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Invited Review

The biology of germ cell tumors in disorders of sex development

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Development of a malignant germ cell tumor, i.e., germ cell cancer (GCC) in individuals with disorders of sex development (DSD) depends on a number of (epi-)genetic factors related to early gonadal- and germ cell development, possibly related to genetic susceptibility. Fetal development of germ cells is orchestrated by strict processes involving specification, migration and the development of a proper gonadal niche. In this review we will discuss the early (epi-)genetic events in normal and aberrant germ cell and gonadal development. Focus will be on the formation of the precursor lesions of GCC in individuals who have DSD. In our view, expression of the different embryonic markers in, and epigenetic profile of the precursor lesions reflects the developmental stage in which these cells are blocked in their maturation. Therefore, these are not a primary pathogenetic driving force. Progression later in life towards a full blown cancer likely depends on additional factors such as a changed endocrine environment in a susceptible individual. Genetic susceptibility is, as evidenced by the presence of specific risk genetic variants (SNPs) in patients with a testicular GCC, related to genes involved in early germ cell and gonadal development.

Conflict of interest

The authors state no conflict of interest.

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Establishment of sex is a critical and crucial part of mammalian development providing the fundamental structure for maintenance of the species, as well as continued generation of genetic diversity. In mammals this process has two sequential stages, being sex determination followed by sex differentiation. Mammalian sex determination is, with a few exceptions, driven by the presence of a specific combination of

sex-chromosomes, either XY or XX. This leads to the development of either a male- or female- gonad originating from an initially undifferentiated bipotential structure. Presence of the Y chromosome, more specifically expression of the *SRY* (sex determining region on Y) gene initiates testis development, while absence of a functional Y chromosome will in-principle lead to the development of an ovary. During the process of sex differentiation, secretion of local and hormonal factors by the fully formed testis or ovary, results in the formation of the internal and external genitalia. A disturbance in gonadal development as well as in its subsequent functionality, can lead to disorders of sex development (DSD), defined as congenital conditions in which development of chromosomal, gonadal or anatomical sex is atypical. Some of these conditions are associated with an increased risk for a germ cell cancer (GCC). Here we discuss the (epi)genetic processes involved in normal early gonadal and (embryonic) germ cell development, and focus on the pathogenesis of the precursor lesions of GCC in the context of DSD.

Normal germ cell development

Primordial germ cells (PGCs) are the precursors of gametogenesis in later life. In the mouse these cells develop in the proximal epiblast, and become detectable at embryonic day E6.5. Specification of PGCs takes place in response to bone morphogenetic protein 4 (BMP) signaling at approximately E6.0 (1). Cells expressing BLIMP1 and PRDM14, induced by BMP signaling, form a cluster of about 40 alkaline phosphatase (AP) positive PGCs at E7.25 (2–4). At E7.5 these PGCs migrate to the developing hindgut and continue at E9.5 through the mesentery and colonize the genital ridges at E10.5-12.5 (5, 6). Once arrived in the genital ridge (i.e. the undifferentiated gonad), PGCs are referred to as gonocytes. During this time, both in the male and female, extensive epigenetic reprogramming takes place.

Germ cell epigenetics before and after gonadal differentiation

The most notable epigenetic event taking place at these early developmental stages is genome-wide DNA demethylation, which is completed at E13.5 (7, 8). As a consequence of this process, parental imprints are erased, and the silenced X chromosome is reactivated in PGCs of female (XX) mouse embryos (9, 10). From E12.5 onwards differentiation of the mouse embryo is sex-specific (11). After PGC reprogramming, establishment of germ cell methylation marks happens in a sex-specific manner, resulting in sperm- and oocyte-specific methylation patterns (9). In males, re-methylation initiates as early as E14.5 in G1 arrested prospermatogonia (12), and is fully established at birth (days 19–21). The methylation patterns are maintained throughout many mitotic divisions before the cells enter meiosis (13). In females re-methylation of the gametes starts at birth during the oocyte growth phase at which time they are arrested in prophase of meiosis I (12). Besides these epigenetic events, multiple signaling pathways are activated in PGCs/gonocytes.

