Journal of Pediatric Surgery 55 (2020) 1201–1210

Contents lists available at ScienceDirect

Journal of Pediatric Surgery

journal homepage: www.elsevier.com/locate/jpedsurg

Cryptorchidism, gonocyte development, and the risks of germ cell malignancy and infertility: A systematic review

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ARTICLE INFO

Article history:
Received 2 May 2019
Received in revised form 18 June 2019
Accepted 28 June 2019

Key words:
Germ cells
Gonocyte transformation
Spermatogonium
Cryptorchidism
Undescended testis

ABSTRACT

Background/Aim: Cryptorchidism, or undescended testis (UDT) occurs in 1%–4% of newborn males and leads to a risk of infertility and testicular malignancy. Recent research suggests that infertility and malignancy in UDT may be caused by abnormal development of the neonatal germ cells, or gonocytes, which normally transform into spermatogonial stem cells (SSC) or undergo apoptosis during minipuberty at 2–6 months in humans (2–6 days in mice). We aimed to identify the current knowledge on how UDT is linked to infertility and malignancy.
Methods: Here we review the literature from 1995 to the present to assess the possible causes of infertility and malignancy in UDT, from both human studies and animal models.
Results: Both the morphological steps and many of the genes involved in germ cell development are now characterized, but the factors involved in gonocyte transformation and apoptosis in both normal and cryptorchid testes are not fully identified. During minipuberty there is evidence for the hypothalamic–pituitary axis stimulating gonocyte transformation, but without known direct control by LH and androgen, although FSH may have a role. An arrested gonocyte maybe the origin of later malignancy at least in syndromic cryptorchid testes in humans, which is consistent with the recent finding that gonocytes are normally absent in a rodent model of congenital cryptorchidism, where malignancy has not been reported.
Conclusion: The results of this review strengthen the view that malignancy and infertility in men with previous UDT may be caused by abnormalities in germ cell development during minipuberty.
Type of study: Systematic review (secondary, filtered)
Level of evidence: Level I.

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https://doi.org/10.1016/j.jpedsurg.2019.06.023
0022-3468/

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Cryptorchidism, or undescended testis (UDT), is common in children, occurring in 1%–4% of full term newborn males [1] and in up to 45% of prematurely born boys worldwide [2,3]. Boys with undescended testes are at risk of testicular neoplasms and infertility; however, the mechanism of these pathologies is unknown [4] . It has been proposed that aberrant transformation of neonatal germ cells in UDT may be due to heat stress which may cause the increased rates of both cancer and infertility in males [5].

Germ cells, as progenitors of gametes, are crucial for the reproductive potential of the individual and the species as a whole. Their journey from pluripotency to the haploid gamete, specialized for fertilization, is long and complex. Owing to their unique characteristics, failure in any stage may lead to both malignancy and infertility in cryptorchidism.

Gonocytes transform into spermatogonial stem cells (SSCs) soon after birth (~2–6 days) in the rodent [6,7] and at 3-6 months of age in humans [9], during what was previously thought to be a quiescent period. Gonocyte transformation is normally completed by twelve months of age [9,10]. SSCs are unipotent stem cells which are crucial for fertility as they are the pool of self-renewing stem cells that eventually produce mature sperm. Spermatogenesis has been studied extensively and many of its processes elucidated in recent reviews. However, the transformation of gonocytes into SSCs has been largely less studied.

This review seeks to detail current knowledge about gonoctye transformation. Much of our understanding stems from animal studies as the molecular and biochemical similarities do give insight into the human condition, although one must be wary of translating findings in murine models to human patients [11]. A better understanding of the drivers and mechanism of gonocyte transformation will have clinical applications to the medical and surgical management of cryptorchidism [12].

1. Methods

Medline (Ovid) and Embase (Ovid) were searched back to 1995 using Thesaurus and/or key words. Pubmed was subsequently searched to retrieve any articles not indexed in Medline. The search strategy used search terms and Boolean operators adapted to fit the search requirements of Embase and PubMed (Appendix 1).

Results were limited to English. We also reviewed reference lists of relevant review articles and hand-searched references of retrieved articles and relevant articles referred by experts in the field.

2. Results

2.1. Origin of the germ cell

Primordial germ cells (PGCs) are the precursors of sperm and oocytes and thus represent the ancestors of the germline. The precursors of PGCs are derived from the epiblast before gastrulation, at 6.5 days' gestation in mice and 2 weeks' gestation in humans. The PGC precursors then rapidly move into the extraembryonic region of the yolk sac wall near the developing allantois and are committed to the germline at embryonic day E7.5 in mice [13] under the influence of Transforming Growth Factor beta (TGFβ) and Bone Morphogenetic Protein (BMP) [14]. These cells must maintain pluripotency and suppress somatic expression to mature into gametes. This process is regulated by a balance of SOX17-BLIMP1 gene dosage in response to WNT and BMP [15]. The expression of Prdm14 [16] and Blimp1/PRMT5 complex activates pluripotent genes and suppresses somatic programming respectively [13,17]. At E7–E11 in mice and the 4–6th week of gestation in humans, PGCs migrate back into the embryo proper along the hindgut and midgut, crossing the dorsal mesentery, and colonize the gonadal ridge [18], where they proliferate to produce the germ cell pool [19]. Specification of PGC involves repression of the somatic program, maintenance of pluripotentcy and reprogramming of unique epigenetic state in both mice and human [20,21].

At E11 to E12 in mice and seven weeks' gestation in human male embryos, the SRY gene on the Y chromosome and signaling factors SOX9, DMRT1, Foxl2, RSPO1 (R-spondin in humans) drive the development of the testis [22,23]. Mesenchymal cells transition to epithelial Sertoli cells to form the testicular cords with PGCs and interstitial cells form Leydig cells [24]. After the testis forms, the PGCs are referred to as gonocytes [25].

