of the percentage of type A spermatogonia. Fractions consisting of about 65 % A spermatogonia were used. The viability of the primary isolated cells was evaluated using LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR, USA) and the total number of A-spermatogonia in the suspension was estimated with a hemocytometer. Type A spermatogonia were distinguished from somatic cell types and more advanced germ cells by their very conspicuous morphology, that is, a big round nucleus with one large central nucleolus and a high nucleus/cytoplasm ratio, as seen in our previous work [5, 6, 9, 10]. In this type of system, contaminating cells are usually Sertoli cells and rarely Leydig cells that have a very different morphology in culture [5, 6]. Cells were seeded in MEM with 10% FCS and fungizone (1 μl / ml), and plated in culture wells at a concentration of 100 cells/μl.

Agar culture system

Two agar solutions were prepared (1 % and 0.72 %) in deionized water to be used as lower and upper layers in the culture wells. For the lower layer, a solution of supplemented MEM 2X (twice the concentration described in the cell isolation section) with 20 % fetal calf serum (FCS) and Fungizone (2 μl / ml), previously equalized in an incubation chamber (37° C, 5 % CO_2), subsequently called MEM 2X, was mixed (1:1) with 1 % agar solution (37° C) and rapidly transferred to culture wells in 96 wells plates. After gelification of the lower layer, culture plates were kept in

the culture incubator at 37º C for 30 min, before addition of the upper layer. The upper layer included the spermatogonial cell suspension. Preequalized MEM 2X was mixed (1:1) with 0.72 % agar solution (37º C). A pellet containing the cells was suspended in this solution to a final concentration of 100 cells / µl.

In both agar layers (lower and upper layers), the solute concentrations drop by 50 %, to yield final agar concentrations of 0.36 % and 0.5 % in the upper and lower layer, respectively. Culture medium elements also dropped in concentration, so that cells were exposed to MEM 1X, FCS 10 % and Fungizone 1:1000 (1µl / ml). Volumes were 100 µl and 200 µl for the upper and lower substrate layers, respectively.

To avoid rapid gelification of the agar at room temperature, the cell suspension was made in small aliquots and progressively transferred to the culture wells. Regardless of the treatment, cells were cultured for 15 days under conditions previously described for liquid media, that is, with a temperature of 37 ºC, with 5 % CO_2 in a humid atmosphere [5, 9, 10]. In cultures with agar, MEM medium was poured to cover the system agar-cells in a volume of 15 % to 25 % of that of the agar system volume. Refreshment of the liquid was done once a week in all cultures (agar and MEM).

Colony number and size evaluation

Colonies of germ cells appearing during culture were studied with a stereomicroscope (Nikon SMZ800, Tokyo, Japan) during the culture period. They were quantified and the morphology of the cultures was compared with that of corresponding cultures in liquid medium (supplemented MEM). For this purpose, three separate experiments were carried out. Each experiment included 64 wells (32 with agar and 32 with MEM). The number of colonies was expressed as colonies per well. Due to the general spherical shape of the colonies, the measurement of their size was based on the areas of the profiles of the colonies in digitalized images. All measurements were done with ImageJ 1.34S software, developed by Dr. W. Rasband at the National Institute of Health, Bethesda, USA, and downloaded from the Internet at http://rsb.info.nih.gov/ij/, with the appropriate calibration.

Statistics

General statistical analysis was performed using the independent-samples Student's T test. All calculations were performed using SPPS software for Windows v11.5.

Results

The set up of the culture

Germ cells in agar culture showed a pattern of development similar to that observed in MEM liquid medium. First, the cells, dispersed in the semisolid medium (agar), reached various levels of depth within the substrate, as observed on day 1 by looking at the cells just seeded and embedded in the agar medium. In the present experiments, somatic cells went to the bottom of the upper layer of agar and these cells made extensive contacts with each other through cytoplasmic extensions, despite the presence of agar (Figure 1). Type A spermatogonia could be seen on top of the monolayer as soon as it was formed (Figure 2). Even though cells were suspended in a semisolid medium, somatic cell-germ cell contacts did occur (Figure 1) and the monolayer eventually reached confluency. Somatic cells grew on top of the more solid bottom agar layer (with a higher concentration of agar), acting therefore as a substrate for these cells. Many germ cells entered apoptosis during the first day of culture as evidenced by their morphology, especially at the peripheral areas of the culture wells (Figure 3). The number of cells with deteriorating morphology (apoptotic) was much higher in agar cultures (530 $\pm$ 64 cells / mm^2) than in liquid medium (MEM, 154 $\pm$ 29), ($P < 0.01$).

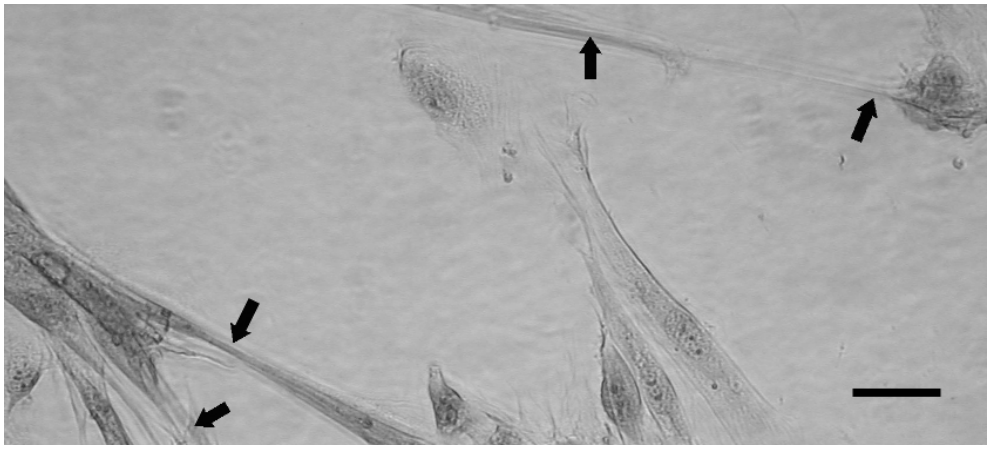


Fig. 1. Monolayer formation during the first week of culture in agar medium. Arrows point to cytoplasmic extensions of somatic cells contacting each other. Bar = 50μm