Signaling pathways in primordial germ cells

It is known that the KIT-KITLG (stem cell factor, SCF) pathway plays an important role in the migration, proliferation and survival of mouse PGCs (14, 15). Recently, a number of additional relevant pathways in embryonic germ cells have been identified. It has been shown that mouse PGCs express the estrogen receptor α and ß at E11.5–12.5, and exposure to estrogens leads to phosphorylation of AKT, ERK and SRC, which regulate (de)differentiation of mouse germ cells (16, 17). Excess exposure to estrogens during the *in vitro* growth of mouse genital ridges, results in upregulation of KITLG and activation of the KIT pathway via AKT/PTEN, which in turn leads to proliferation of PGCs (18). The window of sensitivity for AKT signaling is restricted, as it is absent in germ cells in mitotic arrest and in the beginning of meiosis (17). Knock-out of *PTEN* in human embryonic stem cells (ES), resulted in increased cell survival and proliferation, and these cells had less differentiation capacity due to retained *OCT3/4* and *NANOG* expression levels (19). Both PTEN and P53 independently repress expression of *Nanog* in mouse spermatogonial stem cells. Although these studies give more insight in the signaling pathways involved in the development of PGCs/gonocytes, their relevance for human germ cell development remains to be determined.

Early gonadal differentiation, fetal testis and ovary development.

Differentiation of the early gonad, and development of the fetal testis and ovary are presented elsewhere in this issue, and will only be discussed briefly here. In the early stages of mammalian embryonic development the undifferentiated gonads are bipotential. Formation of the early undifferentiated gonad is influenced by a number of genes, including steroidogenic factor-1 (*SF1/NR5A1*), Wilms' tumor-1 (*WT1*), and chromobox homolog-2 (*CBX2*) (20–22). Studies in mice have shown that these genes play an important role in the developing gonads, and moreover, all have been associated with DSD (see below). Differentiation of the bipotential gonad into a testis begins with the specification of Sertoli cells, triggered by expression of the Y-linked *SRY*, and subsequent *SOX9* (SRY-related HMG-box, gene 9) (23–25). Sertoli cells subsequently coordinate all aspects of testis differentiation and function (26). Anti-Müllerian hormone (AMH), and chemotactic signals produced by the early Sertoli cells causes regression of the Müllerian ducts, and ensure correct cell migration and testis cord formation (26, 27). The appearing fetal Leydig cells start producing testosterone (T) and insulin-like factor 3 (INSL3) inducing masculinization of the fetus (28, 29). PGCs/goncytes colonizing the early testis continue to proliferate until 17–18 weeks GA, at which time

they start to mature into pre-spermatogonia, and start migrating towards the basement membrane. During this time they will lose expression of embryonic markers like POU5F1, NANOG, AP, Ap2gamma and c-KIT, and will enhance expression of amongst others TSPY and VASA (DDX4) (30–33). Occasionally, a few PGCs/gonocytes, expressing embryonic markers, can still be found in the postnatal testis until 6–12 months of age (31). Until recently ovarian development was thought to be the result of a passive or default pathway, i.e. occurring in the absence of SRY expression. However, in recent years, it has been shown that ovarian development requires tightly regulated signaling pathways involving WNT family member 4 (WNT4), Forkhead Box L2 (FOXL2), Catenin beta-1 (CTNNB1) and R-spondin 1 (RSPO1) (34–37). In the fetal ovary the germ cells are called oogonia, they proliferate and their numbers increase to around 7×10^5 oocytes at birth. During this time embryonic markers like POU5F1 and NANOG are downregulated (38). Proliferation, meiotic entry and oocyte formation depend on the interaction with primarily granulosa cells, for which FOXL2 is essential (39). The primordial follicle consists of an individual oocyte, arrested in meiosis I, and surrounded by a layer of pre-granulosa cells (40).

It has become clear that in order to properly develop, the testis and ovary need to suppress the opposing pathways. In the XY gonad WNT4 is suppressed by FGF9 (fibroblast growth factor-9), and loss of *Wnt4* in XX gonads leads to upregulation of *Fgf9* and *Sox9* (41). Moreover, it has been shown that *Ctnnb1*, *Map3k1* (member of the mitogen-activated protein kinase (MAPK) pathway) and *NR5A1* also play a role (35, 42, 43). Interestingly, even in adulthood testes and ovaries need to suppress activation of opposing pathways (44, 45).