After 2–3 months in humans and day 3 after birth in rodents, gonocyte numbers per testicular section are reduced owing to redistribution along the seminiferous cords, which showed continuous growth from E19 to day 5. In mice the gonocytes migrate from the center of the testicular cords to the basal membrane and transform into spermatogonia, which form the stable population of SSCs, from which gametes will be constantly produced after puberty (Fig.1) [9]. In rodents several stages of spermatogonia have been described, including the types A single, A pair, A aligned, A1–4, A intermediate (In), and finally B spermatogonia, while in humans the germ cells are described as A pale, A dark, and B spermatogonia [26]. Spermatogonia have traditionally been identified by morphology and relative position within the developing cords, and more recently with molecular markers such as OCT4 labeling PGCs and gonocytes, PLZF labeling SSC, C-kit, D2-40 (Podoplanin) and PLAP (placental alkaline phosphatase) labeling germ cells in human testis during the first nine months of age (Fig. 2) [27]. This has allowed more accurate identification of cells as they mature [28]. The disappearance of gonocytes and the appearance of SSC are crucial steps for normal development. It has been suggested that in humans with cryptorchidism it is the gonocytes that have escaped apoptosis but also failed to transform into SSC that may cause malignancy, while infertility is caused by deficient SSC [29].

3. 'Minipuberty'

'Minipuberty' is the name given to the transient surge of gonadotrophins, testosterone, as well as inhibin-B and AMH occurring in early infancy that is an important step in the maturation of the hypothalamic–pituitary–gonadal axis [30]. The long-term effects of this occurring in a normal and timely manner are important [31], as it is during this time that Ad spermatogonia appear and gonocytes disappear, as the gonocytes (fetal male germ cells) transform into spermatogonial stem cells (future stem cells to produce sperm) [32]. However, the role of minipuberty in gonocyte maturation is still a subject of debate.

3.1. The possible role of androgens in gonocyte transformation

Androgens are critical for spermatogenesis after puberty [33]; however, there is still debate about their importance in minipuberty and gonocyte transformation [34]. Hadziselimovic et al. have proposed

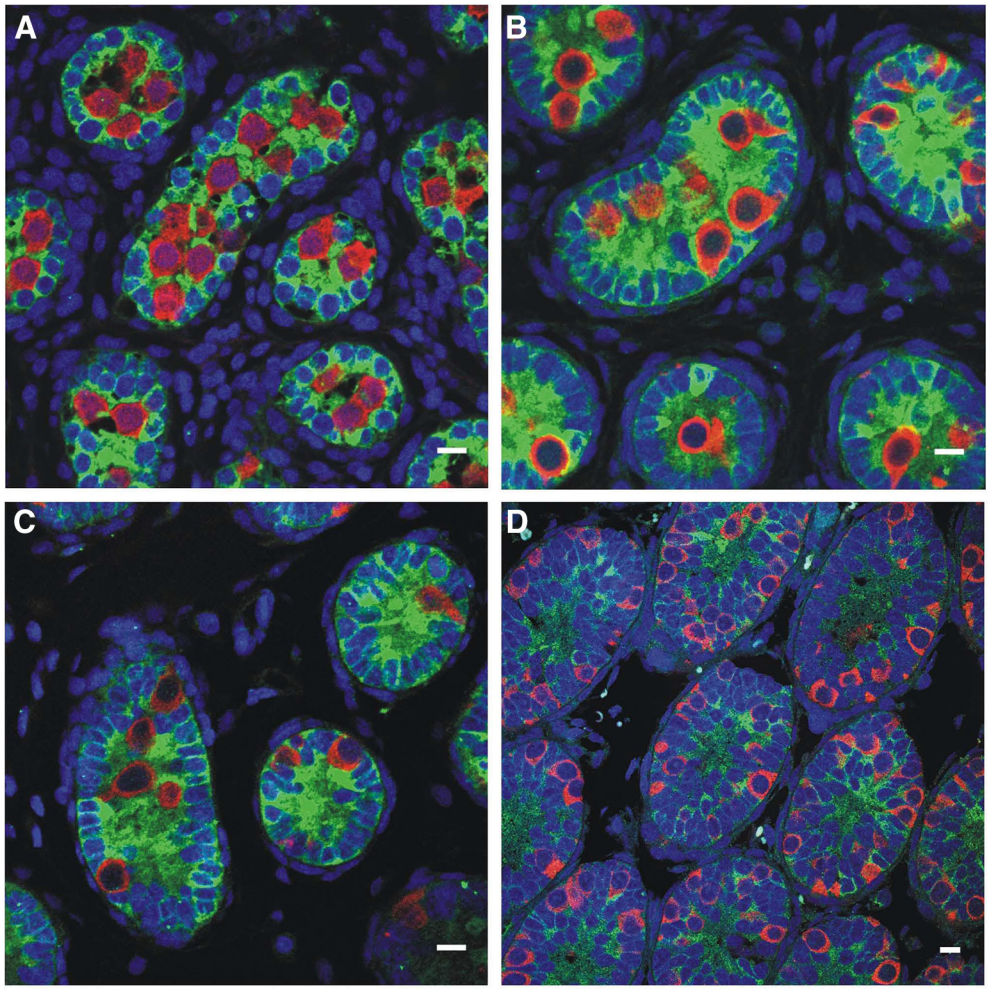

Fig. 1. Morphology and position of germ cells in the first post-natal week in the rat. Germ cells labeled with MVH (mouse homolog of Drosophila Vasa) in red, and the Sertoli cells labeled with AMH in green (bar = 10 μm). The germ cells (gonocytes) are moving to the basement membrane. (A) Day 0, (B) Day 4, (C) Day 6, (D) Day 10. Reproduced with permission [9].