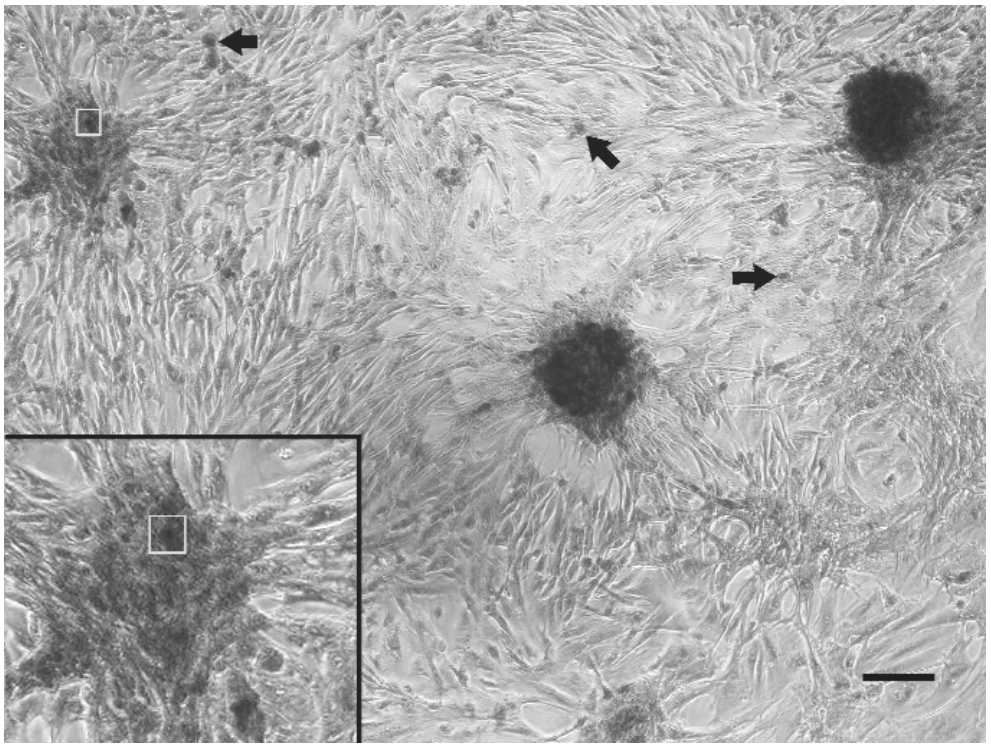


Fig. 2. Bovine germ cell colonies growing on top of somatic cells at day 15 of culture in agar medium. Arrows point to the very few type A spermatogonia that do not become integrated to colonies and remain single in the monolayer. The left bottom corner shows a magnification of a colony with a square framing a bovine type A spermatogonia. Bar = 100μm

Colony formation

Germ cell clusters with a regular spherical shape and composed of more than 64 cells, appeared during culture and were subsequently named colonies [5, 6, 10]. Colonies appeared and developed both in liquid supplemented MEM and semi-solid agar cultures. Colonies in agar had a typical spherical shape, were comparable to those formed in liquid MEM medium, and had a more compact aggregation of cells towards the center (Figure 2). The colonies also tended to protrude upwards rather than spreading over the surface of the culture, in a similar manner as in liquid medium. The colonies were first noticed around day 7 and had their final spherical shape with a compact appearance by day 10. Afterwards and up to day 15 (end of the experiment) the colonies

did not change much in shape. At day 15 of culture, agar colonies were smaller than those present in MEM (23857 ± 2440 μm^2 and 71800 ± 10497 μm^2 respectively, $P < 0.01$). Rarely, single germ cells not associated with colonies, were seen on top of the monolayer in both agar and MEM cultures after day 7. Surprisingly, a much higher number of colonies was seen in the agar cultures than in the liquid (MEM) cultures (17.3 ± 1.4 and 4.8 ± 1.9; $P < 0.01$). There was no difference in the distribution of the colonies, which seemed to be at random within the culture wells.