A graphic overview of normal germ cell – and gonadal development; indicating timing, genes involved, and expression of germ cell markers is shown in Fig. 1.

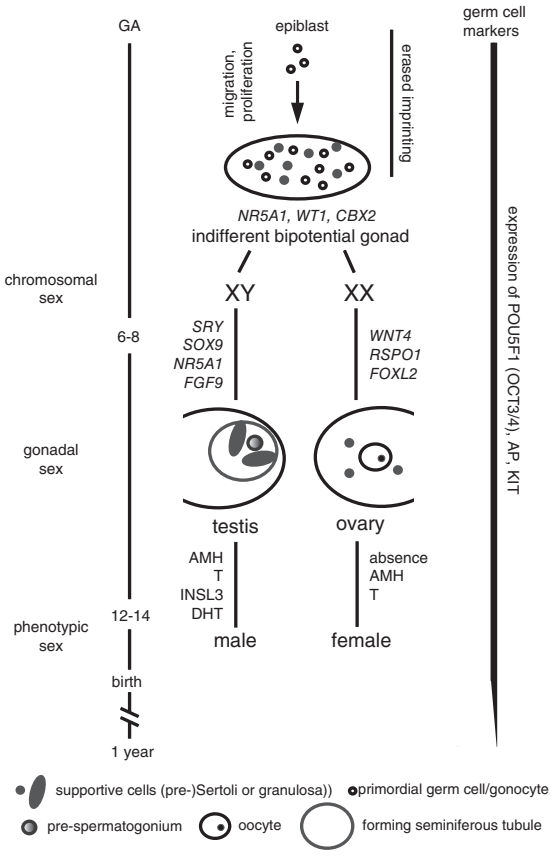

Fig. 1. Schematic representation of the earliest steps in human male and female development. Early in development in both chromosomal males and females the indifferent bipotential gonads are formed under the influence of NR5A1, WT1 and CBX2. In the XY embryo, expression of *SRY* initiates upregulation of SOX9, inducing a signaling cascade involving FGF9 and NR5A1 that leads to the formation of pre-Sertoli cells. These cells will further orchestrate the development of a functional testis, which in turn will, through the action of anti-Müllerian hormone (AMH), testosterone (T), insulin-like 3 (INSL3), and dihydrotestosterone (DHT), direct the differentiation of the male internal and external genitalia. In the XX embryo, the absence of functional *SRY* directs the fate of the bipotential gonad towards the ovarian lineage. Instead of one master gene, several proteins act in parallel in female development. The absence of AMH and T leads to the differentiation of the female internal and external genitalia. The primordial germ cells (PGCs) arise early in development in the proximal epiblast, and migrate towards the genital ridges. During this time the PGCs will undergo extensive epigenetic changes, and by the time they arrive in the gonadal ridges, genomic imprinting is completely erased. A male or female specific pattern of imprinting will be established depending on the presence of a testicular or ovarian environment. In the testis the PGCs/gonocytes associated with pre-Sertoli cells, go into mitotic arrest and differentiate into pre-spermatogonia. In the ovary, gonocytes will differentiate into oocytes. During this process both in the male and female, the germ cells will lose expression of the embryonic markers POU5F1, AP, and KIT, which should be absent after the first year of life. GA: gestational age in weeks. See text for further details.

Disturbed germ cell development

A disturbance in gonadal development as described above, can lead to DSD, which are categorized into three main groups based on karyotype: 46,XY DSD, 46,XX DSD, and chromosomal DSD. Each main group further consists of a spectrum of genetic diseases, hormonal disorders and syndromic forms. Defects in several genes have been shown to result in gonadal malformation and DSD, some of which will be discussed below. DSD patients with Y chromosomal material in their karyotype have the greatest risk of developing the GCC precursor lesion, with a high potential to progress to GCC, see below. We have developed a comprehensive and interactive overview of normal sex development and DSD, publically available (http://www.erasmusmc.nl/47463/51019/4500578/Animation).