that gonocyte transformation into Ad-spermatogonia is caused by androgens, as cryptorchid boys who were treated with human chorionic gonadotrophin (HCG) before surgery for cryptorchidism had higher number of Ad-spermatogonia than cryptorchid boys who had no HCG (to increase androgen level) before surgery [35–40]. In contrast, Li et al. demonstrated that in the mouse androgen receptors are not required for gonocyte movement from the center to the basement membrane of the testicular tubules or for gonocyte transformation [41]. They showed that androgen receptor knock out (ARKO) mice had the same number of germ cells per tubule as wild type mice, both embryonically and postnatally, confirming that androgens had no direct effect on gonocyte migration and transformation. Moreover, in such mice androgen did not influence the gene expression during gonocyte transformation measured by mouse VASA homolog (MVH), anti-Mullerian hormone (AMH), kit oncogene (c-Kit), matrix metalloproteinase-1 (Mt1-mmp), zinc finger, BTB domain-containing 16 (Plzf) and octamer-binding protein 4 (Oct4) [42].

Merlet et al. showed that fetal germ cells do respond to androgens albeit in an unexpected fashion. Fetal mice at E17.5 with an inactivating mutation of AR have significantly greater numbers of gonocytes compared to wild-type mice [43], indicating an inhibitory effect of androgens on proliferating gonocytes However, androgens suppress Sertoli cell production of AMH, and AMH levels are elevated in humans with complete androgen insensitivity syndrome, [44]. In a similar way Su et al. found in a study of human testicular biopsies of patients with undervirilization syndromes that gonocytes appeared to transform earlier, and in greater numbers, than normally. They proposed that a lack of androgens may stimulate nonandrogenic regulators to trigger gonocyte transformation [45].

Hadziselimovic and Dessouky compared patients with steroid 5 alpha-reductase-2 deficiency (a disorder of sexual development; DSD) and UDT to boys with isolated bilateral cryptorchidism. Testes with steroid 5-alpha-reductase-2 deficiency lacked spermatocytes but had Ad spermatogonia and a normal germ cell count. In contrast, boys with isolated bilateral cryptorchid testes had severe germ cell depletion and the majority lacked Ad spermatogonia. It was concluded that in patients with steroid 5alpha-reductase 2 deficiency the impaired second step of germ cell maturation resulted in defective transformation of spermatogonia into spermatocytes. The position of the undescended testis appeared to have no major pathological impact on the development of Ad spermatogonia in patients with steroid 5alpha-reductase 2 deficiency. Rather, it must be a disturbance of the hypothalamic–pituitary–gonadal axis that is a common cause of both UDT and impaired gonocyte transformation which was responsible for the low number of Ad spermatogonia in boys with isolated bilateral cryptorchidism [46]. In some cases unilateral cryptorchidism is a bilateral disease as both the undescended testis and the contralateral scrotal testis showed lack of Ad spermatogonia [47].

3.2. Heat

The testis descends from within the abdomen to the subcutaneous scrotum, enabling the testis to reside in a specialized, low-temperature environment of about 33 °C. As cryptorchidism prevents

