Male Germ Cell Development in Humans

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Key Words

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Abstract

Background: Germ cells are unique cells that possess the ability to transmit genetic information between generations. Detailed knowledge about the molecular and cellular mechanisms determining the fate of human male germ cells still remains sparse. This is partially due to ethical issues limiting the access to research material. Therefore, the mechanisms of proliferation, differentiation and apoptosis of human male germ cells still remain challenging study objectives. **Methods:** This review focuses on using English articles accessible in PubMed as well as personal files on the current knowledge of the molecular and cellular mechanisms connected with human testicular germ cell development, maturation failure and the possibility of fertility preservation in patients in whom there is a risk of gonadal failure. However, since rodents, particularly mice, offer the possibility of studying germ cell development by use of genetic modification techniques, some studies using animal models are also discussed. **Conclusion:** This mini review focuses on the current knowledge about male germ cells. However, the reader is referred to two previous mini reviews focusing on testicular somatic cells, i.e. on Sertoli cells and Leydig cells.

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Introduction

Human Male Germ Cell Development: Prenatal

The establishment of the human male germ cell lineage starts during embryogenesis at 4 weeks' gestation [1]. Studies on mice have shown that primordial germ cells (PGC) originate from the proximal epiblast in the yolk sac in response to bone morphogenetic protein stimulation from the nearby visceral endoderm and extraembryonic ectoderm [2]. However, little is known about the origin of human PGC, though it is believed to be similar to that in the mouse. Recently, it has been shown that bone morphogenetic protein 4 enhances human PGC propagation in vitro [3]. It has long been believed that the PGC gather outside the embryo at the yolk sac wall and then migrate through the hindgut to the genital ridge, which later will become the primitive gonad [4]. The PGC express KIT/c-KIT receptor tyrosine kinase, and the surrounding somatic cells express its ligand, KITLG/SCF/STEEL. The interaction between KIT and KITLG has been suggested to support the migration and survival of PGC [5, 6]. However, recent reports have indicated that PGC migrate very little and their migration from the yolk sac to the genital ridge is aided by hindgut expansion and

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growth movements [7, 8]. Furthermore, it was proposed that human PGC move together with autonomic nerve fibers from the dorsal mesentery to the developing gonad [9, 10]. This hypothesis is supported by the fact that the enteric nervous system of the mid- and hindgut expresses KITLG and therefore could facilitate migration [11]. However, further studies are needed in order to clarify the exact process of PGC migration.

PGC arise from embryonic cells that already have been partly committed to a somatic fate [12]. This means that their genome has already undergone DNA methylation and histone modifications, both of which are epigenetic marks that can regulate gene expression without changing the DNA sequence. During PGC proliferation and re-localization, global DNA methylation erasure occurs [13]. Now the genome is like a blank page and ready for sex-specific de novo DNA methylation. In the male, this methylation occurs in mitotically inactive cells before birth and in growing oocytes in females after birth. In this way the genome of the developing germ cells get distinct methylation profiles of sperm and oocytes [14].

Molecular markers to identify germ cells at various stages of their development are highly warranted, and search for such markers is an active research area. The nascent and relocating human PGC express markers such as *POU5F1/OCT4*, *GAGE*, *MAGE-A4* and *KIT* [9], while mouse PGC have also been shown to express *Prdm1/Blimp*, *Dppa3/Stella*, *Ifitm3/Fragilis* and *Tnap/Alpl/Ap* [15]. In humans, by gestational week 4–6 the PGC enter and colonize the developing gonad located in the genital ridge, and from then on are referred to as ‘gonocytes’ [16]. Gonocytes express genes such as *MAGE-A4*, *DAZ*, *KIT*, *PLAP*, *POU5F1*, *TFAP2C/AP-2γ* and *UTF1* [17, 18]. *DDX4/VASA* is the most widely used marker for germ cells and it is expressed from the gonocyte stage and throughout the whole differentiation process [19].

The decision of gonocytes to enter the female or male germ cell pathway is influenced by the somatic compartment surrounding the germ cells. In males, mesenchymal, Sertoli and Leydig cells are crucial for the formation of the male gonadal paracrine and endocrine interactions (e.g. inhibin B and testosterone) [20, 21]. The initiation of the male path of sexual development is dependent on the activation of several genes such as *DHH*, *FGF9*, *M33*, *DMRT1*, *AMH*, *SRY* and *SOX9* [16, 22].

