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 “naïve” 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
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 Aₜ-model [25]. The Aₜ or single undifferentiated type A spermatogonium is considered to be the “true” SSC. If Aₜ 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 (Aₚₚ) spermatogonia. The production of type Aₚₚ spermatogonia is the first step towards differentiation. Type Aₚₚ spermatogonia divide once more to produce groups of four aligned type A (Aₚₜ) spermatogonia, also connected one to each other. The Aₚₜ cells proliferate, resulting in chains of 8, 16 and occasionally 32 cells. Aₜ, Aₚₚ and
A₂ 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 A₂ spermatogonia will undergo a morphological change and transform into type A₁ spermatogonia. These A₁ spermatogonia are the first generation of differentiating spermatogonia. Next generations include A₂, A₃, A₄, 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 A₅ spermatogonia express β₁-integrin (CD29), α₆-integrin (CD49f), THY1 (CD90), CD9, GFRα1 and E-cadherin, but do not express αᵥ-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, UBE1IY, USP9Y, RBMY, OTT, DDX4, TEX14, USP26, PIWIL2 and PRAMEL1 [26]. New evidence shows that the A₅ 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, A₅ and A₁₅ 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 A₅ model was necessary. Stem cell activity is not limited to SSCs (or A₅ spermatogonia), but A₅ and A₁₅-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 A₄ (or “reserve” stem cells) and the pale A₅ spermatogonia (or “renewing” stem cells). When A₅ spermatogonia divide, they usually remain connected to each other.
by cytoplasmic bridges, forming doublets of A_p spermatogonia. The production of these A_p doublets is the first step towards differentiation. Although most of the type A_p spermatogonia appear in clones of two, four and eight cells, single A_p cells also may exist. Furthermore, A_q and A_p cells can transform into each other. Because A_q spermatogonia are often found in clusters, it was hypothesized that these A_q clusters are the result of a transformation of A_p into A_q at low renewal frequency. Conversely, after cytotoxic injury, the A_q may transform into A_p and start to proliferate. Spermatogenesis is initiated by two divisions of pairs or quadruplets of A_p cells: a first division, after which clones of A_p separate, and a second division, which leads to clones of B spermatogonia as well as pairs or quadruplets of A_p cells. These latter cells are responsible for the maintenance of the original size of the type A population. Because the A_p, which are found in clones of two or four cells, cycle continuously, the “true” stem cells are probably the rarely dividing single A_q and A_p spermatogonia [29] (Figure 1.2B). During the last few years, a lot of progress has been made in the characterization of human SSCs. Human spermatogonia express many markers equivalent to those of rodent spermatogonia, e.g. α6-integrin, GFRA1 and THY1, although other markers are not shared. For example, human SSCs do not express β1-integrin but are positive for TSPY1, CD133 and SSEA4 [30]. From a clinical viewpoint, this model including “reserve” stem cells may explain why both the degree of recovery of spermatogenesis and the time for eventual recovery after gonadotoxic treatment depend on the number of surviving stem cells in the different compartments.

**Spermatogonial Stem Cell Niche**

SSCs are located in specialized niches. The niche can be defined as the microenvironment that regulates tissue homeostasis by controlling the balance between SSC self-renewal and differentiation. The SSC niche in the mammalian testis is mainly located on the basal membrane of the seminiferous tubules, but part of the SSC niche is thought to lie outside the seminiferous tubules. One might assume that all germ cells located at the basal membrane are SSCs, but this is not the case. Stem cell niches are not distributed randomly along the tubule but are thought to be localized in areas near the vasculature, implying an important regulatory function for specific factors transported through the blood or produced by the vascular endothelial cells [31]. In the neonatal testis, follicle-stimulating hormone (FSH) stimulates the proliferation of Sertoli cells. As a result, the number of SSC niches increases, enabling the support of more stem cells, since each niche houses one stem cell. Within the seminiferous tubules, Sertoli cells are the main regulators of SSC proliferation. Sertoli cells have large areas of contact with the differentiating germ cells through desmosome-gap junction complexes, but these intercellular contacts are rarely seen on type A spermatogonia. Therefore, Sertoli cells regulate spermatogonial proliferation by secreting paracrine factors. After stimulation with FSH, Sertoli cells produce and secrete the key regulator of SSC self-renewal, glial-cell-line-derived neurotropic factor (GDNF), which acts on undifferentiated spermatogonia through the RET/GFRA1 receptor complex [32]. Another factor that stimulates self-renewal is fibroblast growth factor 2 (FGF2). This factor is secreted by the Sertoli cells in response to testosterone and acts on both undifferentiated spermatogonia (paracrine) and Sertoli cells (autocrine). As SSCs are in close contact with the basement membrane, this allows the SSCs to respond also to diffusing paracrine factors secreted by Leydig cells, myoid cells or macrophages in the interstitial space. When stimulated by luteinizing hormone (LH), Leydig cells produce testosterone, which in turn stimulates Sertoli cells to produce either self-renewal (FGF2) or differentiation (ABP, oestradiol) factors. LH action on Leydig cells is also responsible for the production of colony-stimulating factor 1 (CSF1), a self-renewal factor acting directly on spermatogonia. The influence of peritubular myoid cells on germ cell regulation has long been questioned, but the interest in this still poorly known cell type is growing. Its role in SSC maintenance was suggested by the fact that CSF1 and GDNF were detected in peritubular myoid cells [33]. Very recently, the niche has been extended with testicular macrophages. In particular, one subset of testicular macrophages, those that are located on the surface of the seminiferous tubules, close to areas enriched for undifferentiated spermatogonia, are found to be involved in the regulation of SSC proliferation and differentiation. These macrophages express spermatogonial proliferation- and differentiation-inducing factors, such as CSF1 and enzymes involved in retinoic acid (RA) biosynthesis [34] (Figure 1.3).