Genetic factors involved in gonadal maldevelopment in DSD

Mutations in the genes involved in formation of the undifferentiated gonad (*NR5A1*, *WT1* and *CBX2*) have been found in patients with DSD. Human *NR5A1* mutations have been associated with adrenal failure, primary ovarian insufficiency, and 46,XY complete or partial gonadal dysgenesis (a complete or partial lack of testicular development resulting in a female or ambiguous phenotype), and even 46,XX (ovo)testicular DSD (43, 46, 47). Specific *WT1* mutations are known to cause

Denys–Drash and Frasier syndrome, characterized by an increased risk for Wilms' tumor and renal failure, respectively, and 46,XY partial or complete gonadal dysgenesis (48, 49). Disruption of *Cbx2* in mice leads to defects in adrenal and splenic development, but also to varying degrees of 46,XY sex reversal, most likely related to reduced expression of *Sry* (50). In humans a loss-of-function mutation in a patient with 46,XY gonadal dysgenesis has been described (51). As mentioned earlier, testicular development starts with *SRY*- and subsequently *SOX9* expression. Eighty percent of 46,XX testicular DSD cases can be attributed to translocation of *SRY* to one of the X chromosomes; and 15–20% of 46,XY gonadal dysgenesis are caused by an inactivating mutation of *SRY* (52–54). Mutations in *SRY* have also been described in some cases with a mosaic sex chromosome constitution, indicating a possible role for SRY in the abnormal gonadal development in these patients (55, 56). Interestingly, a family has been described in which two sisters with 46,XY gonadal dysgenesis had an identical frameshift mutation in *SRY*, which was absent in the brother, but present in the father in a mosaic constitution (57). Moreover, Shahid et al., describe a family in which the father of a DSD patient with a mosaic 45,X/46,XY karyotype displayed signs of testicular dysgenesis syndrome (TDS), and molecular analysis revealed the same deletion in the HMG-box of SRY in both (58). *NR0B1* (*DAX1*) encodes an orphan nuclear receptor; and gene duplications resulting in suppression of the SRY pathway cause 46,XY DSD (59). Mutations in *SOX9* have been identified in individuals with campomelic dysplasia and 46,XY DSD, and duplications of chromosome 17q, including *SOX9* were found in 46,XX testicular DSD cases (60–62). Next to the aforementioned genes, missense mutations in desert hedgehog (*DHH*), a signaling molecule involved in regulation of morphogenesis, cause 46,XY complete or partial gonadal dysgenesis (63). Loss of the 9p region which includes *DMRT1*, a transcription factor expressed in the testis of humans and mice, is associated with gonadal dysgenesis and 46,XY DSD (64). Besides these, mutations in *MAP3K1*, have been found in several cases of 46,XY DSD (65). Mutations in human *GATA4*, a member of the GATA family of transcription factors, have first been associated with congenital heart defects (CHD). However, several cases of 46,XY DSD, sometimes in combination with CHD have been described in families with mutations or copy number variations (CNVs) in *GATA4*, or its co-factor *ZFPM2* (zinc finger protein, FOG family member 2) (66–68). Lastly, in an individual with 46,XY gonadal dysgenesis a deletion spanning multiple exons in the *WWOX* gene was found (69).

Recently, it has been shown that ovarian development involves tightly regulated pathways, and mutations in the genes involved can lead to DSD. Specifically, homozygous null mutations in *WNT4* have been shown to cause 46,XX DSD (70), and heterozygous loss-of-function mutations have been described in rare cases of 46,XX women with Mayer–Rokitansky–Küster–Hauser (MRKH) and mild virilization (71, 72). Loss-of-function mutations of *RSPO1*, a member of the R-spondin family, are associated with *SRY*-negative 46,XX DSD (73, 74). Next to *WNT4* and *RSPO1*, loss-of-function mutations in the gene encoding the transcription factor FOXL2 are associated with BPES (blepharimosis ptosis epicanthus inversus syndrome) with or without ovarian dysgenesis (75). An overview of the genes involved can be found in Table 1, and Fig. 1.