Human Male Germ Cell Development: Postnatal

After the gonocytes have been directed to the male or female path, they either initiate meiosis (female path) or not (male path). Concentrating on the male pathway, the

gonocytes migrate to the basal membrane and become spermatogonia postnatally after approximately 6 months (fig. 1) [23]. By reaching the basal membrane, the gonocytes lose their status of pluripotency, which is depicted by changes in their morphology as well as gene expression profiles (e.g. *POU5F1*, *NANOG*, *TFAP2C* and *KIT*) [24]. As discussed below in the next paragraph, this change in expression levels, mainly for *POU5F1* and *NANOG*, is influenced in testicular germ cell tumors (TGCT) and might be crucial for the survival of these malignant cells within the testis [25]. In contrast to human, spermatogonial stem cells (SSC) described in rodents do express markers such as *POU5F1* or *TFAP2C* throughout their life [26–30].

Normal spermatogenesis is a complex growth and maturation process encompassing three distinct stages: (1) mitotic proliferation and maturation of spermatogonia; (2) meiotic cleavage to form haploid cells, and (3) final maturation into elongated spermatids and spermatozoa (spermiogenesis). In quantitative terms, spermatogenesis starts in early puberty and is clinically recorded as an increased testicular volume. In humans, spermatogonia can be divided into at least two subtypes: (1) undifferentiated (type A_{dark} and A_{pale}; fig. 1) and (2) differentiated (type B). Type A spermatogonia differentiate and form type B spermatogonia, which undergo mitotic proliferation until puberty, when meiosis is initiated [16, 31]. A multitude of genes are related to the different types of spermatogonia (fig. 2), and many of those have been suggested as potential SSC markers [16, 32]. Recently, isolation of human putative SSC has been performed by selecting GPR125-positive cells coexpressing ITGA6, THY1 and GFRα1 [33].

The meiotic process gives rise to haploid spermatocytes and can be grouped into preleptotene, leptotene, zygotene, pachytene and diplotene stages. After the second meiotic division, haploid round spermatids are formed, which turn into elongated spermatids during spermiogenesis, which is a morphogenic process without further proliferation [34]. The whole process of human spermatogenesis takes 74 days and gives rise to 16 spermatids, if at full efficacy, from each SSC [35]. The sperm is finally released into the lumen of the seminiferous tubules and transferred to the epididymis for final maturation.

Since the processes of spermatogenesis and spermiogenesis are highly complex, a failure in any crucial gene is immediately affecting the outcome of both. Below we will focus on genes expressed by male germ cells related to dysfunctional spermatogenesis.

Fig. 1. Histological overview of early human germ cells. Different germ cells [gonocytes; prespermatogonia (Pre-Spg); A_{dark} spermatogonia; A_{pale} spermatogonia] are shown in a cross-section of seminiferous tubules from a 9-month-old boy and highlighted at higher magnification and as cartoons. According to the literature, the cell types can be characterized as follows. Gonocytes: located in the lumen; cells are roughly spherical with a high nucleocytoplasmic ratio; nuclei in the center; binuclear forms are occasionally seen (preserved without evidence of degeneration); nucleoli have characteristic filamentous structure (uniform electron density) and are usually not attached to the inner nuclear membrane; double nuclear membrane has no unusual features; cytoplasm contains 2 types of mitochondria (one with parallel cristae and another with dilated vesicular cristae); some of the mitochondria are connected to the outer nuclear membrane [94]. Prespermatogonia/fetal spermatogonia: located at the basal membrane; frequently chromatid body in cytoplasm; large cell body; round-to-oval nucleus (about twice as large as that of Sertoli cells); large nucleolus in the middle of the nucleus (consisting of loose reticular substance and 2–3 amorphous bodies); chromatin is distributed over the entire nucleus in form of fine granules; large mitochondria connected with intermitochondrial substance (mainly tubule type, but also crista type); Golgi complex is concentrically located around the