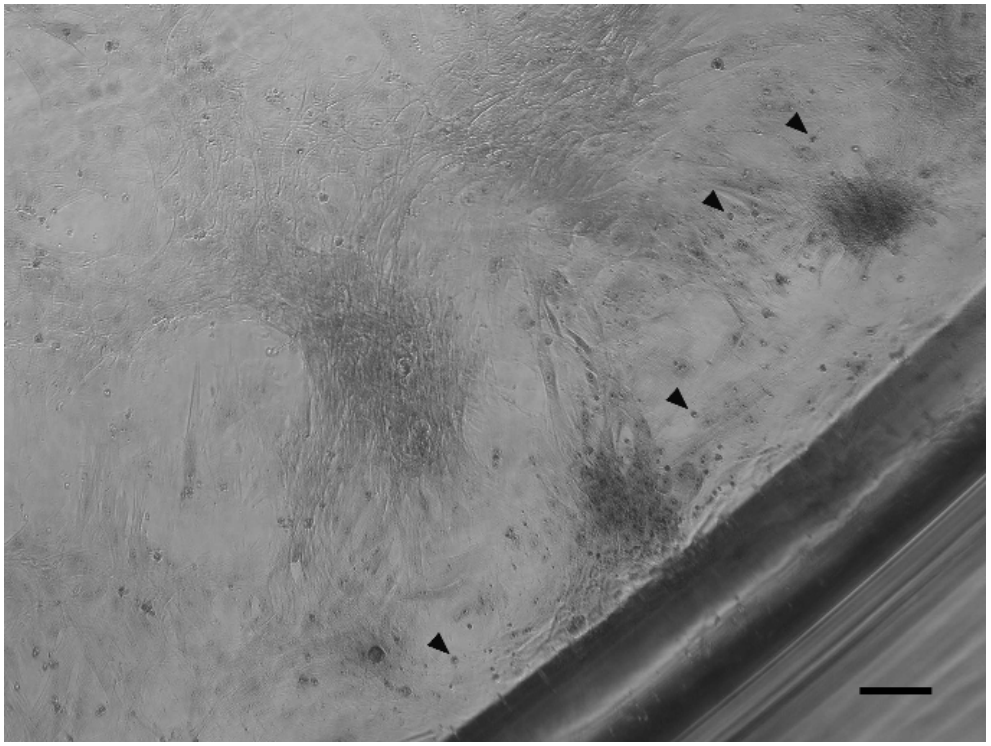


Fig. 3. Peripheral area of a culture well showing apoptotic type A spermatogonia (arrowheads). This illustrates the apoptotic wave that occurs during the first two days of culture in agar medium. Bar = 100μm

Discussion

Bovine testis cell (germ and somatic cell) colonies which appear in vitro constitute an interesting model to study germ cell differentiation and stem cell biology. In the present work we examined the growth of germ cell colonies in a semi-solid culture medium, a system generated as an adaptation from existing prokaryotic cell methodology for the study and evaluation of tumor colony formation by cancer stem cells [26, 27, 30, 31]. Bovine SSCs in culture grow in a clonogenic way, and form colonies in association with the somatic cells in liquid media [5, 6, 10].

We now show that bovine germ cell colonies grow and can be maintained in a semisolid medium with agar. The development of these colonies in agar was similar to that of colonies appearing in liquid medium (random positioning on top of a somatic cell monolayer and comparable timing). Interestingly, a 4-fold higher number of colonies was formed when germ cells were cultured in agar medium compared to in liquid medium (MEM). Furthermore, despite the fact that colonies developed in agar or liquid medium following a very similar pattern of formation and similar morphology, agar colonies had a smaller size than liquid MEM colonies. The higher number of colonies can be explained in terms of the prevention of SSCs to move and aggregate because of agar acting as a barrier. Somatic cells in our cultures did form a monolayer by contacting each other, which means that despite the physical barrier formed by the agar, cytoplasmic extensions of the cells could make their way through the agarose protein pores. Apparently, SSCs embedded in agar medium, had difficulties contacting each other when they were close enough. This phenomenon probably impedes neighbouring colonies to merge. This would explain the large differences we saw between colony number and size, comparing colonies grown in liquid or semisolid media. If agar immobilizes SSCs, preventing more than one SSC to become involved in the formation of one colony, young developing colonies will not aggregate and develop with less starting cells and colony size will be smaller than of colonies grown in liquid medium. In addition, during cell cultures, in which liquid media are used and refreshed at regular intervals, some cells will always be lost during the refreshment of the medium, including SSCs and even early colonies. Indeed, spermatogonia tend to detach from the culture when a liquid medium is used [5], while in agar medium cells would not be able to leave the system, as seen in our cultures, where a higher number of dying cells was seen in comparison to MEM cultures. Alternatively, it

is also possible that colonies in semi-solid medium are restricted from expansion, adding to the explanation of the smaller size.

Apoptotic phenomena can also contribute to the understanding of the kinetics of colony formation in culture. Stuckenborg et al., (2008), reported about 40 % of apoptotic spermatogonia during the first day of culture in agar medium with a tendency of less apoptosis in co-cultures with somatic cells [29]. In our work, the higher number of apoptotic cells in agar medium than in liquid medium, may not only indicate the impossibility of dead cells and debris to be eliminated through refreshment but also other factors inherent to the wellbeing of cells in such a system. On the other hand, it will have to be analyzed whether the higher numbers of colonies in agar can be the result of better survival rates of spermatogonia in that medium in comparison with liquid cultures, because it is also possible that apoptotic cell numbers are underestimated in liquid cultures because of cell loss during refreshments.

Taken together, our results show that a semisolid medium of agar promotes a higher number of small colonies than in liquid medium. The property of agar to immobilize cells can prove useful in the future to produce a spermatogonial stem cell assay in vitro, to study bovine spermatogenesis, as recently shown for the mouse [32]. So far, the identification and quantification of SSCs has been a challenging task relying on a time-consuming technique of transplantation of these cells into a seminiferous tubule environment [14, 15]. The transplantation technique consists of transplanting a germ cell suspension into the seminiferous tubules of a recipient mouse testis with depleted endogenous spermatogenesis, through the efferent duct. SSC among the transplanted germ cells migrate to the basal membrane of the seminiferous tubules and subsequently start to form a repopulating colony of donor spermatogenesis. The only existing in vitro assay to quantify mouse SSCs relies on the fact that colonies were formed and the number of these colonies was shown to be unequivocally related to the number of SSCs identified through the transplantation technique [32]. However, bovine SSCs proliferate but do not form colonies when long-term cultured under optimal conditions for SSC self-renewal [6], suggesting important differences between mammalian species regarding SSC behavior in vitro, and again, the need for an in vitro assay for the bovine species.

The present work opens an interesting research path that includes the characterization of bovine testis cell colonies that appear in agar medium and the determination of SSC kinetics in

such a system, which will prove useful in the development of an in vitro spermatogonial stem cell (SSC) assay for such important species.

