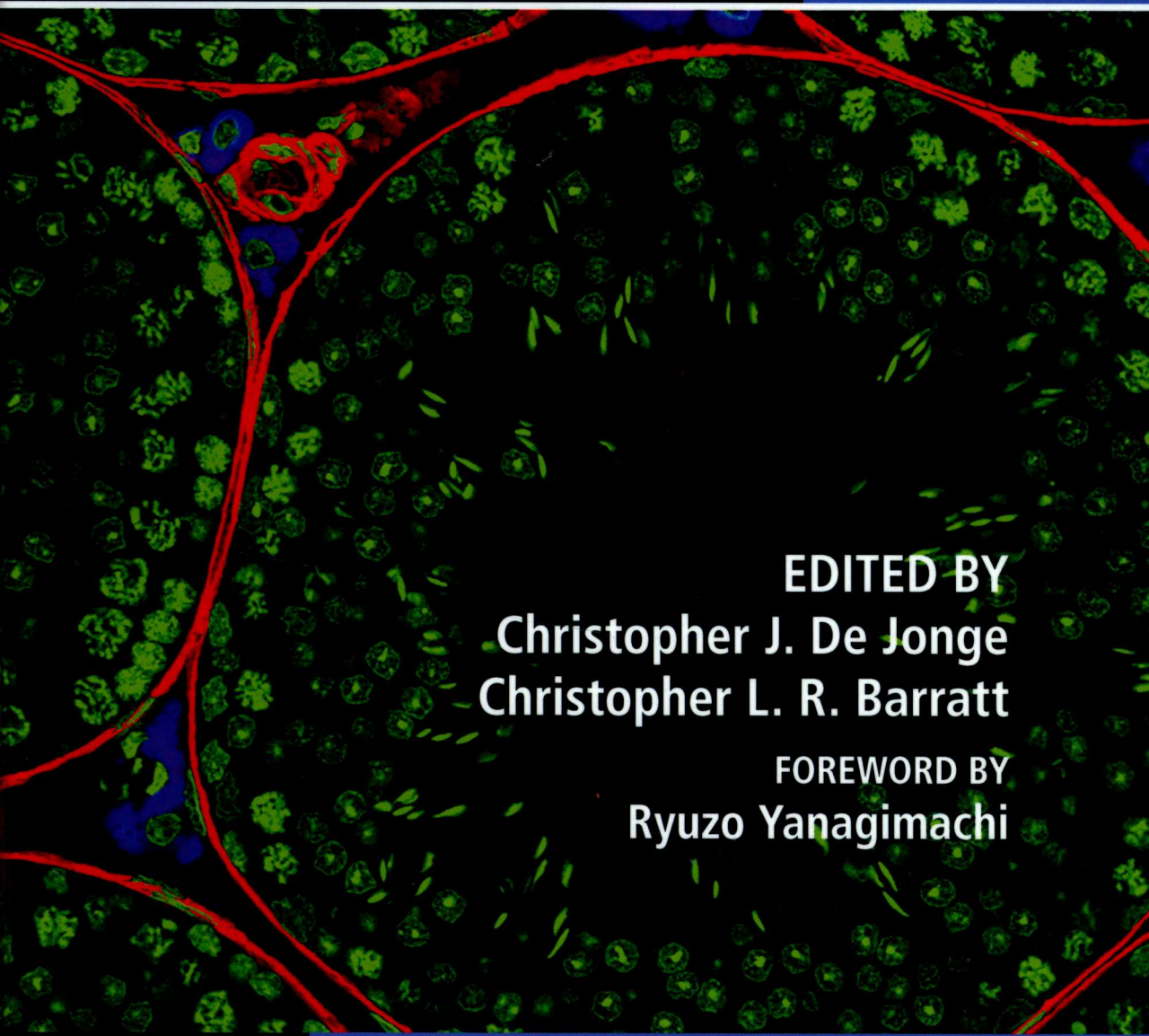
The Sperm Cell

PRODUCTION, MATURATION, FERTILIZATION, REGENERATION

Second Edition



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CAMBRIDGE

Medicine

The Sperm Cell

Production, Maturation, Fertilization, Regeneration
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The Sperm Cell