nucleus; chromatid bodies are frequently observed in the vicinity of the nucleus [95–97]. A_{dark} spermatogonia: located at the basal membrane; characteristic 'lighter zone' (rerarification) generally located in the middle of the nucleus; peripherally located nucleoli, rarely connected to the nuclear membrane; crista-type mitochondria are connected to the nuclear membrane and often to each other by intermitochondrial substance (cement); rougher endoplasmic reticulum concentrically around the nucleus than A_{pale} spermatogonia; ribosomes, microtubules and glycogen granules in the cytoplasm; 'crystalloid of Lubarsch' (1896) in the vicinity of the nucleus [95, 98]. A_{pale} spermatogonia: located at the basal membrane; 2–4 times bigger than an S_a Sertoli cell (developed from fetal Sertoli cells directly after birth and roughly twice as big as an adult erythrocyte); excentrically located round or polygonal nucleus; bleb formation can be observed in the nucleus; fine and homogeneously distributed chromatin; 1–3 nucleoli attached to the nuclear membrane; no mitochondria are attached to the nucleus; cytoplasm is of fine granular type; occasionally large vacuoles in the cytoplasm; crista-type mitochondria; slightly developed Golgi apparatus; ribosomes, microtubules and glycogen granules in the cytoplasm; 'crystalloid of Lubarsch' (1896) in the vicinity of the nucleus [95, 98, 99]. BM = Basal membrane; N = nucleus; n = nucleolus; SC = Sertoli cell; PC = peritubular cell.

Reasons for Failure of Human Male Germ Cell Development

There are many different reasons for failure of male germ cell development. Overall, mutations in genes related to PGC, gonocytes and spermatogonia are often accompanied by tumor formation, early spermatogenic ar-

rest and/or lethality in early embryonic development of the offspring [16]. Conditions like Klinefelter syndrome (KS), cryptorchidism and androgen insensitivity syndrome can lead to problems with fertility [36, 37]. Furthermore, gonadotoxic cancer treatment of boys at various ages can result in male infertility [38]. This may be of major concern especially for prepubertal boys, who do

Fig. 2. Schematic overview of genes expressed during different stages of human male germ cell development.

not yet produce mature sperm and therefore are not able to supply mature gametes for cryopreservation before starting oncological treatment [38].

Testicular Germ Cell Tumors

The incidence of TGCT is 1% of all cancers in men, and 95% of those arise in men between 15 and 34 years of age. Interestingly, the precursor cells for TGCT, carcinoma in situ or gonadoblastoma cells seem to arise during fetal life and are silent until they transform into TGCT cells later in life [24].

TGCT are known to be related to mutations in several genes including *KITLG*, *SPRY4*, *BAK1*, *TERT*, *DMRT1* and *ATF7IP* [39]. Detection of these malignant cells on the protein level can be achieved by showing positive expression mainly for *POU5F1*, *PLAP*, *KIT*, *TFAP2C/AP-2γ* and *NANOG* [25]. Thus, these cells express many markers which have been described for early germ cells

like gonocytes and PGC [24, 25]. Recent studies are in line with previous ones, demonstrating the important role of *KITLG* and *DMRT1* gene alterations as main factors involved in TGCT formation [40, 41]. As a third important player in TGCT formation, *TSPY* has to be mentioned. *TSPY* is expressed in spermatogonia in the healthy adult testis, but in immature germ cells in testes with TGCT [24]. *TSPY* has no functional homolog in mice and might thereby explain why no type II TGCT (classic testicular seminoma, dysgerminoma as well as various types of nonseminoma/nondysgerminoma) has been described in these animals so far [42].

Even if the detailed physiological role of *TSPY* is not totally understood, an overexpression seems to contribute to the survival and proliferation of germ cells, even in a nonoptimal environment [43]. This antiapoptotic effect has also been described for *POU5F1* and *KITLG* [10, 24, 44]. However, *POU5F1* is mainly expressed in early

germ cells up to the stage of gonocytes, whereas TSPY is expressed mainly in early spermatogonia when gonocytes have reached the basal membrane and formed prespermatogonia [18, 45]. Therefore, a double expression of POU5F1 and TSPY in germ cells at the basal membrane of the seminiferous cord/tubule might be a strong indicator for malignant cell development in the testis [46, 47]. These findings are also in line with a recent study by Som et al. [48], which suggests that the origin of seminoma is more likely in the undifferentiated than in a dedifferentiated germ cell. In addition to the germ cell origin, the influence of paracrine and endocrine pathways on the development of abnormal functions of somatic and germ cells in the testis has been discussed in several studies and a delayed function has been associated with the formation of testis cancer [24, 49, 50]. A reduced androgen production might act via the Sertoli cells on the germ cells and be the reason for the increased risk of patients with androgen insensitivity syndrome to develop TGCT [24, 25, 50].