Table 1. Genes involved in disorders of sex development

Gene	Disorder of sex development (DSD)	References
CBX2	46,XY gonadal dysgenesis	(51)
DHH	46,XY complete and partial gonadal dysgenesis	(63)
DMRT1	46,XY DSD	(64)
GATA4	46,XY DSD	(66, 67)
MAP3K1	46,XY DSD	(42, 65)
NR0B1 (*DAX1*)	46,XY DSD	(59)
NR5A1 (*SF1*)	46,XY gonadal dysgenesis; 46,XX ovotesticular DSD	(43, 46, 47)
RSPO1	46,XX DSD	(73, 74)
SOX9	46,XY DSD; 46,XX testicular DSD	(60–62)
SRY	46,XY gonadal dysgenesis; chromosomal DSD	(52–58)
WNT4	46,XX DSD	(70–72)
WT1	46,XY DSD, Denys–Drash and Frasier syndrome	(48, 49)
WWOX	46,XY gonadal dysgenesis	(69)
ZFPM2	46,XY DSD	(68)

Although the number of genes implicated in DSD is growing, a molecular diagnosis cannot be made in about 75% of DSD individuals. New technologies like next generation sequencing will likely improve on this, and will uncover multigenic cases (76).

Next to these genetic factors, environmental factors play a role in gonadal and genital development. These can be due to placental insufficiency, possibly related to intra-uterine environment and endocrine disruptors, and should be considered as contributing factors to DSD-related conditions such as cryptorchidism and hypospadias (77, 78).

Disturbed microenvironment for embryonic germ cells

Gonadal maldevelopment as described above, or lack of hormone production or action, results in a disturbed nourishing microenvironment for the PGCs/gonocytes. In these conditions, supportive Sertoli/granulosa cells and/or Leydig/theca cells cannot provide a proper niche for the germ cells, causing a delay or block in the necessary maturational steps for full functionality as described above. This can ultimately result in infertility or even GCC, and the combination of both. Immature germ cells can be identified by the expression of the aforementioned embryonic markers (POU5F1, NANOG, etc.) at a stage beyond their normal expression window. The precursor lesions of GCC are the germ cell neoplasia *in situ* (GCNIS, formerly termed carcinoma *in situ* – CIS) or gonadoblastoma (GB), as discussed below.