Second Edition

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Foreword

Eggs are made for sperm. Sperm are made for eggs. All other (body) cells are made to support, directly or indirectly, the development of eggs and sperm and the survival of their united product: the zygote – the next generation. The prime function of spermatozoa is to deliver the male genome safely into eggs. Any errors during sperm formation, maturation and union with eggs will result in serious problems in the male's fertility and in the wellbeing of the offspring.

This book covers our current knowledge of (1) the formation of spermatozoa, (2) the preparation of spermatozoa for fertilization, (3) the union of spermatozoa with eggs, (4) the awakening of 'sleeping' eggs by spermatozoa leading to embryo development, (5) genomic and nongenomic (e.g. environmental) factors affecting the development and fertility of spermatozoa, and (6) the challenges of overcoming male (sperm) fertility problems. Information compiled in each chapter should be considered a stepping stone to better understanding and better control of male fertility and infertility.

The very first chapter of this book mentions the possible production of 'artificial human spermatozoa' from pluripotent stem cells such as human iPSCs. Obviously, it is not appropriate to use live animals or get assistance from live animal cells to achieve this goal. To eliminate or minimize the stress and risks these cells would face during their transformation into haploid cells, we must learn much more about what is really happening in the natural environment of spermatogenic cells, within the testes. The last chapter considers the value of the mouse as a model for the study of mammalian fertility and infertility. Is the mouse a perfect animal model to use for the study of fertility and infertility of all mammals, including humans? Although the mouse is certainly one of the most heavily used model animals for studying mammalian fertility and reproduction, we must remember that each animal uses species-specific tactics to produce its offspring. What is found in one species must be extrapolated to other species with caution.

Today, it is theoretically possible to reproduce any mammals without males. In fact, hundreds of cows have already been produced by somatic cell nuclear transfer. Clearly, males are not essential for animal and human reproduction. Why are there males? At the beginning of life on Earth, there were no males. Females reproduced by themselves. During the course of evolution, a bisexual mode of reproduction emerged, and it has been maintained in most animals, including humans. Compared with animals propagating unisexually (females only), animals using a bisexual mode of reproduction seem to be less vulnerable to extinction in the face of constantly changing, competitive environments. Technically, human cloning (nonsexual reproduction) is possible today. In other words, humans can reproduce without males. Is this what we desire? A few years after the birth of Dolly (a cloned sheep) and many cloned mice, I gave talks to groups of people about animal and human cloning. At the end of my talk I asked the audience if they wanted to live in a world without men. With no exception, women did not want to live in the world without men. 'It would be boring. We cannot use men? That would be horrible.' Men are needed by women, and we will stay that way.

When I started research as an undergraduate student, I thought everything written in books and research papers was a fact. I now know that what is written is authors' interpretations or just a part of the whole story. Many things written in books and reported in original papers will be modified and even discarded during the next 40–50 years. Science progresses that way.

The comprehensive collection of topics that compose this new edition of *The Sperm Cell* provide readers with a map and compass to chart a course for future investigations. It is the readers' task after reading these highly topical research areas to determine what

subjects are left unclear and compelling, what next courses might be important to follow and what burgeoning questions are yet to be studied.

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Preface

Ten years have quickly passed since the publication of *The Sperm Cell – Production, Maturation, Fertilization, Regeneration*. When published in 2006, this unique book provided a comprehensive introduction to the formation, generation and function of the human male gamete. Over the past 10 years science and technology have advanced remarkably and so similarly has advancement in understanding and characterizing the sperm cell. Thus, it is now very timely that we present a completely revised and much expanded second edition of *The Sperm Cell*.