Acknowledgements

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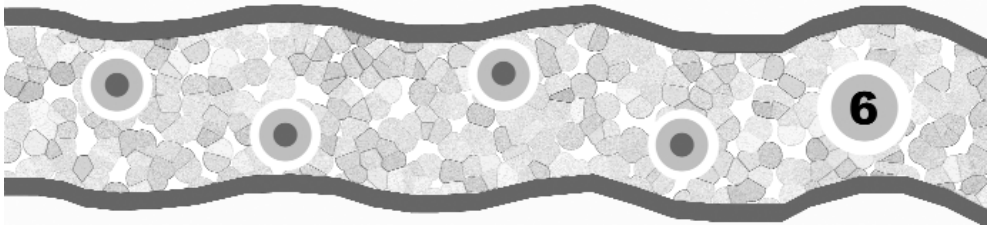
Summarizing discussion

Chapter



General

In cattle production systems, males are used to genetically introduce traits of interest into herds. Genetic selection can lead to an improvement of the production of meat and milk when appropriate male genes are introduced into the females of the herd. Furthermore, cows are often artificially inseminated with semen obtained from genetically evaluated sires at breeding centers. Therefore, artificial insemination is an effective technology to deliver new genes into an established ever improving genetic pool. However, the cells involved in this technology, sperm, are terminally differentiated and thus their availability is limited in number and restricted to a physiological window occurring from and after puberty.

Alternatively, spermatogonial stem cell (SSC) technologies show promising features. SSCs, as male gene carriers, can be collected in high numbers early in life and manipulated in vitro to renew themselves. One testis from a 5 month old calf contains about 10^9 cells with the morphology of type A spermatogonia [1]. In this population, 1.3 % would be SSC [2], thus a 5 month old testis can potentially contain 13 x 10^6 SSCs. Every stem cell can theoretically produce millions of sperm while still holding the ability to maintain itself through self-renewal. This amazing property opens the way for the future development of in vitro spermatogenesis systems to produce sperm for experimental and commercial uses.

This thesis explores the area of basic behavior of bovine SSC in vitro, looking forward to future practical applications in the reproduction field. It also provides means to continue the study of the regulation of SSCs at the molecular level. Many basic physiological aspects of the bovine testis in vivo have received attention in the past [1, 3-8], but information about bovine germ cell behavior in vitro is more limited, especially in comparison to reference species like rodents. Nevertheless, some important progress has been made in the field [9-17]. With this progress as a baseline, the next logical step was to improve the existing culture systems to stimulate SSC self-renewal in bovine species.

The testis of Brahman bulls

In chapter two we described testis development in Brahman bulls, a commercially important tropical breed (Figure 1). This is one of several breeds originating from Asia, belonging to the *Bos indicus* or Zebu group, while *Bos taurus*, another important group, encompasses European breeds. *Bos indicus* animals adapt particularly well to tropical conditions, as opposed to most European breeds. The male reproductive biology of Zebu breeds is less known than that of European breeds. Of all reproductive phenomena in bulls, the acquisition of the pubertal state is perhaps one of the most relevant because of economical implications in cattle operations. Puberty is the time when a bull is capable to participate in reproduction [3], or more accurately, the age when a bull produces an ejaculate with at least 50 x 10^6 sperm with at least 10 % of progressive motility [18]. From a morphological point of view, the onset of puberty is marked by the presence of sperm in cross-sections of seminiferous tubules [19-23]. One physiological definition of puberty assumes that, a pubertal animal will be able to produce 0.5 x 10^6 sperm per gram of testicular parenchyma per day [1]. If herds can be managed in such a way that bulls reach puberty early, reproduction can start sooner and more offspring will be produced during one life cycle. However, while the establishment of spermatogenesis and onset of puberty is well known for European breeds [1, 3, 4, 6], this has rarely been studied in *Bos indicus* animals. The main rational for our research was the previous observation that *Bos indicus* bulls reach the pubertal state later than their European counterparts [24-27]. As expected, the kinetics of germ cells in the testis of the Brahman bull progressed according to the mammalian model, but real differences with respect to European bulls were foundn terms of the timing of the appearance of the different cell types. We found Brahman bulls have a delayed onset of puberty because the start of spermatogenesis (progression from gonocytes to type A spermatogonia) occurs several months later than in European bulls (Figure. 2). One of the possibilities opened by this article is to accurately choose the best time window to intervene or collect certain cell types for biotechnological purposes in this type of animals. It also opens a research field to discover the reason why *Bos indicus* bulls have a belated start of spermatogenesis in comparison to European breeds and whether this can be manipulated.

Fig 1. Brahman cattle grazing in a tropical farm

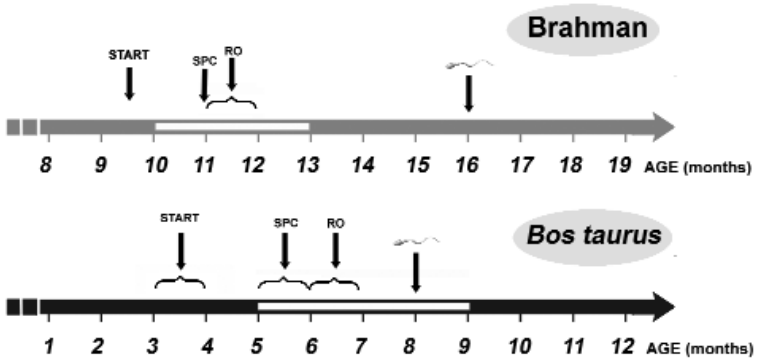


Fig 2. Comparison of cellular events in the seminiferous epithelium during the prepubertal period in Brahman (*Bos indicus)* and *Bos taurus* bulls. Numbers indicate age in months. Braces cover the time when specific cell types appear for the first time. The start of spermatogenesis, as evidenced by the formation of A spermatogonia for the first time (start), the appearance of spermatocytes (spc), round spermatids (ro), and sperm (sp), occur earlier in *Bos taurus* bulls than in Brahman bulls. Sertoli cell differentiation occurs from 10 to 13 months of age in the Brahman bulls while from 5 to 9 months in the *Bos taurus*, (white line). *Bos taurus* information was inferred from data presented by Curtis and Amann [1] and Amann [3].