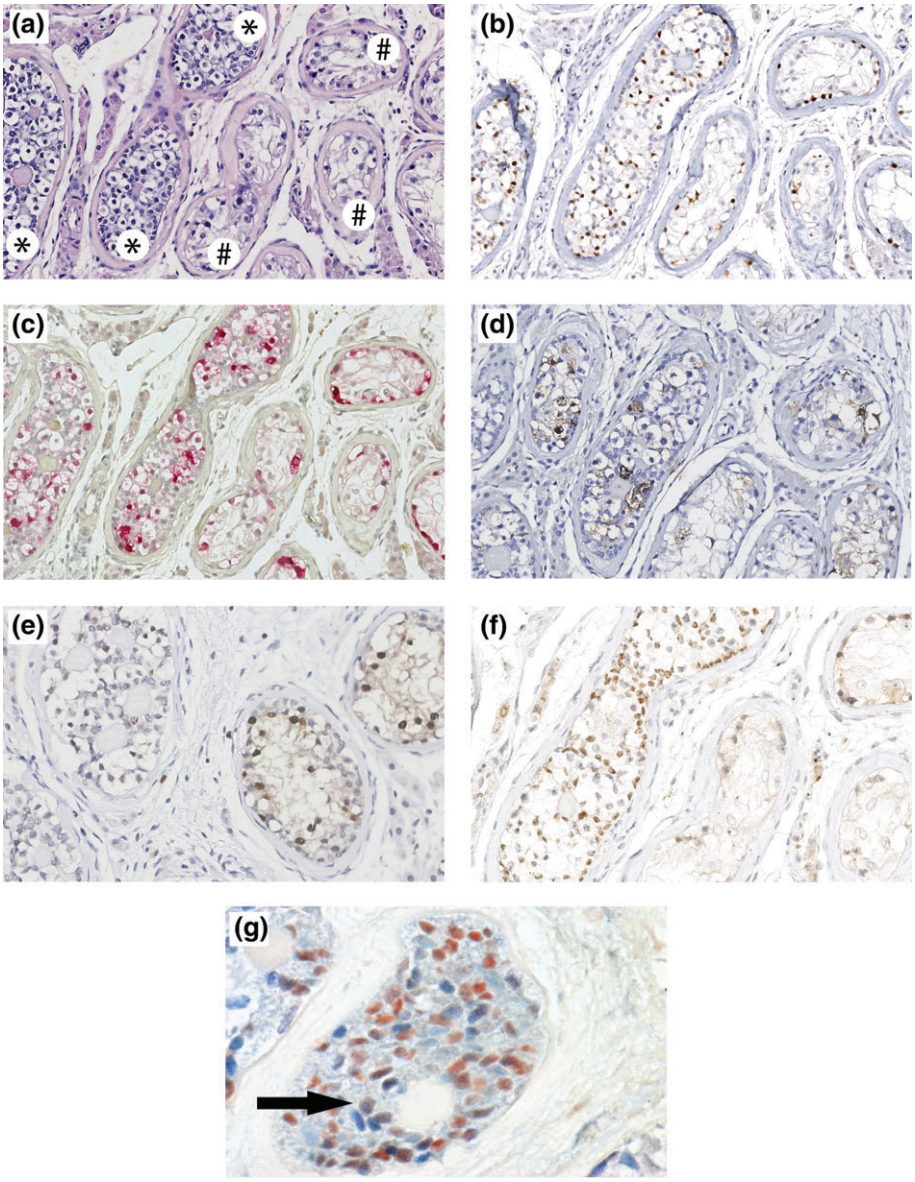

Fig. 2. Immunohistochemical staining pattern of GB and GCNIS. (a–f) Presence of GB and GCNIS in a single DSD gonad. (a) Representative hematoxylin and eosin staining. On the left side of the image the GB lesion (indicated with *), embryonic germ cells mixed with granulosa-like supportive cells, can be seen. On the right of the image GCNIS (indicated with #), is present, with embryonic germ cells on the basal lamina associated with Sertoli cells. Both GB and GCNIS cells stain positive for (b) POU5F1 (brown staining), (c) TSPY (red staining), and (d) KITLG (brown staining). (e, f) The supportive cells in the GCNIS lesion are SOX9 positive (brown staining), and are negative for FOXL2. In the GB lesion the supportive cells stain positive for FOXL2 (brown staining) and are negative for SOX9. Adapted from (104). (g) As evidenced in the GB lesion found in another DSD patient, next to this FOXL2 only staining pattern, also the presence of both markers, sometimes even within one cell (indicated by the arrow), is observed (FOXL2: brown staining, SOX9: blue staining). Magnification: (a–d) ×200, (e–g) ×400. Slides (b–g) are counterstained with hematoxylin.

Germ cell cancer

The focus of this review being on the earliest steps in the pathogenesis of GCC, invasive GCC will only be discussed briefly. If untreated, the precursor lesion GCNIS will progress into an invasive GCC in an estimated 70% of cases, whereas around 50% of cases with GB will progress to dysgerminoma/seminoma (79–81). There are, in principle, five types of germ cell tumors, but only the type II, the seminomas/dysgerminomas and non-seminomas, are relevant in the context of DSD. In the testis, these cancers are now referred to as GCNIS-related germ cell tumors according to the latest WHO classification (82). Diagnostic markers for the invasive components can in fact be predicted based on the expected expression pattern, and knowledge of the precursor lesions either, GCNIS or GB. These are POU5F1 for both seminomas and embryonal carcinoma, independent of stage of the disease at clinical presentation and anatomical localization. In addition, SOX17 is present in seminomas and SOX2 in embryonal carcinomas, allowing a straightforward way of diagnosing these components (83). Of interest