In *The Sperm Cell*, second edition, we have again focused on providing the reader with a tapestry of topics that reveal a more comprehensive characterization into the generation and function of the spermatozoon and that encompasses both basic and clinical aspects. Up-to-date information on subjects where there has been very recent and rapid progress in our understanding – sperm cell epigenetics, proteomics and basic genetics and the consequences of such as potential markers of sperm function – is included. New topics have been added where novel data have revealed fascinating insights into the biology of reproduction, such as the role that seminal plasma may play in modifying both the female tract and the fertilising potential of sperm. Additionally, the book provides two chapters that present competing mechanisms for the process in which a sperm activates an egg. Importantly, a chapter on sperm ultrastructure is included. The application of electron microscopy for scrutinizing ultrastructural components provides amazing insights into the structure and function of the cell that are having an impact on clinical diagnoses.

There has been breathtaking progress in our knowledge base of the human spermatozoon, yet there is still much to learn, and many areas remain relatively poorly explored. For example, ICSI is still regarded as the primary treatment option for men with presumed sperm dysfunction. Insights provided in these chapters will hopefully stimulate investigations that will make less uncertain the structural and functional potential of sperm for fertilization and embryogenesis.

The remarkable cover art for *The Sperm Cell*, bears some similarity to the cover art of the first edition. However, a difference between the images can clearly be seen. For the latter, a somewhat foggy, less distinct cross-sectional image of the seminiferous tubule was used – reflecting, in essence, the 'scratching at the surface' knowledge base of the field at the time. The present cover shows an image of a seminiferous tubule that is sharp and distinct, reflecting greater clarity – clarity in our understanding and characterization of this most unique cell, the spermatozoon.

Our hope is that the collective contributions in this book will inspire and encourage the next generation of research and clinical scientists to the field and, perhaps, reinvigorate older and experienced scientists to think anew from the fresh perspectives offered in *The Sperm Cell*, second edition.

Christopher J. De Jonge
Christopher L. R. Barratt

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Chapter 1

Spermatogenesis
Clinical and Experimental Considerations

Ellen Goossens and Herman Tournaye

Introduction

Spermatogenesis is a complex process that starts in early foetal development and continues through a man's entire lifespan. The process involves cell specification, cell migration, mitotic and meiotic cell division, differentiation and eventually maturation. Only when all these events take place in a correct sequence, in a specific setting and without any errors, will enough mature haploid spermatozoa be produced to enable both fertilization of an oocyte and embryonic development. A single error can hamper sperm production and render a man infertile. The severity of infertility depends on the specific time point when the error occurs during spermatogenesis. Errors in the first steps of establishing spermatogenesis in foetal life cause more severe infertility than errors happening in later phases of spermatogenesis.

Male infertility is a health problem with a dramatic impact on both individuals' and couples' psychosocial wellbeing, as well as a significant healthcare cost. Worldwide, at least 45 million couples are suffering from infertility [1]. In about 50% of couples, a male factor is involved, either alone or in combination with a female-related problem [2]. Since the cause for infertility cannot be identified in all patients, especially in men, most infertile men suffer from unexplained infertility. Yet in recent years, a genetic background for male infertility conditions is being established more and more in these patients.

In patients with oligozoospermia or obstructive azoospermia, spermatozoa can easily be retrieved either from the semen or by surgery from the epididymis or the testis. This sperm can be used for intracytoplasmic sperm injection (ICSI). In about half of patients with nonobstructive azoospermia, sperm can be obtained through testicular biopsies, however; when no sperm are retrieved, there are currently no therapeutic options available for these patients to father a biological child. However, several therapeutic approaches are under investigation. In cases where undifferentiated cells are the only germ cells present in the testis, in vitro or in vivo strategies aiming to generate sperm from spermatogonial stem cells (SSCs) or their daughter cells are to be established. On the other hand, if germ cells are lacking, induced pluripotent stem cells (iPS) derived from the patient's own somatic cells may be the only possible source to generate patient-specific gametes.