**Gonadotoxic Treatment During Puberty**

One child in every 600 is diagnosed with cancer. As the majority of childhood cancers are systemic cancers...
(leukaemia, lymphoma, tumours of the central nervous system), high (and multiple) doses of chemotherapy and/or total body irradiation have to be administered to cure the patient. These treatments are now very efficient, and therefore 80% of the children eventually will survive their disease. Unfortunately, when these patients grow up they may encounter an important life-quality-threatening problem: fertility. Since spermatogenesis are also extensively proliferating before puberty [35], the prepubertal testicular tissue is highly sensitive to damage by chemo- or radiotherapy, which can lead to destruction of the SSC pool. Some diseases such as acute lymphoblastic leukaemia even cause decreased gonadal function independent of cancer therapy. Besides cancer patients, children affected by non-malignant diseases such as sickle cell disease or drepanocytosis may require gonadotoxic treatments as a conditioning therapy for their curative bone marrow transplantation. As these patients are treated with high doses of chemotherapy and sometimes also total body irradiation to destroy their bone marrow, their chances for spontaneous fertility restoration are virtually nonexistent.

If the SSC pool is not completely destroyed, spermatogenesis can recover from the surviving SSCs. After chemotherapy, the chance of spermatogenic recovery mainly depends on the received dose and the gonadotoxic agent. All alkylating agents (cyclophosphamide, ifosfamide, nitrosoureas, chlorambucil, melphalan, busulphan, procarbazine) are gonadotoxic. On the other hand, antimetabolite agents such as methotrexate and cisplatin-based regimens such as bleomycin do not have a long-term effect on fertility [36]. For radiotherapy, recovery depends on the received dose, the scattered radiation and the fractionation of the radiation bundles. Some patients need treatment for testicular cancers, and therefore the gonads are irradiated directly. Radiotherapy applied to other sites in the body can cause a scatter dose that damages the testes. Whereas, in adults, low doses only cause transient infertility, both low and high doses are harmful to prepubertal SSCs, because these stem cells have a much higher mitotic rate. Also, their shorter stature causes a greater risk for scattered radiation in children compared with larger (adult) individuals. Hence, children are much more vulnerable to the effects of chemotherapy and irradiation than adults (see section on Gonadotoxic Treatment During Adulthood).

As gonadotoxic treatments affect the proliferating A Germ spermatogonia, restoration of the SSC pool depends on the survival of quiescent A Germ spermatogonia. As soon as niches become available due to SSC loss, these A Germ spermatogonia start dividing to replenish the stem cell pool. After a first mild treatment with a gonadotoxic agent, most of the patients show a higher germ cell number than untreated individuals [37]. However, when these patients have to be treated for a second time, the risk of losing their SSCs is even higher, as these cells are now more proliferative. Other testicular cell types are much less susceptible to damage by chemo- and/or radiotherapy than germ cells. Sertoli cells, peritubular myoid cells and Leydig cells have a lower turnover rate. To cause damage to the Leydig cells of prepubertal boys, doses of at least 2400 cGy are needed, potentially causing delay or arrest of pubertal maturation and problems in developing secondary sexual characteristics. Once the SSC pool is completely lost, fertility cannot be restored. Therefore, although still experimental, several fertility centres have started a preventive fertility preservation program for prepubertal boys [38]. Preferably before the start of any gonadotoxic treatment, testicular tissue containing SSCs has to be biopsied. During the time of therapy and recovery, the testicular tissue is stored in liquid nitrogen.