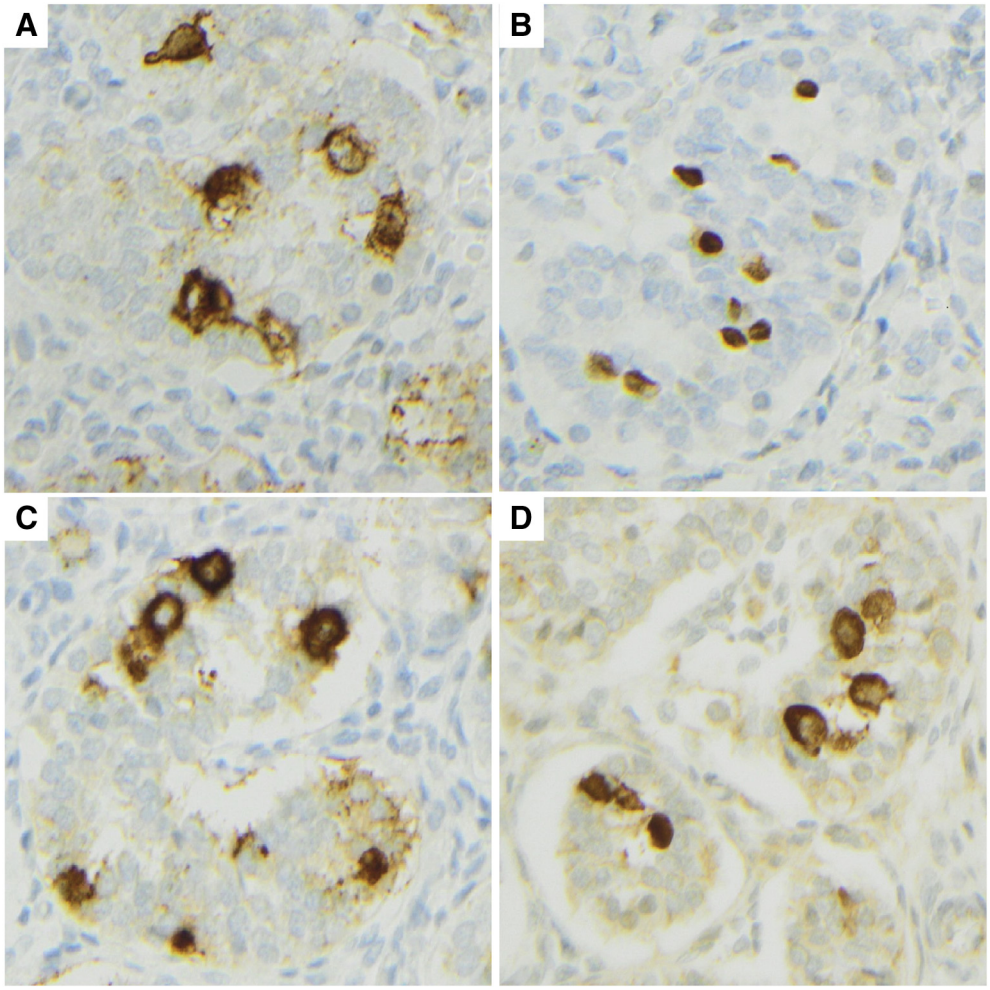

Fig. 2. Immunohistochemical stainings of normal human testicular tubules. The biopsies are from forensic medicine material and therefore not optimal quality. A and B are specimens from a 10-day-old mature normal boy showing germ cells positive for D2-40 and Oct3/4 respectively in the center of tubules and therefore probably gonocytes. C and G are specimens from a 60-day-old mature normal boy showing germ cells positive for PLAP and C-Kit respectively in the periphery of tubules and therefore probably spermatogonia stem cells. Reproduced with permission [27].

the testis reaching the lower scrotal temperature, heat stress has been proposed as a major factor in the resulting testicular dysplasia [48]. Elevated temperatures are known to trigger cell death, and excessive death of gonocytes and spermatogonia has been proposed as being the cause for infertility seen in UDT [39,49]. In a seminal work Setchell [50] reviewed the effects of heat on the physiological, histological, biochemical and functional properties of the testis. This work revealed much information about the negative impact of elevated temperature on an otherwise healthy testis, including increased cell death. However, most of the studies cited were performed on mature testes, thereby bypassing the critical period of gonocyte transformation and providing little information on the cause of aberrant transformation of gonocytes while in the higher temperatures of the abdomen or groin in UDT. Likewise, studies on the impact in adults of lifestyle factors that result in increased scrotal temperatures [51] miss the critical window in which postnatal gonocyte transformation occurs. It is not known when the descended human testis adapts to the scrotal environment and ambient temperature of 33 °C, but it has been suggested that this normally occurs shortly after birth [52]. In contrast, as the rodent testis is not fully descended into the scrotum until puberty, adaptation of the rat/mouse testis to the scrotal temperature of 33 °C probably does not occur until early puberty [53].

Recent studies looking at elevated scrotal temperatures in the prepubertal rat model have pointed towards the role of oxidative stress in apoptosis [54], although here too cryptorchidism was only induced at 10 days postpartum, missing the period of minipuberty and gonocyte transformation at 2–6 days [55] . Similarly, COX-2, an inhibitor of apoptosis was found to be elevated in cryptorchid testis, perhaps as a mechanism of preserving germ cell numbers [56]. Furthermore, 'prepubertal' rats exposed to transient elevated temperatures, had dying germ cells within the tubules [57]. However, this is unrelated to gonocyte transformation, as all gonocytes are expected to be gone well before 30 days postpartum when this experiment took place. Detailed mouse studies examining the genetic and proteomic changes induced by heat found a decrease in Rbm3, a cold-shock protein of Sertoli cells that must be expressed for germ cell maturation to occur [58]. In addition, mtLR1, a testis-specific gene that is normally upregulated during sexual maturation, is downregulated by heat stress *in vitro* [59].

Huff et al. proposed that the abnormalities found in UDT were not owing solely to a thermal injury, as their study of almost 800 human testicular biopsies of both the undescended and contralateral descended testes showed abnormalities of gonocyte transformation and subsequent meiosis to be delayed and defective in both testes, even within the normally descended testis that was at normal scrotal temperatures. However, it may be that in unilateral UDT the abnormal testis produces some abnormal signal that damages the normally descended, contralateral testis. Evidence for postnatal signaling between the testes is provided by the phenomenon of compensatory hypertrophy of the single, scrotal testis after perinatal torsion of the contralateral testis. However, studies from the Copenhagen group [10] indirectly support the aforementioned skepticism by Huff et al. [8]. Already at term around 20% of

fetuses with cryptorchid testes had germ cell hypoplasia, with the number of germ cells per tubular cross section below the lowest normal value for gestational age [60]. Furthermore, normal number of Ad spermatogonia was found in 43% of cryptorchid testes from infant boys with normal germ cell number, but in none from testes of age-matched cryptorchid boys with germ cell hypoplasia [10]. So, the abnormalities of gonocyte transformation may probably be related to both the congenital status of the seminiferous tubules and the subsequent thermal injury.

3.3. Role of apoptosis

Apoptosis, or programmed cell death, is a necessary process in the formation of the stable population of SSCs that confers fertility on the adult male. A series of proliferative and apoptotic phases during embryonic and postnatal development ensures a reservoir of healthy stem cells. Apoptosis acts to eliminate cells with genetic aberrations and thus preserves the integrity of the germline and protect against germ cell malignancies [61]. It is proposed that germ cell neoplasms develop in human UDT as a result of gonocytes escaping apoptosis.

It is thought that a possible mechanism of infertility in UDT is the inappropriate apoptotic destruction on germ cells [62]. In unilateral UDT, the testicular weight is significantly decreased on the affected side, although testicular weight on the normal control side increased in parallel with body weight. Germ cell death was significantly more frequent on the affected side in all age groups. Certainly, germ cells and their derivatives continue to undergo 'necroptosis', or pathological cell death if they remain in an environment of higher temperatures and this may impact on fertility, thereby suggesting the need for early intervention to enhance fertility in patients with cryptorchidism [63,64].