In this chapter, we will summarize the main events during normal spermatogenesis, along with potential errors that may arise in the specific stages. In addition, the resultant fertility problem(s) will be described, together with potential treatments either already available or still under investigation.

Primordial Germ Cells

Physiology

The primordial germ cells (PGCs) are the bipotential ancestors of the germ line. These cells can differentiate to either spermatozoa or oocytes. In the mouse embryo, around 6.25 days postcoitus (dpc), six PGC precursors are specified in the posterior proximal epiblast cells near the region where the primitive streak will form. This specification is induced by the transforming growth factor β1 (TGFβ1) superfamily, namely, bone morphogenetic protein (BMP) 8a, BMP4 and BMP2. Like all other somatic cells, these PGCs are diploid [3]. In human embryos, PGC precursors can already be observed in the primary

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ectoderm (epiblast) in the second week after conception. In the third week, PGC precursors migrate outside of the embryo proper into the extraembryonic ectoderm, where they become PGCs. A cluster of 30–50 PGCs can be observed near the dorsal wall of the yolk sac at the basis of the allantois. During the fourth week, when the embryonic disc undergoes a folding process, PGCs incorporate passively into the embryo and are set aside as single cells among the endodermal cells of the primitive hind- and midgut epithelium. By this time, their number has increased, reaching up to 100 PGCs. From week 5 onward, the PGCs travel along the developing nerve fibres from the wall of the hindgut via the dorsal mesentery to the midline of the dorsal wall and laterally into the gonads [4]. The mechanisms by which PGCs migrate to the gonadal ridges include contact guidance with somatic and/or extracellular matrix molecules and chemotactic and repulsive signals. It has been shown that mouse PGCs may use various types of integrins for dynamic adhesive interactions with extracellular matrix molecules such as fibronectin, laminin and collagen IV [5]. Stromal-derived factor 1 (SDF1) and stem cell factor (SCF) have been proposed as chemoattractants for human PGCs [6, 7]. During migration, and after their arrival in the gonadal ridges, PGCs keep on proliferating. It has been estimated that the total number of PGCs increases from 1000 (in week 5) to 150,000 (in week 9) [7]. Once the PGCs arrive in the genital ridges, the somatic cells (early Sertoli cells in a male embryo) will enclose the PGCs to form primitive seminiferous sex cords. From this moment onward, PGCs are called "gonocytes".

While migratory PGCs can be identified by their expression of the pluripotency markers OCT4, NANOG, SSEA1 and c-KIT, the PGCs that have reached the gonadal ridges lose these pluripotency markers and start to differentiate into sex-specific gonocytes.

During their development and migration, PGCs undergo extensive epigenetic reprogramming. While the majority of the genes are demethylated in migratory PGCs, a number of CpG islands (short stretches of DNA in which the frequency of the CG sequence is higher than in other regions) in imprinted genes, X-linked genes and genes involved in meiosis and gamete generation become demethylated once the PGCs enter the gonadal ridge. At this point, the epigenome has reached its most "naive" state. During later stages in gamete development, new epigenetic marks and genomic imprints will be acquired [8].

Sertoli-Cell-Only Syndrome

Mouse PGCs lacking β1 integrins or the c-KIT receptor fail to migrate normally to the genital ridges. Mutations in the human c-KIT gene have not been reported. However, some reports suggest that variants within the nucleotide sequences of the c-KIT and SCF genes are associated with Sertoli-cell-only (SCO) syndrome (Figure 1.1A), also known as Del Castillo syndrome, after the author who first described this condition characterized by germ cell aplasia [9].