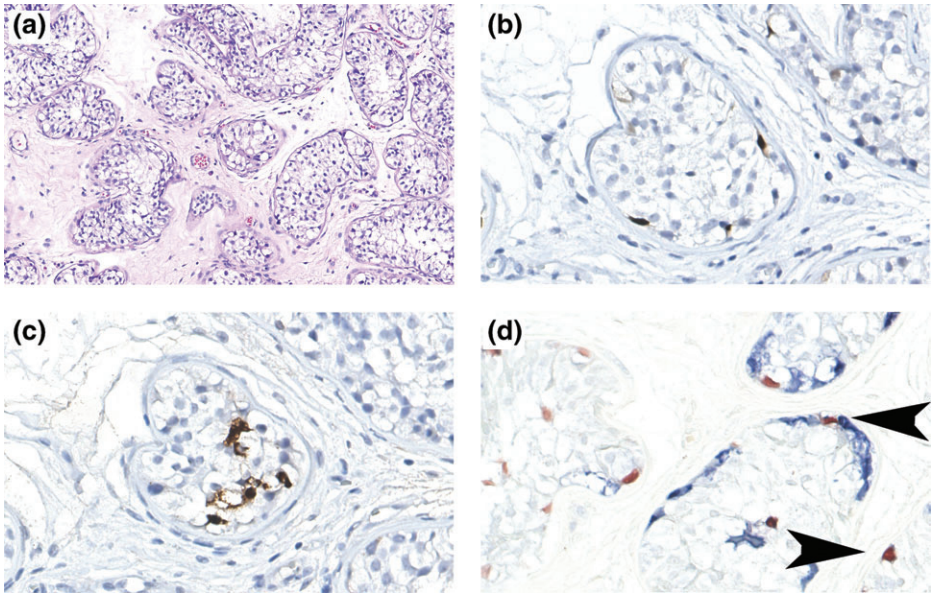

Fig. 3. Immunohistochemical staining of pre-GCNIS present in the testis of a 12-year-old presenting with severe hypospadias and cryptorchidism. (a) Representative hematoxylin and eosin staining of the area containing pre-GCNIS cells. (b) Germ cells, both pre-malignant and with maturation delay, stain positive for POU5F1 (brown staining). (c) In the staining for KITLG only pre-GCNIS cells stain positive (brown staining). (d) Double staining for POU5F1 (brown staining) and TSPY (blue staining). Note that the majority of cells are positive for either TSPY or POU5F1, and only a few cells show staining for both (arrowheads). Magnification: (a) ×200, (b–d) ×630. (b and c) are counterstained with hematoxylin.

is that the switch from SOX2 in embryonic stem cells (ES) towards SOX17 in PGCs has indeed recently been proven to be the driving force in the differentiation of PGCs from ES (83, 84). There seems to be a regulatory mechanism in which POU5F1 switches from SOX partner thereby targeting specific loci to determine cell fate (85). Based on actual data, there is no indication that genomic anomalies affecting these loci are driving cancer development, it just are persisting embryonic signaling programs. Interestingly, a defined set of micro-RNAs (miRs) show a characteristic pattern of expression in GCC (86). A functional role has been identified for miR-371–373, specifically the inhibition of *LATS2*, a downstream target of the P53 pathway (87), providing an explanation for the unusual presence of wild type P53 in GCC. The miR cluster 371–373 is normally only expressed in the embryonic germ/stem cells during embryogenesis, indicating that the origin of GCC lies in early embryonic development (88). Recently, the elevated presence of this cluster of miRs in serum from testicular GCC patients has been described (89–91). However, they cannot detect patients with GCNIS only (92). A further breakthrough in understanding GCC pathogenesis came from genome-wide association studies (GWAS) in which a selected number of high risk single nucleotide polymorphisms (SNPs) were identified (93–97). None of the GWAS showed a difference in effect in seminoma *vs* non-seminoma cases, despite sufficient sample size, supporting the presence of common oncogenic pathways in both groups. Interestingly, one SNP (rs4590952) residing in the P53 binding site of *KITLG*, thereby modulating its expression, is located in the same linkage disequilibrium block as the SNP found by Kanetsky et al. in 2009. (93, 98). Studying promoter methylation of several of the associated genes found in the GWAS showed that increased promoter methylation of *PDE11A*, *SPRY4*, and *BAK1*, and decreased promoter methylation of *KITLG* were significantly associated with familial testicular GCC risk (99). Another intriguing finding pointing towards susceptibility, is the presence of heterozygous germline mutations in *LRCC50* in familial seminoma cases. Expression of the wild type allele is at least partially lost in these familial seminomas (100). These findings open up possible novel ways for the diagnosis and follow-up of GCC patients and for genetic susceptibility screening in GCC risk populations such as DSD (101).