Zogbi reported that in mice initially after birth apoptosis of germ cells did not occur but rather the lengthening of the seminiferous cords acted to dilute gonocyte numbers that can be detected in tubule cross sections. This, coupled with the transformation into Ad spermatogonia, explains the low cell count [65]. In humans, the testis grows from birth to 3 months of age and the testes at 3 months of age were larger and serum hormone levels of inhibin B, FSH and SHBG (Sex hormone-binding globulin) were higher in Finnish than in Danish boys. Inhibin B was significantly positively correlated to testicular volume [66]. This is in agreement with the observation of the number of Sertoli cells in humans [67]. During the first 3 months of life, the number of Sertoli cells increased by more than a 5 fold, and then was constant thereafter until the 10th year, followed by a two fold increase during puberty [67]. Furthermore, in humans the total number of germ cells (including gonocytes) reached a maximum around the third month of life in the period from week 28 of gestation until the third year [68].

3.4. Genetic influences

Human primordial germ cells express KIT, MAGE-A4, GAGE and OCT4 [69]. When they localize to the gonadal ridge, and while in the newly developed testes where they mature into gonocytes, their gene expression changes and they then express KIT, MAGE-A4, POU5F1, UTF and VASA. When the gonocytes transform into spermatogonia there are some distinct differences in gene expression: KIT, POU5F1 and PLAP are undetectable and expression of DMRT1 is upregulated [70]. Kubo et al. performed extensive genome-wide analysis on cells of the germ-line lineage in mice, comparing differential gene expression profiles as they mature and transform. They found that as cells matured from PGC (embryonic day 16.5) to gonocytes (postnatal day (PND) 0.5) to SSC (PND 7.5 KIT^+) to differentiated spermatogonia (PND 7.5 KIT^-) they had unique gene expression patterns [71]. As gonocytes transformed to spermatogonia 1865 genes were upregulated and 893 genes were downregulated in PND 7.5 (KIT^-) spermatogonia compared with PND 0.5 gonocytes, indicating the complex changes occurring within the cells at this time.

POU5F1 is the gene encoding the transcription factor OCT4, known for maintaining pluripotency in both human and rodent embryonic stem cells [72,73]. Rajpert-De Meyts' group quantified the variable expression of OCT4 by immunohistochemistry throughout human germ cell maturation [74]. They found that OCT4 is expressed strongest in the earlier stages of development in PGC and gonocytes. Levels of OCT4 gradually decreased from 15 weeks' gestation and rapidly dropped off after 20 weeks. No spermatogonia expressed OCT4 after the first few postnatal months. This is constant with downregulation of Oct4 during the gradual transformation of gonocytes into SSCs. Similarly in mice, gonocytes within the center of the lumen as well as those having just made contact with the basement membrane were OCT4-positive at postnatal days 0–4 [7]. After this, when transformation into SSCs is thought to occur, numbers of OCT4-positive germ cells decreased significantly but were still present, albeit with less intense staining. These findings are in accordance with the results of immunohistochemical staining of biopsies from normal testes of forensic medicine material from 69 boys aged 1–690 days [27]. Positive staining for D2-40 and OCT3/4 was demonstrated up to 6 and 9 months respectively. The D2-40 antibody recognizes the M2A antigen, a marker for adult testicular cancer. D2-40 expression is downregulated during the movement of gonocytes towards the basement membrane and was accordingly the first to disappear in the germ cells. PLAP appeared stably expressed throughout the ages studied. The likelihood of a positive reaction for c-Kit waned with increasing age within the study period [27].

Integral to gonocyte transformation is their migration from the center of the seminiferous cords to the basement membrane. It is known that this is mediated by the tyrosine kinase receptor c-Kit and its ligand SCF. SCF-c-Kit interaction is also implicated in maintaining pluripotency and avoiding apoptosis [75]. Once gonocytes take up residence in the basement membrane (at day 3–8 in the rat), and are proposed to transform into SSCs, they lose expression of c-Kit. Blocking SCF-c-Kit interaction with the chemotherapeutic agent imatinib mesylate inhibited this migration and the subsequent formation of Ad spermatogonia and the stem cell pool [76].

In a well-designed and comprehensive study Vigueras-Villasenor's group analyzed these same proteins OCT4, c-Kit and PLAP in a sample of 70 human cryptorchid testes compared to normal controls [77]. They found that patients with UDT had significantly prolonged expression of all three proteins, even into their teenage years. As these proteins confer pluripotency and antiapoptotic qualities on cells, abnormal expression of these proteins may inhibit gonocytes from transforming and may be involved in the possible development of GCNIS (Germ Cell Neoplasia *In Situ*). These findings are in agreement with our recent published study [78]. We did immunohistochemical staining with PLAP, anti-OCT3/4, anti-c-Kit and anti-D2-40 in 1521 consecutive testicular biopsies from 1134 boys aged 1 month to 16½ years operated for UDT. OCT3/4 and D2-40-positive germ cells, probably gonocytes, were found throughout the first two years of life, with declining frequency. Even though the maturation seemed delayed, after two years they should have disappeared and might indicate neoplasia. PLAP-positive cells were seen in 57%–82% and c-Kit-positive in 5%–21% of cryptorchid testes between 4 and 13 years. Not until puberty did PLAP and c-Kit-positive undifferentiated SSCs vanish. When positive they had weak expression and were placed at the basement membrane, and when OCT3/4 and D2-40 negative they could not be classified as dormant gonocytes having escaped from apoptosis. Obvious prepubertal germ cell neoplasia *in situ* (GCNIS) [79] was only seen in 0.3% of the material and all the affected boys had syndromic cryptorchidism [78] . Such prepubertal GCNIS histological pattern with positive Oct3/4 and D2-40 gonocytes was described by Osterballe et al. [80] in 5½ and 13½ year-old boys with later invasive seminoma and teratocarcinoma of 20 and 27 years old respectively. But the first patient was syndromic with microcephaly, high imperforate anus with sacral anomaly and hydronephrosis and in the latter patient teratoma cells are known to have malignant potential.