A significant group of men with this syndrome have microdeletions in the azoospermia factor (AZF) region of the Y chromosome. This region contains three important genetic domains (AZFa, AZFb and AZFc). Though a definitive genotype/phenotype correlation does not exist, deletions spanning multiple AZF regions or deletions restricted to AZFa usually result in patients with SCO, whereas deletions restricted to AZFb or AZFc can result in patients with phenotypes ranging from SCO to moderate oligozoospermia [10]. AZFa contains three genes: USP9Y, DBY and UTY. Deletions or mutations in USP9Y may cause severe oligozoospermia. DBY is frequently deleted in infertile patients, and its absence leads to severe oligozoospermia or azoospermia due to SCO. Moreover, all patients in whom both the USP9Y and the DBY gene are deleted show a testicular histology of SCO [11].

Artificial Gametes

Currently, couples with the man suffering from SCO can undergo TESE for retrieving testicular spermatozoa eventually to be used for ICSI. However, spermatozoa can be observed after TESE only in about half of these men [12]. These men represent a subgroup referred to as "incomplete SCO". In the other half, no spermatozoa can be found, even after multiple biopsies. At present, these men with "complete SCO" can have children only via gamete donation. However, most couples prefer to raise their genetically own child. Therefore, several investigators address the question of whether artificial gametes could become a possible alternative. In order to produce gametes for these patients, induced pluripotent stem cells (iPSCs) have to be developed from the patient's own somatic cells. Subsequently, these iPSCs should be differentiated into functional gametes (Figure 1.1B).

The most promising strategy for the generation of patient-specific human iPSCs is the reprogramming of

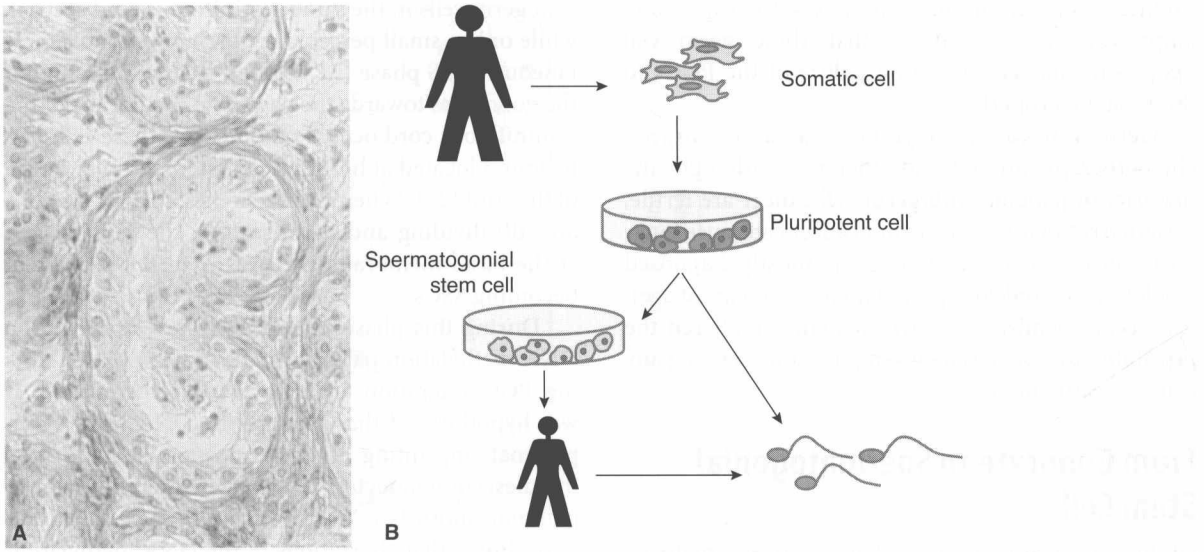

Figure 1.1 Fertility restoration in SCO patients. The testes of patients with SCO lack germ cells (A). Gametes might be produced from patient-specific somatic cells (B). The somatic cells (e.g. skin) are reprogrammed to pluripotent cells by overexpression of Sox2, Oct4, cMyc and Klf4. The pluripotent stem cells are then differentiated to spermatozoa in vitro, which can be used in assisted reproduction. Alternatively, pluripotent stem cells are differentiated to spermatogonial stem cells, which can be transplanted to the testis to further differentiate in vivo. (A black and white version of this figure will appear in some formats. For the colour version, please refer to the plate section.)

differentiated somatic cells by forced overexpression of the pluripotency genes Sox2, Oct4, cMyc and Klf4. However, one must be aware that these induced PSCs may retain their somatic epigenetic memory, which could affect their eventual differentiation into gametes.