GDNF in bovine spermatogonial cultures

The work presented in Chapter 3 is the logical continuation of previous efforts in our laboratory to set up a system for the study of bovine spermatogenesis in vitro [9, 13, 14]. Accordingly, we started primary cultures of about 80 % pure type A spermatogonia and 20 % somatic cells (mostly Sertoli cells) and treated some of them with glial cell line-derived neurotrophic factor (GDNF). This growth factor has been shown to enhance SSC renewal in the mouse [28] and we investigated the possible role of GDNF in SSC renewal in bovine species. We found that type A bovine spermatogonial numbers dropped during 25 days of culture, independently of GDNF addition, but this decrease was significantly less steep than in control cultures. Therefore, GDNF at least enhanced the maintenance of the type A bovine spermatogonial population. In this type of culture system, colonies consisting of type A spermatogonia and Sertoli cells, appeared around day 15. These colonies might well provide a supportive structure for type A spermatogonia, because the numbers of A spermatogonia, residing at the periphery of the colonies, were significantly higher in the GDNF treated cultures. Since SSCs are only a small subpopulation of the type A spermatogonial population, it was necessary to evaluate their stem cell properties by means of transplantation into nude mice. Thus, we found a two-fold enhancement of the percentage of mouse seminiferous tubules containing bovine SSCs, two months after transplantation of cells coming from GDNF treated cultures in comparison to those from control cultures.

SSC activity enhancement via GDNF can be theoretically explained in three ways: increase of proliferation, better survival rate or blockage of differentiation. Our studies rendered evidence that GDNF supports the maintenance of the type A spermatogonial population by blocking differentiation. Between days 7 and 15 of culture there were about two-fold more aligned type A spermatogonia (cells widely accepted as spermatogonia committed to differentiate) in the control cultures than in those treated with GDNF. It seems possible that differentiation is the default choice of SSCs and that GDNF or other factors produced by Sertoli cells and released in the niches, stimulate self-renewal pathways [29]. Self-renewal is mediated by GDNF upon interaction with a complex formed by the receptor GFRA1 and the co-receptor tyrosine kinase (RET) [30]. KIT is a differentiation marker in the seminiferous tubule since differentiating spermatogonia but not SSCs express this protein on their plasma membrane [31]. Plzf, the first gene discovered to be associated with SSC renewal [32, 33] is a repressor factor which downregulates Kit [29].

Moreover, knocking out of Gfra1, the gene codifying the receptor for GDNF, with small interference RNA (siRNAS) induces the phosphorylation of its co-receptor RET and the upregulation of Kit [34]. Clearly, the details of the selfrenewal and differentiation pathways are only starting to become known. Protein kinases are involved in the cascade of phosphorylation events similarly to other stem cell systems [35]. Briefly, GDNF seems to mediate the activation of either Src family kinases or AKT [36] and these kinases exert their action through a PI3K/Akt-dependent pathway [35, 37] .

Furthermore, we found less apoptotic cells in the outskirts of the colonies in GDNF treated cultures than in controls, suggesting a survival enhancement mechanism as previously discovered for the nervous system [38, 39] and recently suggested for the testis [36]. Proliferation of SSCs in our culture system was not observed, probably due to a slow cycling of SSCs. We also observed that Sertoli cells from the prepubertal calf were able to proliferate in vitro and survive as a monolayer until at least day 30, possibly allowing the maintenance of the SSCs during that time.

A more advanced culture system

In Chapter 4 we used our bovine culture system to test a highly enriched stem cell medium previously optimized for the growth of SSCs in a mouse system [40]. Moreover, we studied the effects of other growth factors in our bovine culture system, in addition to GDNF: EGF (Epidermal growth factor), FGF2 (Fibroblast growth factor 2) and LIF (leukemia inhibitory factor). These factors are all linked to the promotion of the proliferation or survival of cells from the male germ line at some point of development (EGF [41, 42], FGF2 [43, 44], LIF [45-48]). They were added individually or in a combination of all four.

The use of stem cell medium resulted in the formation of bigger colonies with internal areas with a round shape in which type A spermatogonia were located. This in contrast to colonies arising in cultures with the regular cell culture medium (MEM), which appeared internally disorganized with few scattered type A spermatogonia.

The effect of the use of various growth factors in our experiments was also visible in the external appearance of the colonies, individual growth factors inducing different colony shapes, as depicted in figure 3.

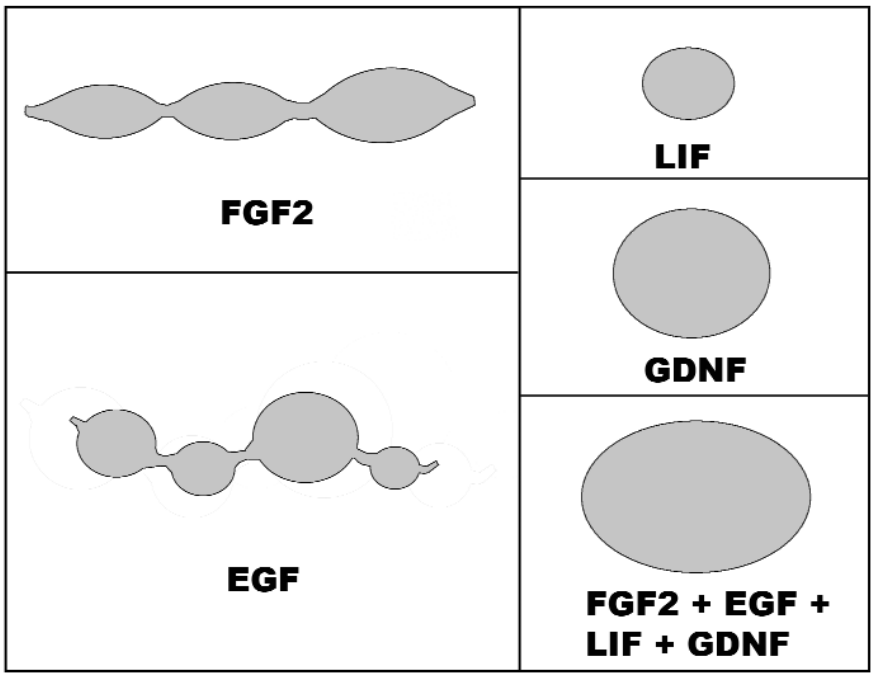


Fig. 3. Typical shapes of colonies appearing in cultures between 10 and 15 days exposed to various growth factors (FGF2 = Fibroblast growth factor 2; EGF = Epidermal growth factor, LIF = leukemia inhibitory factor, GDNF = glial cell line-derived neurotrophic factor). FGF2 induced the formation of large elongated colonies; EGF treated cultures had spherical colonies connected by bridges. LIF, GDNF and the combination of the four growth factors produced spherical to ovoid colonies, with differences in size, as approximately shown in this figure.