Several cryopreservation protocols are in use, but slow freezing using dimethyl sulphoxide as cryoprotectant is preferable. Two different protocols were developed in animal models to restore fertility after thawing: transplantation of cell suspensions containing SSCs into the seminiferous tubules (aka SSC transplantation) and testicular tissue grafting. Although
in mice fertility can be re-established in 50% of transplanted animals, the colonization efficiency of transplanted SSCs is rather low (12%), probably because the natural niche contacts have been lost. Since a very large number of SSCs are required to recolonize an adult testis, increasing the amount of SSCs by in vitro culture could solve this problem. Although it was described that in vitro SSC culture could increase SSC numbers 18,000-fold, these findings have not been widely confirmed [39; 40]. It might be more efficient to transplant the tissue as a whole, as the grafting technique retains the natural SSC niche and was reported to yield a 100% success rate in mice [41]. On the other hand, this technique cannot be used in patients suffering from certain malignant disorders because of the risk of reintroducing malignant cells back into an otherwise cured patient [42]. Although, in rodents, it was reported that sorting malignant cells out of a testicular cell suspension did not reintroduce tumour formation after transplantation, it remains unknown whether the same strategy could be used in humans [43]. The culture system developed to propagate SSCs in vitro could be an alternative strategy for depleting malignant cells, since a pilot study showed that this culture system does not support the growth of leukemic cells [44].

Whereas at present transplantation of SSCs or tissue is not yet ready for clinical application in patients because of unresolved safety issues, patients who...
suffered from a non-malignant disease will probably be the first to have their stored testicular tissue transplanted back given the absence of the risk for malignant contamination (Fig 1.4).

Environmental Factors Reduce Niche Numbers

Apart from chemotherapeutic agents and irradiation, other environmental and lifestyle factors can influence the number of SSCs. Our lifestyle may indeed affect sperm quality in a negative way, both during foetal and neonatal life and in adulthood (see section on Environmental and Lifestyle Effects During Adulthood). During foetal and neonatal life, testosterone levels increase, promoting important early events in spermatogenesis: testicular development is initiated, PGCs differentiate into spermatagonia and Sertoli cells proliferate. As the number of Sertoli cells is related to the number of SSC niches, and hence the number of SSCs, factors that interfere with androgen function may influence Sertoli cell proliferation and via the indirect effect on SSC numbers future sperm output. Sertoli cells stop proliferating shortly after birth, followed by another proliferation phase at puberty. However, the effects that arise during foetal and neonatal life are indirectly important in determining their final number at adulthood [45].

Together with changes in lifestyle, our exposure to a wide range of environmental chemicals (PCBs, phthalates, pesticides) has increased during the last decades. Several of these chemicals accumulate in the fat and have anti-androgenic activity. During pregnancy and lactation, fat cells are broken down in response to a higher energy need. This process may cause the release of accumulated PCBs, which are passed on to the foetus. In the male foetus, androgen activity may be hampered, resulting in lower proliferation rates in Sertoli cells and thus a reduced sperm output in adulthood. The impact of these environmental chemicals may be even higher among obese women (more fat, thus higher accumulation) and women who become pregnant later in life (longer exposure to these compounds) [46; 47]. Several research groups investigated the associations between maternal exposure to environmental chemicals present in cosmetics, toiletries or medications during pregnancy and the risk for testicular dysgenesis syndrome in their sons, but little consistency was found. Therefore, it was hypothesized that effects on the developing foetal testis are likely to occur as the result of exposure to a mixture of environmental chemicals.

Foetal exposure to dioxin, which is a highly toxic byproduct of incineration, and polycyclic aromatic hydrocarbons (PAHs) that are constituents of exhaust fumes, smoke and cooking processes are also hypothesized to result in a lower sperm concentration in the adult. These molecules interact with the aryl hydrocarbon receptor. The activation of this receptor can antagonize androgen-mediated action, leading to a reduction in Sertoli cell number and lower sperm counts in adulthood [48; 49]. Dramatically lower sperm counts were found in men whose mothers had smoked heavily during pregnancy, probably caused by the interaction of PAHs in the cigarette smoke with the aryl hydrocarbon receptor [50]. A similar pathway might explain the significant reduction in sperm counts in sons of mothers who consumed a lot of beef during pregnancy. However, this could also be due to saturated fats or anabolic steroids [51].

In general, any environmental compound that affects testosterone production and/or function in the foetus may theoretically have consequences in terms of reduced number of SSC niches, and therefore reduced sperm production and reduced sperm counts later in life [for review, see 52].

Niche Deficiencies

Mumps Orchitis

Thanks to international vaccine programs, mumps has become less common in children. However, if postpubertal men get infected, mumps may be complicated by orchitis in about 20–30% of patients [53]. The mumps virus infects the testicular glands within the first days of infection, leading to parenchymal inflammation, separation of seminiferous tubules and perivascular interstitial lymphocyte infiltration. As the tunica albuginea forms a barrier against oedema, the intratesticular pressure rises, resulting in pressure-induced testicular atrophy.