So far, production of artificial gametes from PSCs has been achieved only in mice [13]. These gametes were able to fertilize oocytes, resulting in viable offspring, although some of the pups showed tumour formation in the neck region, which could be related to imprinting problems.

Another group also succeeded in obtaining live offspring from haploid cells produced from iPSCs, but the offspring died prematurely, probably due to aberrant imprinting [14].

To date, complete in vitro spermatogenesis from human iPSCs has not been demonstrated. Panula et al. found that ~5% of human iPSCs can differentiate into PGCs after stimulation with bone morphogenetic proteins. In response to the overexpression of DAZ family proteins, germ cells entered meiosis and differentiated into haploid cells [15]. Recently, human iPSCs were shown to differentiate directly into haploid spermatidlike cells when cultured for 10 days under conditions used for culturing spermatogonial stem cells [16]. These results may indicate that human iPSCs derived from adult somatic cells can develop germ line cells, but the efficiency of the process needs further improvement, and eventually quality and safety tests have to be conducted.

In addition to the many scientific hurdles that must be overcome before this method may become clinically available, many ethical concerns associated with this procedure need to be addressed, e.g. what kind of preclinical safety studies have to be conducted, and which results will be considered safe enough to make the step towards clinical trials.

Germ Cell Tumours

As PGCs follow the sympathetic nerve fibres on their way towards the gonads, PGCs failing to exit the nerve branches at the gonadal site may continue along the sympathetic trunk, ending up in other organs where, under normal circumstances, they are eliminated through apoptosis [17]. However, PGCs that have migrated aberrantly might survive in the ectopic location if they overexpress c-KIT [18]. If that is the case, these PGCs may form germ cell tumours. Germ cell tumours have been described in the head, neck, mediastinum, pelvis and testis. These tumours can be benign (teratoma) or malignant (teratocarcinoma). Two main germ cell cancers have been described: seminomas and nonseminomas. The

finding that seminomas show OCT4 expression emphasizes the hypothesis that these germ cell neoplasms may result from a failure of the PGCs to differentiate properly.

Germ cell cancers can be treated by surgery, chemotherapy and/or radiotherapy. Although the majority of patients with germ cell cancer are fertile, certain treatments for testicular cancer can cause long-term sterility. As these tumours are mostly diagnosed in adolescents and young adult men (<35 years of age), it is recommended that these patients be offered the possibility to store a semen sample before starting any cancer treatment [19].

From Gonocyte to Spermatogonial Stem Cell

Germ cell stages between PGCs and spermatogonial stem cells (SSCs) are usually named gonocytes, suggesting that these cells represent a single developmental stage. Nevertheless, several studies have indicated that rodent and human gonocytes in fact encompass a number of consecutive stages, described as mitotic (M), quiescent (Q) and transitional migratory (T) gonocytes. During the first trimester of gestation, gonocytes are mitotically active, but during the second trimester, most but not all gonocytes progressively lose mitotic activity, together with their pluripotency and PGC markers. In rodents, there is a second mitotic phase in the early neonatal period, but in humans, the gonocytes remain quiescent until two or three months after birth. By that time, the gonocytes start to express more advanced germ cell markers such as melanoma antigen-A4 (MAGE-A4), and they reexpress c-KIT, which mediates their migration from the centre of the seminiferous cord towards the basal membrane. When the gonocytes attach to the basal membrane, they start differentiating into spermatogonia. The only clear differences between neonatal gonocytes and spermatogonia are their morphological appearance (large spherical gonocytes versus smaller half-moon-shaped spermatogonia) and their different locations within the seminiferous cord [20].