Type A spermatogonia were always located inside structures resembling seminiferous tubules within the colonies. Surprisingly, flat cells surrounding these structures, expected to be peritubular myoid cells were stained mainly with vimentin, a specific marker for Sertoli cells. Only a few of these lining cells were positive to α-smooth muscle actin, specific for peritubular myoid cells, (Figure 4). Sertoli cells and peritubular myoid cells have been recently characterized in vivo through the afore mentioned markers during the bovine postnatal development [49]. Although not exactly matching the morphology and cell composition of a seminiferous tubule, the round structures found inside the colonies reveal an important degree of tissue organization under the effect of stem cell medium.

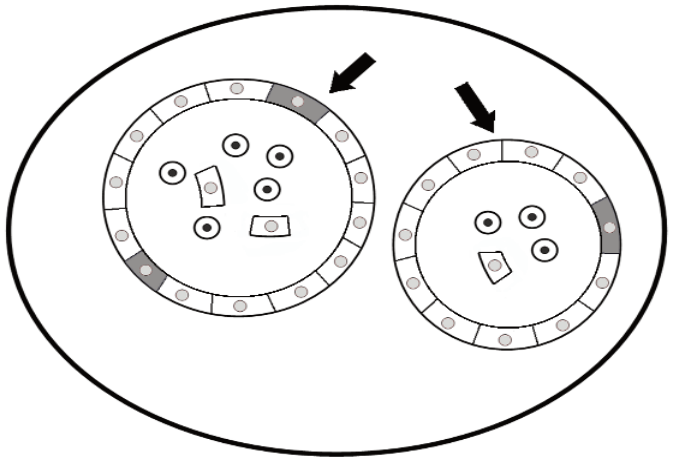


Fig. 4. Schematic representation of a sectioned colony. Cross-sections of seminiferous tubule-like structures can be seen in the core of the colony (arrows). These structures are lined mainly by flattened Sertoli cells (white cytoplasm in the figure) and occasionally tubular myoid cells (black cytoplasm in the figure). Sertoli cells are also located inside the "lumen" of the round tubule-like structures. Type A spermatogonia (round cells in the figure) are always located in the "lumen" of the tubule-like structures.

Interestingly, the highest number of type A spermatogonia located inside the colonies occurred in those cultures where GDNF was present, i.e. GDNF or the combination of all four growth factors. This strongly suggests a process of SSC renewal inside the colonies as early as 15 days of culture under the afore mentioned conditions.

Once we learned growth factors could increase the numbers of SSC during short term cultures, the next step was to start cultures for longer periods of time including sub-culturing bovine spermatogonia, as long-term, multi-passage culturing optimized the propagation of mouse SSC [40, 50]. Culturing for long periods of time is necessary for SSC to grow in numbers as it seems that these cells, similarly as in the in vivo situation, double their numbers every 5.8 days, a considerably longer time than in the case of non-stem cells [51]. Serial passaging implies diluting the harvested cells before re-plating them, so that the cell density becomes lower, creating a higher probability that single SSC remain single and express their full clonogenic capacity [40]. Furthermore, after passaging bovine germ cell cultures, the Sertoli cell monolayer develops all over again both by an

increase in cell numbers and by forming cytoplasmic extensions. These extensions connect with nearby germ cells during colony formation, while many type A spermatogonia also show contacting cytoplasmic extensions. During this process, new germ-somatic cell contacts are established in such a way that multiple new niche sites are formed [52]. Therefore, the enzymatic dissociation of the cultured cells at every passage and subsequent formation of new and more niche sites in the cultures probably enhances the SSC self-renewal process.

In summary, our experiments allowed the expansion of the SSC population during long term culture, serial sub-culturing and multi-growth factor approach (EGF, FGF2, LIF and GDNF). By using this special culture system, we accomplished a 365-fold increase of type A spermatogonia after 26 days of culture. As allegedly, some of the aforementioned type A spermatogonia are SSC, this had to be investigated and quantified by means of the transplantation technique. These experiments, using nude mice, showed the presence of SSC before and after culture, with an estimation of about a 10000 fold increase in the number of SSC after 30 days of culture. This represents about 13 cell divisions in 30 days and an average duration of the cell cycle in our experiment of about 2.3 days. This is comparable to what has been estimated for the mouse in vivo, in which there are probably 2.5 SSC divisions in 8.6 days, that is, a cell cycle of about 3.4 days [2]. Mouse SSCs in a foreign environment (culture [51] or busulphan depleted recipient testis[53]) have an estimated cell cycle duration of 5.6 days [51, 53]. Apparently, SSC in our culture system cycled faster than normal, probably due to the specialized medium and growth factors used. GDNF enhances SSC self-renewal through the GFRA1/RET receptor complex. [30]. The nature of the pathways that lead to self-renewal of SSCs is constantly being addressed in the literature. There is growing evidence that activated self-renewal pathways prevent cells of attaining a differentiating phenotype, for instance the expression of Kit [29, 34]. Probably self-renewal is enhanced beyond physiological levels in our culture system due to the high amounts of GDNF present in the medium. A contribution of endogenous secretion of GDNF by the Sertoli cells present in the system can not be precluded. However, as GDNF secretion is mediated by the presence of FSH [54], and the probable source of FSH in our cultures can only be serum which was used in both control (MEM) and growth factors-stem cell medium cultures, we do not expect a very important contribution of this pathway in our cultures. GDNF is probably saturating the pool of its receptors on SSCs in culture, mediating the very strong self-renewing response ob-

served. This somehow emulates what occurs in vivo in GDNF overexpressing mice in which SSC accumulate in clusters inside the seminiferous tubules [28]. Apparently SSCs self renew but can also survive in the specialized environment. It is suggested that one of the pathways activated via GDNF stimulation, involving AKT, promotes the survival of SSCs [36]. Other growth factors present in the medium might as well be exerting a stimulation of SSC renewal through redundant pathways or others yet to be characterized. These results demonstrate that bovine SSC self-renew in an enhanced way in our specialized bovine culture system and that this system can be used for the propagation of these cells.