Spermatogenic Arrest

Spermatogenic arrest is defined as an interruption to male germ cell development at any stage before complete maturation. Reasons for spermatogenic arrest are primarily genetic or a result of secondary influences from other diseases (e.g. severe liver or kidney insufficiency, sickle cell anemia), or iatrogenic (radiation, chemotherapy or certain antibiotics) [38]. To date there are no therapies available for cure of complete (azoospermia) or partial (oligoasthenoteratozoospermia) arrest of spermatogenesis. As mentioned before, impairment or failure of even one gene related to germ cell differentiation may cause spermatogenic arrest. This arrest can be observed at different developmental levels (spermatogonia, primary or secondary spermatocytes and round spermatids) and can be roughly defined as premeiotic, meiotic and postmeiotic spermatogenic arrest (fig. 3).

At the start of spermatogenesis, genes of importance for SSC renewal and differentiation are often regulated by Sertoli cells [16, 21]. Some of those which are involved in these regulations and expressed in human SSC are *GFR α 1*, *THY1/CD90*, *PLZF*, *MAGE-A4* and *GPR125* [32]. Failure related to these and to genes expressed by Sertoli cells (e.g. *ERM*) has been studied in mice, and it results in spermatogonial arrest or Sertoli-cell-only syndrome [51].

In mice, *mUtp14b*, an X-linked gene, is expressed from the level of zygotes up to round spermatids [52]. Failure of this gene causes a spermatogonial arrest after the first

wave of spermatogenesis. A similar spermatogenic failure is observed in juvenile spermatogonial depletion (jsd) mice mutants [53, 54]. This mutation is causing spermatogonial arrest at the level of type A spermatogonia, after the first wave of spermatogenesis in mice. From 4 to 20 weeks after birth, these animals show increased levels of follicle-stimulating hormone (FSH) but otherwise remain endocrinologically normal after 1 year.

In humans, *BOULE*, a member of the *DAZ* family, regulates the translation of *CDC25* [55] and was first identified in azoospermic men with Y-chromosomal deletion [56]. Together with cyclin B, *CDC25* is driving meiosis as a maturation factor, and nonfunctional *CDC25* results in disrupted spermatogenesis [57]. Genes of the *DAZ* family are expressed by differentiated spermatogonia type B, preleptotene and zygotene spermatocytes. In rats, *DAZ* is involved in early stages of spermatogonial differentiation, while *DAZL* is connected to early spermatogenesis as well as the maturation of elongated spermatids during spermiogenesis [58].

Another main player is *CREM*, a gene of importance for the formation of haploid cells and therefore for the completion of spermatogenesis. *CREM* expression is modulated by FSH and GDNF, and a null mutation is causing spermatogenic arrest at the stage of round spermatids [59]. However, a deficiency of *CREM* specifically in Sertoli cells is not related to spermatogenic arrest.

After meiosis, disintegration of histones and the integration of protamines are crucial for the maturation process of the spermatozoa and are performed by the transition protein. Mutations in the transition protein influence the condensation of chromatin, causing breaks in the DNA structure of late spermatozoa.

Inhibin B has been described as an early indicator of testicular damage [60]. An early postnatal increase in inhibin B correlates with the activation of the hypothalamic-pituitary-gonadal axis and seems to parallel the changes in testosterone and luteinizing hormone levels. The highest levels of inhibin B are found at the age of 3 months postnatally during mini-puberty [61]. During puberty, inhibin B reflects Sertoli cell proliferation and FSH action. After completion of Sertoli cell differentiation, the levels of inhibin B decrease and remain at low levels. Changes in inhibin B after puberty are related to changes in germ cell proliferation. Here, the androgen receptor (AR) might be the driving force [60]. AR is not expressed in germ cells, but a lack of AR in Leydig cells has a major influence on the steroidogenic function and results in a spermatogenic arrest at the stage of round spermatids. In terms of spermatogenic outcome, inhibin B directly cor-