Although M, Q and T gonocytes express different levels and combinations of proteins, at a given time, subsets of cells positive and negative for specific markers do exist. In mice, the various germ cell subsets are not restricted to specific time frames in development. Rather, there seems to be an overlap of subpopulations in time, with the presence of both mitotic and quiescent germ cells in the same seminiferous cord section, while only a small percentage of gonocytes are simultaneously in S phase [21]. Moreover, the migration of the gonocytes towards the basement membrane of the seminiferous cord occurs randomly, as these cells can be found located at both the periphery and the centre of the cord [22]. Whereas cells in the centre of the cord are still dividing and premigratory, the cells located at the basal membrane are already in the process of becoming SSCs.

During this phase in germ cell development, the DNA methylation patterns that had been erased during PGC migration are now being remethylated. It was hypothesized that most of the DNA involved in paternal imprinting and transposons is methylated in quiescent gonocytes [23]. Correct establishment of paternal imprints is of major importance, as studies have shown that aberrations in the DNA methylation pattern and inactivation of proteins involved in this process can lead to embryonic lethality in rodents. In humans, defective DNA methylation of imprinted genes has been associated with oligozoospermia (see the section on *Oligozoospermia* under *Spermiogenesis and Epididymal Sperm Maturation*) [24].

Spermatogonial Stem Cell Proliferation and Differentiation

Physiology

Spermatogonial Stem Cell Proliferation and Differentiation

SSCs are single triangle-shaped cells located on the basement membranes of the seminiferous tubules, in close contact with the Sertoli cells. The population of SSCs is a small subpopulation of the spermatogonia. In rodents, the prevailing model is the A_s-model [25]. The A_s or single undifferentiated type A spermatogonium is considered to be the "true" SSC. If A_s spermatogonia divide completely, they usually migrate separately and retain stem cell activity. If, upon division, they remain connected to each other by a cytoplasmic bridge, they become paired type A (A_{pr}) spermatogonia. The production of type A_{pr} spermatogonia is the first step towards differentiation. Type A_{pr} spermatogonia divide once more to produce groups of four aligned type A (A_{al}) spermatogonia, also connected one to each other. The A_{al} cells proliferate, resulting in chains of 8, 16 and occasionally 32 cells. A_s, A_{pr} and