In search for an in vitro SSC assay (semisolid cultures: agarose)

In vivo techniques for the evaluation of SSC activity still remain in wide use despite the high amount of labour and time involved. We tested a semisolid medium of agar (agarose) to culture bovine SSCs, as an alternative for the use of liquid medium with the perspective of developing an in vitro SSC assay, based on the clonogenic properties of these cells. Since SSCs produce colonies in vitro, we hypothesized agar would prevent active cell migration while still permitting the access of molecules to and from the culture medium, so that finally each colony would represent the progeny of one original SSC. We found that germ cells isolated from prepubertal bulls and cocultured with Sertoli cells in an double layer agar system could proliferate in a short term culture. Somatic cells grew on top of the bottom agar layer as a monolayer. Germ cells remained on top of the forming monolayer and engaged in colony formation in interaction with somatic cells. Colonies, appeared around day 7 and grew up to day 10 regardless of the culture medium used (liquid vs agar). The presence of more colonies in the agar in comparison with liquid medium may suggest that there were more original immobilized SSC in the semisolid medium. Furthermore, the size of the colonies being smaller when grown in agar reinforces the idea that SSC are prevented to migrate and thus only single clonogenic SSCs would contribute to the process of colony formation, thus generating smaller colonies. In addition, we propose cultured SSCs are prevented from being washed away during the refreshment of the agar cultures in contrast with what occurs when these cells are cultured in liquid media.

More work needs to be done to validate this methodology, especially verifying the presence

of SSCs in the system with the help of the transplantation assay. Our experiments show that a SSC agar system can prove useful to study SSC kinetics in a more realistic way, thus allowing to study the self-renewal and/or differentiation pathways of SCSs through the stimulation of appropriate growth factors. This contribution has the potential of widening our knowledge of bovine SSCs physiology and their use in biotechnological applications.

Final remarks

The possibility to propagate bovine SSC in vitro represents an important breakthrough for cattle production systems and in the reproductive biology field in general. It will be necessary to further optimize our culture system enhancing its usefulness. Transgenesis represents one of the most recently developed aspects of reproductive biotechnologies [55] and SSC technologies may well result in highly efficient ways to deliver genes into livestock species, as current transgenic techniques are not widely used in large domestic animals. When higher numbers of bovine SSC can be routinely obtained by using our methodology, a better understanding of bovine spermatogenesis and new biotechnological developments in the cattle industry will become available.

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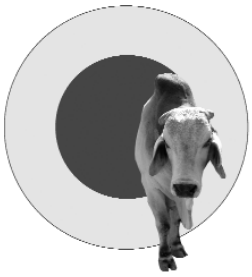
Samenvatting



Samenvatting

In de testis vind het proces plaats van de zaadcelvorming, ook wel spermatogenese genoemd, waardoor millioenen zaadcellen per dag worden gevormd. Aan de start van de spermatogenese staan de spermatogenetische stamcellen (SSCs), die het vermogen hebben om te delen waarbij zij ofwel twee nieuwe stamcellen vormen, een zelfvernieuwende deling, of twee dochtercellen voorbestemd om zich verder te ontwikkelen tot uiteindelijk spermatozoën, wat een differentiërende deling genoemd wordt.

Stamcellen zijn aanwezig in alle andere weefsels in het lichaam maar SSCs zijn de enige stamcellen die in staat zijn genetische informatie door te geven aan toekomstige generaties. Dit opent de mogelijkheid om SSCs in kweek te manipuleren, bijvoorbeeld met het doel belangrijke genen te verspreiden onder kuddes runderen. Aangezien runderen een relatief lange generatietijd hebben, zou een systeem waarmee genen aan runderen kunnen worden doorgegeven via SSCs zeer tijd-efficiënt kunnen zijn. SSCs en de eerste generaties differentiërende dochtercellen (spermatogoniën) verschillen uiterlijk niet van elkaar en worden type A spermatogoniën genoemd. Daarom zijn wij gestart met te onderzoeken op welke leeftijd van kalveren, het beste type A spermatogoniën kunnen worden geïsoleerd. Allereerst hebben wij het begin van de spermatogenese onderzocht in Brahman stierkalveren, een algemeen gebruikt runderras van Aziatische oorsprong. Dit bleek later te zijn dan bij Europesche runderassen. De beste tijd voor de isolatie van A spermatogoniën van Europesche en Aziatische runderkalveren bleek te zijn de periode tussen de start van de spermatogenese (het moment waarop de eerste spermatogoniën verschijnen) tot het moment waarop de eerste spermatocyten worden gevormd. Gedurende deze leeftijdsperiode kunnen A spermatogoniën optimaal gezuiverd worden omdat er nog geen verder ontwikkelde spermatogenetische cellen aanwezig zijn. Daarna hebben wij een kweeksysteem ontwikkeld om A spermatogoniën, waaronder stamcellen, te vermeerderen. Gebruikmakend van StemPro medium met daaraan toegevoegd een aantal groeifactoren konden wij de cellen een aantal weken succesvol kweken. Het nut van de toevoeging van de groeifactoren genaamd GDNF, EGF, LIF and FGF2 werd bestudeerd. Elk van de groeifactoren bleek een specifiek effect te hebben in de kweek. De factoren EGF, LIF en FGF2 hadden een specifiek effect op de niet-spermatogenetische, zogenaamde somatische, cellen in kweek. Daarentegen had GDNF een duidelijk effect op de aantallen alleen liggende A spermatogoniën, de waarschijnlijke stamcellen. Over het geheel genomen wer-