RAP1 was found in rats to be exclusively expressed in gonocytes in the neonatal period and downregulated as they moved towards the basement membrane and underwent transformation. It has a known role in cell survival and differentiation and to have protective effects against reactive oxygen species. Mature rats treated with methoxyacetic acid (MAA) demonstrated increased apoptotic activity in the testis, and the localization of RAP1 to the nucleus of developing sperm cells [81]. It may be that RAP1 is acting to protect against apoptosis of the affected cells and conversely it must be down-regulated within the gonocyte population to allow the successful removal of redundant cells not undergoing transformation.

While studying the expression of PAX7, a known marker of stem cells, Aloisio et al. found a rare subset of A_{single} $PAX7^+$ spermatogonia in adult mouse testes that functioned as stem cells. These cells made up a much larger percentage of germ cells in the neonate as they rapidly proliferated by PND3. These results are concordant with the transformation of gonocytes into SSCs. However, Pax7 knockout mice were still fertile and therefore it is not essential for the establishment of SSCs but merely a marker of such cells [82]. Similarly, SALL4 is a known pluripotency factor that was found to be differentially expressed in gonocytes during the first week of life in mice. It was expressed by a subset of gonocytes at PND 0 but by all gonocytes at PND3 and all basement membrane-bound germ cells at PND 7 [83]. Perhaps here too, SALL4 is a driver of gonocyte transformation and it was only the subset of $SALL4^+$ cells that underwent transformation to take up residence in the basement membrane as SSCs while the others underwent apoptosis.

3.5. Epigenetic changes

During the development and migration of sexually undifferentiated PGCs, epigenetic markers including DNA methylation and histone modifications are erased, and the DNA is ready for sex-specific markers to drive development into spermatozoa or oocytes [84]. Subsequent epigenetic imprinting is also crucial for the transformation of the gonocytes into SSCs [85]. DNA methyltransferase DNMT3A and its cofactor DNMT3L are highly expressed in gonocytes, and therefore they display genome-wide methylation. Disruption of either DNMT3A or DNMT3L results in loss of germ cells [86], which is evidence of their importance in gonocyte transformation.

Kubo's group performed extensive studies using whole-genome bisulfite sequencing (WGBS) examining how the methylation profile of murine cells changed as they transformed from gonocytes into undifferentiated stem cells, and then into spermatogonia [71]. They found a significant increase in methylation levels between E16.5 cells (30.1%) and P0.5 cells (76.1%) which was maintained from then on until mature spermatozoa. Also of note, regions of DNA that were differentially methylated were found to correlate to specific genes involved in cell movement and proliferation and stem cell development and maintenance, providing further evidence for epigenetic changes in gonocyte transformation.

Recently studies [36] with biopsies from human infant cryptorchid testes found that expression of genes that regulate apoptosis (FASLG) and proliferation (GDNF) was increased after gonadotrophin-releasing hormone agonist stimulation. The authors proposed that gonodotrophins support Sertoli cell proliferation and gonocyte transformation to Ad spermatogonia. Hormonal stimulation, however, also induced a transcriptional repression of both inhibin-B and activin-A, indicating that Ad Spermatogonia development in infant boys was independent of the direct activation of a pathway in Sertoli cells.

Blimp1 is required to suppress somatic programming of PGCs [13]. The expression in fetal gonocytes in humans [17] is in concordance to the observations made in mouse [87] indicating a conserved role of BLIMP1/PRMT5 complex between mouse and man. In murine germ cell development Blimp1 associates with PRMT5, an arginine methyltransferase, to initiate histone methylation. The BlimP1/PRMT5 complex must subsequently be downregulated to lose or eliminate H2A/H4 arginine 3 dimethylation for gonocytes to transform into Ad spermatogonia. Failure to downregulate Blimp1, and therefore constitutive histone methylation, may allow cells to escape the regular differentiation program resulting in their persistence into adulthood, contributing to the premalignant changes seen in germ cell neoplasia *in situ* (GCNIS) [79]. This provides evidence for the theory that GCNIS cells are derived from gonocytes.

3.6. UDT, gonocyte development and germ cell malignancy

The association between cryptorchidism and testicular neoplasia has been recognized for more than a hundred years but the molecular biology underpinning it has only recently begun to be unraveled [88]. While the understanding that GCNIS [79] is a precursor lesion to testicular malignancies has been established for decades, the link between germ cell malignancy and aberrant gonocyte transformation has only recently come to light. The rationale for this hypothesis is the morphological similarity between both cells and, the similarity in the molecular expression profiles of gonocytes and GCNIS cells and the host of shared stem cell-like markers [89]. Boys with cryptorchidism are approximately three-to-five times more likely to develop testicular cancer than the general population [90,91] and 5%–10% of men with testicular cancer have a history of UDT [92]. In a large study of almost 17,000 men it was found that the relative risk of developing testicular cancer increases when the orchidopexy was performed after puberty [93]. Interestingly, a large thirty-year retrospective study in Jordan of almost 3000 patients with a history of surgically corrected UDT found no evidence of malignancy later in life in any patient [94]. However, it relied on self-reporting of cancer and sought no radiological or pathological studies. This may help explain the anomalous finding.