Mumps orchitis may also affect Leydig cell function, as low testosterone levels, elevated luteinizing hormone levels and an increased pituitary response to luteinizing-hormone-releasing hormone have been reported. Testosterone concentrations return to normal levels after several months, but follicle-stimulating hormone and luteinizing hormone concentrations remain significantly increased until at least one year
later [54]. Azoospermia is a rare possible consequence of mumps orchitis and mainly linked to severe cases of bilateral orchitis with testicular atrophy. However, even azoospermia is not necessarily associated with the complete absence of spermatozoa in the testes. A testicular biopsy often yields a few spermatozoa and fertilization and pregnancy can thus be achieved by intracytoplasmic sperm injection [55].

Klinefelter Syndrome
Klinefelter syndrome (KS) is a sex chromosomal syndrome, affecting 1/600 newborn males. This syndrome is characterized by the presence of one or more extra X chromosomes and is among the most common genetic causes of human infertility. It is estimated that less than 10% of the cases are diagnosed during childhood; the majority are diagnosed during infertility counselling or remain undiagnosed [56]. Infertility in KS patients is caused by the loss of germ cells, which starts in early infancy and accelerates at the onset of puberty. Before puberty, the testicular architecture is normal, but some studies claim that germ cell numbers are already reduced during childhood. The adult KS testis often shows extensive fibrosis and hyalinization of the seminiferous tubules and hydroplasia of the interstitial tissue [57]. Only in a minority of KS patients can sperm cells be found in the ejaculate; most patients are azoospermic. However, intratesticular residual foci of spermatogenesis can be present in adult azoospermic KS patients. The introduction of intracytoplasmatic sperm injection provided hope for fertility in Klinefelter patients. Successful recovery of spermatozoa by testicular sperm extraction is reported in about half of the azoospermic KS patients referred to centres specializing in assisted reproductive techniques [58]. Currently, the other half of the patients do not have any option to father their genetically own children. As it is impossible to predict which patients will be fertile at adulthood, the banking of prepubertal tissue containing SSCs is an attractive strategy [59]. Whenever azoospermia with failed retrieval of testicular spermatozoa is a fact at adulthood, frozen–thawed SSCs could hypothetically be differentiated in vitro and subsequently used for ICSI (Figure 1.4).

While the cause of the germ cell loss in KS patients is still unexplained, it is also not clear from which SSCs the testicular spermatozoa (that are found in half of adult men) originate. It is still a matter of debate whether degeneration of the testis is a consequence of meiotic errors, the changed testicular/endocrine environment or altered X-linked gene expression. Unraveling the mechanisms playing a role in KS-related infertility could provide important insights into preventing sterility in these patients. While some reports claim that 47,XXY germ cells are able to complete meiosis, it is also hypothesized that spermatogenesis in KS patients exclusively arises from 46,XY germ cells [61]. If this were supported by further evidence, the feasibility of in vitro differentiation would become questionable. Another question that needs to be elucidated is whether the 46,XY germ cells eventually generating spermatozoa in adulthood are already present at birth or arise during development, as this has important implications for future fertility preservation strategies. Detailed histological study on testes from peripubertal KS patients (aged 13–16 years) showed that all displayed a significant loss of SSCs and no meiotic differentiation, with widespread fibrosis and hyalinization of the seminiferous tubules [62].

In vitro Spermatogenesis
The first studies on in vitro spermatogenesis (IVS) date back to the beginning of the twentieth century. Although simple culture techniques were employed, promising results were obtained that formed the basis for later research. Several attempts were undertaken to improve incubation parameters, feeders or coatings of dishes, but all of these failed to accomplish complete IVS [for review – 63]. The most successful study was performed by Cremades et al., who cultured immature germ cells from infertile men with arrested germ cell development [64]. Only when starting from postmeiotic germ cells (round and elongating spermatids) could fertilization–competent gametes be derived. Studying spermatogenesis in situ has led to the understanding that the spatial arrangement of the testicular cells is very important for the regulation and completion of germ cell maturation. In line with this, Stukenborg et al. hypothesized that under improved culture conditions (low temperature, appropriate endocrine and paracrine milieu, 3D-structures supporting cell-to-cell contacts), germ cells would be able to enter and pass through meiosis and spermiogenesis in vitro. Eventually, morphologically normal spermatozoa from mouse spermatagonia were obtained in such a 3D culture system [65]. A few years later, in vitro sperm production was accomplished in neonatal mouse testes using an organ culture method [66]. These sperm were able to fertilize