den de beste resultaten bereikt in kweken waaraan alle vier de groeifactoren waren toegevoegd. Na een kweek van vier weken werd een 365-voudige toename gevonden van het aantal A spermatogonien. Om te bewijzen dat GDNF inderdaad de vermeerdering stimuleerde van SSCs, werd een spermatogoniale stamceltransplantatie uitgevoerd, waarbij de gekweekte cellen werden ingespoten in testes van ontvangermuizen. De getransplanteerde SSCs kolonizeerden de testes van de ontvangermuizen en de mate van rekolonisatie werd genomen als een maat voor het aantal getransplanteerde SSCs. Een 10.000–voudige toename in het aantal SSCs na een kweek van 4.5 weken werd gevonden.

Gedurende een kweek in vloeibaar medium, gaan veel cellen verloren gedurende de verversingen van het medium. Zoekend naar een mogelijkheid om dit celverlies te voorkomen hebben wij een niet-vloeibaar medium getest, te weten agar. Inderdaad vonden wij vier maal meer kolonies van spermatogenetische cellen wanneer agar medium werd gebruikt. Dit betekent dat het nuttig is om de voordelen van het gebruik van agar- houdend medium in verder detail te bestudeen.

Samenvattend, wij hebben een kweeksysteem ontwikkeld wat het mogelijk maakt SSCs van runderen te vermeerderen. Dit vergemakkelijkt verdere experimenten aan SSCs en de genetische manipulatie van deze cellen. Dit zal uiteindelijk leiden tot protocollen waardoor genetische verbetering van veepopulaties vergemakkelijkt zal worden.

Summary

In the testis a complex process, called spermatogenesis, generates millions of spermatozoa per day. At the start of this process there are spermatogonial stem cells (SSCs) that have the ability to divide either into new stem cells (self-renewal) or daughter cells committed to develop into spermatozoa (differentiation). SSCs are the only cells among the adult stem cell systems capable of transmitting genetic information to future generations. This offers possibilities for in vitro SSC manipulation for instance with the goal of transferring relevant genes across bovine herds. As bovine species have a long generation interval, a system through which genes could be delivered to cattle through SSCs would prove effective and time-saving. SSCs and the first generations of differentiating spermatogonia are morphologically indistinguishable and are called type A spermatogonia. Therefore, we started with finding out at which age type A spermatogonia could be best isolated. We first investigated the onset of spermatogenesis in Brahman bulls, a widely used breed of Asian origin, and compared our results with the available information on European breeds. The best time to obtain A spermatogonia from European and Asian breeds was found to be the age period between the start of spermatogenesis (appearance of the first spermatogonia) to the age at which the first spermatocytes are formed. During this period the purity of the spermatogonia is optimal since no other differentiating germ cells have yet appeared. We then developed a culture system to propagate these A spermatogonia. Using StemPro medium and adding a number of growth factors we could successfully culture the cells for weeks. The usefulness of adding the growth factors GDNF, EGF, LIF and FGF2 was studied. Each of these growth factors was found to exert a specific effect on the culture. EGF, LIF and FGF2 specifically affected the somatic cells in culture while GDNF had a pronounced effect on the numbers of single type A spermatogonia, presumably SSCs. Overall the best results were obtained when the culture medium contained all four growth factors. A 365-fold increase in the numbers of A spermatogonia was found after 4 weeks of culture. To proof that GDNF actually stimulated the propagation of SSCs, a spermatogonial stem cell transplantation assay was carried out in which the cultured cells were transplanted into recipient mouse testes. The transplanted bovine SSCs colonized these testes and the extent of the colonization was taken as a measure of the numbers of bovine SSCs transplanted. A 10000–fold increase in SSC numbers was found after a culture of 4.5 weeks. During culture in a liquid medium, many germ cells are lost during medium re-

freshments. To prevent this cell loss, we tested a semisolid culture medium (agar). Indeed, with an agar medium four times as many bovine spermatogonial colonies were able to grow. This indicates that it will be worthwhile to study the advantages of using an agar medium in further detail. In conclusion, we have developed a culture system for the propagation of bovine SSC which will allow further experimentation/manipulation and will eventually lead to more efficient ways of genetical improvement of cattle populations.

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other than zebra fish, one of these days, preferably in Belo Horizonte.

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Nancy, Amael and Adriana

Curriculum vitae

Pedro M. Aponte was born in Anaco, Anzoategui state, Venezuela, the 12th of December of 1965. After graduating in high school in Caracas, in 1982 he attended an extra-year of highschool in Lockport, NY, USA, were he obtained a second highschool diploma, while being a exchange student there. Back in Venezuela, he attended the Faculty of Veterinary Sciences of the Central University of Venezuela to obtain his Veterinary Medicine Diploma (Medico Veterinario) in 1990. In 1992 he was appointed with a teaching position in the same Faculty. Meanwhile, he did a Master Course in Animal Reproduction, leading to graduation in 2001. During his Master studies, he worked under the supervision of Dr. Pedro Bastidas in the field of bovine Andrology, with emphasis in the quantification of spermatogenesis of Venezuelan native cattle.

After obtaining his Master degree he started to work as a PhD student at the University of Utrecht, first in the Department of Cell Biology at the Utrecht Medical Center and later in the Department of Endocrinology at the Faculty of Biology, always under the supervision of Prof. Dr. Dirk G. de Rooij. During his PhD project he participated in the Utrecht Graduate School of Developmental Biology. The results of his PhD project are presented on this Thesis. He will continue to work at the Central University of Venezuela, Faculty of Veterinary Sciences, Department of Biomedical Sciences, where he will do teaching/research in the Anatomy and Animal Reproduction fields.

List of Publications

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