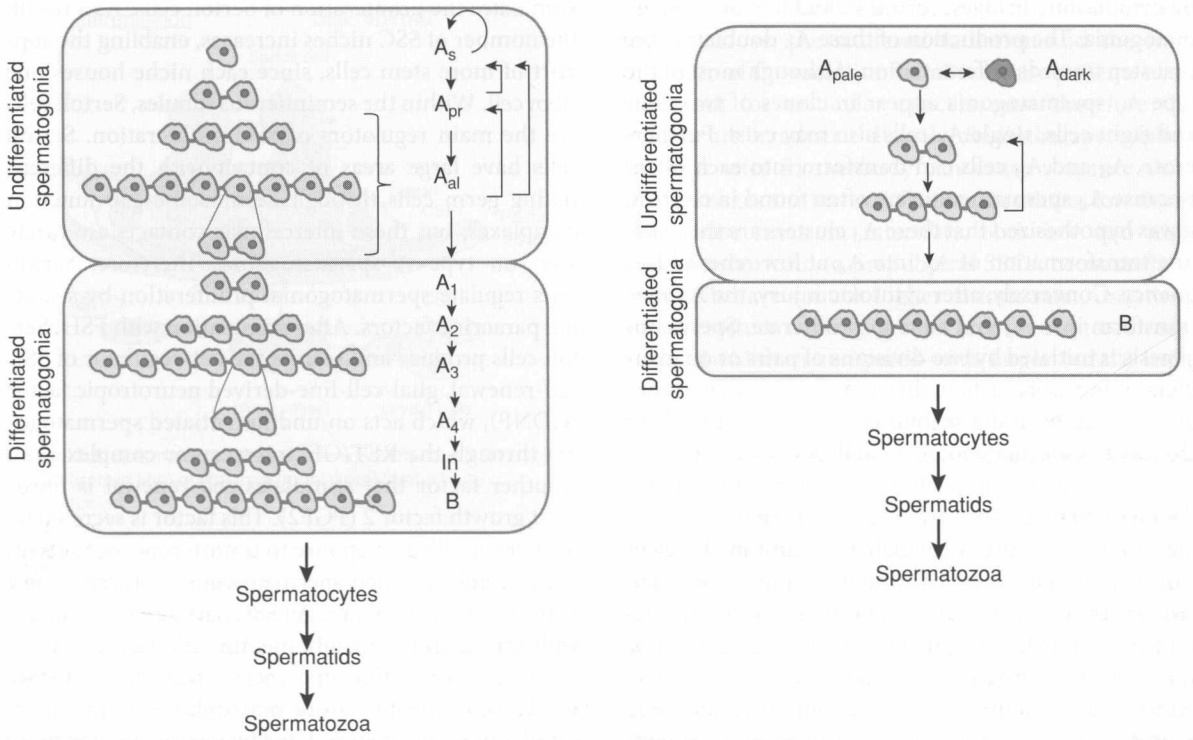

Figure 1.2 Model of spermatogonial stem cell proliferation in rodents (A) and humans (B).

A_{al} spermatogonia have the same morphology and can be distinguished only according to their topographical arrangement on the basement membranes of the seminiferous tubules. Most of the A_{al} spermatogonia will undergo a morphological change and transform into type A_1 spermatogonia. These A_1 spermatogonia are the first generation of differentiating spermatogonia. Next generations include A_2, A_3, A_4, intermediate and B spermatogonia. A lot of research has been done to characterize rodent SSCs. The combination of fluorescence-activated cell sorting with SSC transplantation has revealed that A_s spermatogonia express β_1-integrin (CD29), α_6-integrin (CD49f), THY1 (CD90), CD9, GFRα1 and E-cadherin, but do not express α_v-integrin (CD51), MHC-I, C-KIT and CD45. Cells expressing OCT4 showed higher stem cell activity than the OCT4$^-$ cells. Also, PLZF, SOX3, NGN3, NANOS2 and STRA8 were determined in undifferentiated spermatogonia. Other genes that are expressed in spermatogonia, but not in somatic cells, are MAGE-A4, UBE1Y, USP9Y, RBMY, OTT, DDX4, TEX14, USP26, PIWIL2 and PRAMEL1 [26]. New evidence shows that the A_s population and spermatogonial chains of the same length are heterogeneous in respect to their gene expression. Inhibitor of DNA binding 4 (ID4), for example, has the most restricted expression pattern observed to date, but not all single spermatogonia express this marker [27]. Others showed that NGN3 expression was heterogeneous, since only 11% of transplantable SSCs were NGN3-positive. The implications of this heterogeneity for SSC function are largely unknown. However, recent findings have elucidated that the NGN3 positive subpopulation is destined for differentiation, implying that not all SSCs act equivalently as stem cells. Moreover, A_{pr} and A_{al} spermatogonia were found not to be committed unidirectionally to differentiation but to be capable of reverting to shorter chains by fragmentation [28]. As a consequence of the progress made by these characterization studies, a revision of the A_s model was necessary. Stem cell activity is not limited to SSCs (or A_s spermatogonia), but A_{pr} and A_{al}-spermatogonia also have the potential to self-renew (Figure 1.2A).

In primates, two morphologically different classes of type A spermatogonia are observed: the dark A_d (or "reserve" stem cells) and the pale A_p spermatogonia (or "renewing" stem cells). When A_p spermatogonia divide, they usually remain connected to each other

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