Fig. 3. Schematic overview of different stages in differentiation of human spermatogenesis: complete spermatogenesis (a), meiotic arrest (b), premeiotic arrest (c) and 'Sertoli cell only' (d). Different cell types, including cells of the germ and somatic cell compartments, present in the healthy adult testis are shown in a schematic

overview on the left side (color code). Impairments of spermatogenesis are shown schematically in the middle column of the figure. Reference histological samples, stained for periodic acid-Schiff reagent, for the different types of spermatogenesis are shown on the right side of the figure next to the schematic overviews.

relates with sperm counts [62]. In addition to inhibin B, AMH is another crucial factor for Sertoli cell maturation, testicular formation and germ cell differentiation [49]. AMH is a dimeric glycoprotein hormone which belongs to the transforming growth factor- β super family. During gonadal development, AMH is one of the first genes expressed by Sertoli cells [49, 63] and is maintained as long as they stay in their immature status until puberty. A recent study by Dennis et al. [64] demonstrated that serum levels of AMH reflect the number of cells producing AMH. Therefore, AMH may be used as a marker of gonadal developmental status in humans.

Germ Cell Consequences of Cryptorchidism

With a prevalence in full-term newborns of 1–3%, cryptorchidism is the most common congenital malformation in boys [37]. Well-recognized consequences of cryptorchidism include impairment of germ cell maturation, subsequent infertility in adulthood as well as a higher risk for development of TGCT [65]. As early as 6 months of age, a reduction in germ cell numbers starts, dependent on the position of the testes. In general, the higher the testicular position at the time of treatment, the fewer the number of germ cells [66, 67]. A lack of germ cells is an important predictor of future infertility and can be identified by testicular biopsy, taken at the time of or-

chiopexy [68]. The incidence of azoospermia in unilateral cryptorchidism is 13%, whereas an increase up to 89% in untreated bilateral cryptorchidism was described [69]. However, due to the rarity of spontaneous descent after 1 year of age and the possible improvement in fertility that early intervention may confer, cryptorchidism should be treated before the patient is 1 year old [65, 67, 70]. Furthermore, to preserve fertility in patients with bilateral orchiopexy, freezing of sperm or testicular tissue with preserved spermatogenesis has also been suggested [71].

Germ Cells in Klinefelter Syndrome (KS)

The estimated prevalence of KS in the general male population is around 0.2% [72]. In spite of the wide variability in clinical appearance, patients with KS suffer from absolute or relative hypergonadotropic hypogonadism as well as impaired spermatogenesis. During childhood, boys with KS have lower testicular volumes compared with age-matched controls [72]. In KS patients, the growth of the testes is increasing temporarily until midpuberty. After midpuberty it declines, and low serum testosterone as well as elevated gonadotropin levels can be observed [73]. It has been described that spermatogenesis in these patients is often arrested at the level of spermatogonia or early spermatocytes. Spermatogonia seem to be hindered to enter the meiotic pathway and undergo apoptosis at the onset of puberty [74]. A gradual deterioration in seminiferous tubules can be observed over time. This is accompanied by tubular hyalinization as well as Leydig cell hyperplasia, which is characteristic of testes in adult KS patients. Isolated foci of spermatogenesis can exist in the testes of KS patients. Therefore, successful pregnancies resulting from rare ejaculated sperm in the semen of men with KS have been reported [75, 76]. Recent studies recommend early sperm and/or semen retrieval or, for prepubertal patients, cryopreservation of testicular tissue, due to the progressive decline in steroidogenic and spermatogenic functions during puberty in KS patients [77–79]. Attempts at surgical sperm retrieval were reported to be successful in up to 66% of men included in a study by Ramasamy et al. [80]. In combination with advanced assisted reproduction technology (ART), these sperm retrieval rates have improved the ability of KS patients to father their own biological children. However, no generally accepted recommendations for cryopreservation of testicular tissue in KS adolescents exist so far.

Fertility Preservation

It is well recognized that germ cells are difficult to culture in vitro and are sensitive to conditions that apply outside the gonadal microenvironment. However, methods for retrieving, processing and maturing germ cells are highly warranted for clinical purposes. Pediatric cancer patients exposed to gonadotoxic therapy are an increasing group which attracts such attention in order to preserve their future gonadal function.