The genes identified to be highly expressed in gonocytes and be downregulated after their transformation into spermatogonia have been linked to GCNIS and germ cell tumors [70,95–97], providing evidence that aberrant gonocyte transformation may result in malignancy [98]. Retinoblastoma protein 1 (RB1) is a well-known tumor suppressor gene and regulator of the cell cycle, and is implicated in many cancers [99]. Yang et al. demonstrated the critical role it has in gonocyte transformation [100]. RB1 knockout mice cannot form the pool of SSC required for fertility and are found to develop GCNIS, similar to undescended testes. Claudins are a family of cell adhesion molecules highly expressed in rat gonocytes at day 3 and downregulated by day 8 at the conclusion of transformation and minipuberty. It is thought they play a role in the migration of gonocytes to the basement membrane and transformation into SSCs. These same genes that are highly expressed in neonatal rat germ cells are seen in human testicular neoplasia, supporting the idea of a gonocytic origin of GCNIS and cancer [101].

However, there is only good evidence that abnormal gonocytes cause neoplasia in humans with syndromic UDT and especially DSD cases. Besides the two cases in the series of Osterballe et al. [80] described above, only 1 of 13 boys with immunohistochemical GCNIS marker staining of prepubertal biopsy of UDT, who developed testicular cancer at 25 to 37 years old, had any positive staining of the original biopsy. The 14 year-old patient who developed seminoma at 36 years old had originally a weak PLAP positive staining of germ cells at the basement membrane, which is often seen in prepubertal biopsies of nonsyndromic UDT. These findings can explain why the relative risk of developing testicular cancer increases when the orchidopexy was performed after puberty. The long-term thermal injury on any SSCs during childhood causing progressive germ cell loss/apoptosis [78], and the hormonal changes occurring during puberty may be an important additional factor to be associated with the occurrence of testicular cancer later in adult cases. The adult GCNIS may be a new histology pattern before invasive germ cell cancer is demonstrated and not especially related to the early infant gonocyte transformation. Alternatively, lack of persistent gonocytes in prepubertal biopsies in patients who later

developed cancer may be just related to the very low frequency of the persisting gonocytes, so that they may be missed by sampling error. The role of persistent PLAP and c-Kit positive SSCs in prepuberty still needs to be determined.

Why is malignancy not reported in rodent models of UDT, when infertility is well known and similar to that of humans with cryptorchidism? Studies with 3 rodent models of UDT, Sprague–Dawley and Buffalo rats made cryptorchid by surgery at 20–22 days of age [102], and congenital transscrotal (TS) rats with suprainguinal ectopic testes [103] demonstrated infertility with UDT but not testicular cancer. The TS rat has UDT in about >75% of animals, and the testis is located in the groin similar to the human. The cause of UDT is likely to be an abnormality of the genitofemoral nerve (GFN), rather than androgen deficiency [104–106]. In both the congenital and surgically-created model of UDT, there was significant germ cell apoptosis from the onset of puberty (3–4 weeks of age) and UDT failed to grow. Despite UDT in these and other rodent models triggering widespread germ cell death and testicular atrophy, there is no reported rodent model of UDT causing testicular cancer.

We have proposed that the absence of malignancy in rodent models of UDT is related to the timing of testicular descent in rodents, which is different from humans. The rodent testis does not reach the scrotum until after 10 days of age, so that this is after the gonocytes have transformed into stem cells for spermatogenesis or undergone apoptosis. This means that by the time the rodent UDT is first exposed to an abnormally high temperature at 10–20 days of age, there are no gonocytes present. We can only study the temperature effect on early germ cells indirectly, and have previously shown that artificially lowering the temperature of the prepuburtal rodent testis by placing it in organ culture at 33 °C triggers immediate development of spermatogonia into the primary spermatocytes [107]. Similarly, orchidopexy of congenital UDT in the TS rat prevents the subsequent germ cell death and infertility, consistent with the apoptosis being temperature-dependent. Taken together, these results suggest that the rat testis adapts to the lower scrotal temperature of 33 °C at 10–20 days of age, after the testis is normally descended into the scrotum and after gonocyte development is complete. Gonocyte transformation in the first week of life has been shown to be not affected at all in TS rats, where both normal and future UDTs are still at the higher temperature in the abdomen/groin [53].

3.7. UDT, gonocyte transformation and infertility

The hormonal surge of minipuberty is crucial for fertility, and in cryptorchid testes, this process is deranged and as a consequence there are disturbed transformation of gonocytes and a lack of Ad spermatogonia. Patients with UDT experience impaired fertility by any measure; paternity rates are decreased, sperm concentrations are low, germ cell numbers are decreased and SSCs are deficient [108–110]. Those with bilateral cryptorchidism have a six times greater risk of infertility compared to the general population [111]. Furthermore, about 10% of infertile males have a history of cryptorchidism [112]. Infertility experienced by patients with UDT may be related to the underlying etiology of the disease or as a consequence of the malpositioned testis. These effects in humans are the same as in the rodent models, as shown above. In a recent population study of 350, 835 boys conducted in Western Australia there was a clear link between UDT and increased risk in testicular cancer and infertility. For every 6 months of delay (beyond the upper limit age for orchidopexy of 18 months), there was a 1% reduction in paternity, as well as a 6% increase in the risk of testicular cancer and 5% increase in risk of future use of assisted reproductive technology [113].