Methods for Preserving Male Germ Cells

Postpubertal males who produce fully mature sperm can cryopreserve their semen with a possibility to father children upon desire later in life by in vitro fertilization. Young prepubertal boys do not produce mature sperm and thus do not have the option of cryopreserving sperm [38]. Prepubertal patients can be offered testicular biopsy which contains SSC. Potentially, the immature testicular tissue or isolated SSC can be matured either in vitro or by placing them back into the normal somatic microenvironment (to be discussed below). The idea is that these cells can later in life be used for in vitro fertilization and give these patients the option of fathering their own biological children. Since testicular tissue or isolated germ cells need to be stored for a long time, cryopreservation techniques need to be optimized. Currently, one of the procedures for cryopreservation used most often is to add ethylene glycol and dimethyl sulfoxide together with a slow freezing program [38].

Methods for Maturation of Male Germ Cells

Major efforts have been put into optimizing in vivo and in vitro methods for maturation of immature germ cells, but these techniques still remain experimental (fig. 4). For the in vivo methods, the immature testicular tissue or SSC could be transplanted back to the patient after cancer treatment, but in such a case there is a potential risk of introducing cancer cells back to the patient [81]. Transplanting the tissue or the isolated SSC to other animal species for maturation is also an option, but then there is a potential risk of introducing the germ cells to xenogenic tissue hosting unknown viruses such as retroviruses. Therefore, such a methodology would presently not be considered acceptable in clinical practice. For the in vitro methods, the SSC could be differentiated in a monolayer on a supporting feeder layer or on extracellular matrixes [82, 83]. Also, it has been reported that premeiotic germ cells from mice can be differentiated in a soft agar culture system where the cells have 3D support

Fig. 4. Strategies to explore molecular and cellular mechanisms important for human spermatogenesis and needed for the establishment of an in vitro system to differentiate early male germ cells into mature sperm for potential use of ART in the future. These

experimental strategies involve cultures of human induced pluripotent stem (iPS) and human embryonic stem (ES) cells as well as testicular cells obtained from biopsies of prepubertal testes. ICSI = Intracytoplasmic sperm injection.

[84, 85]. Another promising system currently showing a successful differentiation of early germ cells into mature sperm in mice is the organ culture [86, 87].

All of these methods are still technically suboptimal and need to be further improved in order to be employed for clinical purposes. This optimization research is difficult due to limited access to human prepubertal testicular material. Further, much research of this kind is still done on animal models, which is problematic as there are species differences. A novel and promising strategy is to employ human embryonic stem cells (hESC) and human induced pluripotent stem cells (hiPSC) in order to try to optimize in vitro differentiation [88–90]. These stem cells are pluripotent but originate from different sources; hESC are derived from the inner cell mass of an embryo

at the blastocyst stage, while hiPSC are generated by reprogramming somatic cells to a pluripotent stage by using factors of importance for pluripotency, e.g. the Yamanaka factors (POU5F1, KLF4, SOX2 and MYC) [91–93]. In theory, these pluripotent stem cells are able to differentiate in vitro into all the different cell types of the body, including germ cells if the right culture and differentiation methods are found.

Stem Cell Approach to Mature Germ Cells to Rescue Future Fertility

Because research material from prepubertal boys is sparse, hESC and hiPSC would be ideal for optimizing an in vitro model for differentiation of immature germ cells into mature sperm. Being able to differentiate hESC and

hiPSC as well as immature germ cells in vitro would help in understanding the basics of germ cell development as well as aid in optimizing a protocol for fertility preservation (fig. 4). Furthermore, if possible, it would be a huge breakthrough if hiPSC obtained from an infertile male could be differentiated into morphologically, genetically and epigenetically normal and functional spermatozoa. These hiPSC would be patient specific, and thus immune rejection and graft-versus-host reaction could be avoided. In theory, these spermatozoa could be used in combination with advanced ART.

Concluding Remarks

Germ cells are the most fundamental cells in life, complicated and fragile. Increasing knowledge has been obtained about the control of proliferation and differentia-

tion of human male germ cells, but a lot yet remains unknown. Techniques are now rapidly developing to expand and mature germ cells in vitro, and such approaches have produced apparently normal offspring in experimental animal models. Although there are still ethical, safety and efficiency concerns with humans, we anticipate that it will not take long until such methods, combined with advanced ART, are introduced at highly specialized medical centers.

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