Hadziselimovic et al. propose that the appearance of Ad spermatogonia is important for future fertility potential of the boy by establishing the pool of stem cells. They have shown that having Ad spermatogonia present in a testicular biopsy at the time of orchidopexy results in significantly higher sperm counts [35,40,114]. Furthermore, they found a correlation between number of Ad spermatogonia per tubular transverse section at orchidopexy and sperm concentrations after puberty [115]. Kraft et al. reported a significant association between abnormal number of Ad spermatogonia per tubular transverse section at orchidopexy and decreased sperm density [116]. Similarly, Cortes et al. found a correlation between adult semen concentrations and number of germ cells present at orchidopexy for UDT in childhood [117]. Thorup et al. also studied human testicular biopsies taken at orchidopexy and found a correlation between the number of PLAP-positive germ cells and increased fertility potential. They hypothesized that PLAP-positive germ cells present the greatest potential for transformation into spermatogonia and subsequent fertility compared to PLAP-negative cells. This was demonstrated by patients with the highest number of PLAP-positive germ cells having the greatest number of germ cells per tubule.

Hadziselimovic et al. compared gene expression in boys with UDT and high risk of infertility (HIR), defined as those with no Ad spermatogonia, to that of boys with UDT and low risk of infertility (LIR), defined as having more than 0.1 Ad spermatogonia per tubule, to elucidate the genetic mechanism behind transformation. Using microarray data and immunohistochemistry they found that in addition to the differential expression of hundreds of gene transcripts involved in the reproductive axis, those in the HIR group did not express any EGR4, a known master regulator of genes integral to fertility. Deficient EGR4 in UDT disables the transformation of gonocytes and impedes the establishment of a pool of SSCs for spermatogenesis and impacts fertility potential [118].

The mechanism that leads to abnormal germ cell transformation and/or apoptosis remains incompletely understood, despite the advances already described. Most studies have concentrated on testosterone during minipuberty, and recently on FSH, but few studies have examined a possible role of inhibin-β. The inhibin-B has a maximum value at around 3 months of age, and the elevated inhibin-B persists for a longer period of time than the elevated FSH, LH and testosterone [119]. It has been found that in cryptorchid patients at 0.5 to 1 year age LH was positively associated to inhibin which may be important for the transformation of gonocytes to A-adult dark spermatogonia [120]. However, in accordance with the aforementioned findings by Gegenschatz-Schmid et al. gonadotrophin-dependent increases in FASLG and GDNF expression drove Sertoli cell proliferation and germ cell self-renewal and supported the transition of gonocytes to Ad spermatogonia, independent of inhibins [36].

4. Conclusion

Although much has been learned about gonocyte transformation in recent years, and many of the processes driving this crucial step have been discovered, there are still large gaps in our knowledge. Many of the studies on cryptorchidism in both murine and human subjects have looked at the long-term sequelae of the disorder and have focused on the downstream effects of deficient Ad spermatogonia and inappropriate apoptosis, at a time period beyond that of the gonocyte transformation. This means there is a deficit in knowledge pertaining to this important period. The hypothalamic–pituitary–gonadal axis matures and becomes increasingly more important during minipuberty but the mechanism by which it controls gonocyte transformation is not yet understood. The similarities in the genetic and molecular profiles between gonocytes and malignant cells together with clinical observations support the theory that germ cell malignancies stem from an arrested gonocyte primarily in syndromic UDT. In addition, the link between dysfunctional gonocyte transformation and the establishment of the SSC pool and infertility is becoming clearer. Despite this, there is still much to discover about the precise mechanism of gonocyte transformation in the postnatal period and its clinical application to ideal timing of orchidopexy and role of hormonal treatment in patients with UDT to prevent testicular cancer and infertility later in life.

Author contributions

ML and RL discussed the literature and conceived the outline of the manuscript, ML wrote the first draft followed by RL, JMH, DC, JT, and ECL critical revision. All authors reviewed the manuscript and provided critical discussion and input.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

Acknowledgments

This study is supported by the Australian National Health and Medical Research Council (Grant Number 049014 and APP1127109) and the Victorian Government's Operational Infrastructure Support Programme.

Appendix 1

1. (embryo* or pre-pubert* or prepubert* or infant* or neonat* or newbor* or postnatal or child* or pre-schooler* or preschooler*).tw,kf,hw.
2. *germ cells/ab, ah, cy, gd, me, pa or *spermatogonia/ab, ah, cy, gd, me, pa.
3. *Spermatogenesis/
4. *dna methylation/ge
5. ((cell adj differentiation) or (gene adj expression)).tw,kf.
6. ((stem adj cell*1) or stemcell or (germ adj1 cell*1) or prospermatogonia or prespermatogonia or gonocyte* or (primordial adj germ adj cell*1) or spermatogoni*).tw,kf.
7. (transform* or differentiat* or matur* or spermatogenesis).tw,kf.
8. testis/ab, ah, cy, gd, me, pa or ((gonad* and male) or testis or testes or testicle* or spermato*).tw,kf,hw.
9. (non-descend* or nondescend* or maldescen* or undescend* or cryptorchid*).tw,kf.
10. (cancer* or neoplas* or malignan* or infertil*).tw,kf.
11. CRYPTORCHIDISM/
12. (exp mice/ or exp rats/ or (mouse or mice or murine).tw.) and male.tw,hw.
13. temperature.tw,kf. or body temperature/
14. (2 or 6) and 13 and (9 or 10 or 11) and 1
15. (2 or 6) and 13 and (3 or 4 or 7 or 8) and 1
16. (2 or 6) and (3 or 4 or 5 or 7) and 8 and 12 and 1 and (9 or 10 or 11)
17. (2 or 6) and 7 and 8 and 1 and (9 or 11)
18. 14 or 15 or 16 or 17
19. limit 18 to english language
20. limit 19 to yr = "1995 -Current"

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