Germ cell cancer precursor lesions

The precursor lesion of GCC in the testis is GCNIS (82, 102). In DSD, GB can also be found in the dysgenetic gonad, and sometimes both precursor lesions are present within the same gonad (Fig. 2) (79, 103). Presence of GB, possibly combined with GCNIS, found in a scrotal testis, always indicates an underlying DSD condition (104). GCNIS and GB can be distinguished by the characteristics of the supportive cells, being exclusively Sertoli cells (SOX9 positive) in GCNIS, and granulosa cells (FOXL2 positive) or a combination of both cell types, in GB (Fig. 2) (105). The level of testicular development (testicularization), as evidenced by overall morphology and SOX9 and FOXL2 expression determines the type of precursor. Malignant transformation of the germ cells requires, next to other factors described below, a susceptible environment. If the gonads have fully developed in either the female (ovary) or male (testis) direction, risk

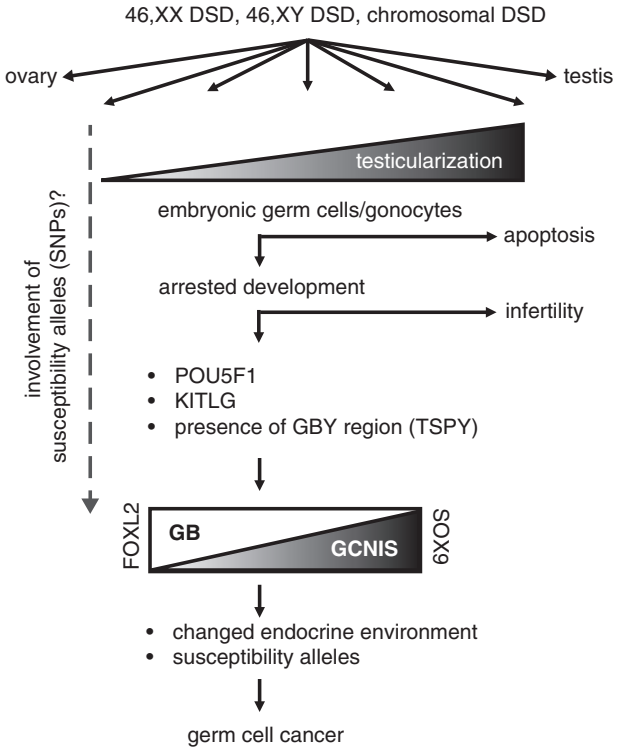

Fig. 4. Pathobiology of germ cell cancer in DSD. Individuals with DSD can have a disturbed gonadal development in which the function of the supportive Sertoli/granulosa cells is impaired. Germ cells residing in this disturbed microenvironment can undergo apoptosis, or be arrested in their development, the latter leading to infertility, or alternatively, these cells can give rise to the precursor lesions. Prolonged expression of POU5F1, KITLG and increased TSPY expression enhances the chances of survival of these germ cells. Formation of gonadoblastoma (GB) or germ cell neoplasia *in situ* (GCNIS) depends on the level of testicular development (testicularization), as evidenced by the expression of FOXL2 and SOX9 in the supportive cells. The expressed embryonic markers and epigenetic profile of GB and GCNIS reflect the developmental stage at which these cells are blocked, and are most likely not a driving mechanism. Development of these precursors later in life into germ cell cancer depends on a changed endocrine milieu in a susceptible individual. This susceptibility might depend on the presence of the risk SNPs (single nucleotide polymorphisms) identified in the genome-wide association studies.

for malignant transformation of the germ cells is low, as is the case in ovotesticular DSD where development of a precursor lesion is rarely seen.

GCNIS cells resemble PGCs/gonocytes both phenotypically and ultrastructural; they show the same gene and miRNA expression profiles, have a similar epigenetic status, and express the same proteins, such as POU5F1, AP, c-KIT and KITLG (31, 106–113). The germ cell component of GB and GCNIS are identical, both morphologically as well as with regard to protein expression, providing further evidence that the cell-of-origin for both GCNIS and GB is a blocked PGC/gonocyte (114, 115). There are a number of diagnostic markers for both precursor lesions, including, historically AP and c-KIT. It must be noted that routine use of c-KIT detection nowadays, given the sensitivity and automated detection methods, can lead to over-diagnosis (116). Interestingly, in ovarian GCC without any signs of DSD a high frequency of c-KIT activating mutations (up to 50%) was found (117), while individuals with DSD with a specific part of the Y chromosome present, the so-called GBY region (118), show expression of TSPY (testis specific protein on the Y chromosome, see below), suggesting that the c-KIT pathway is bypassed by the presence of TSPY (117, 119). One of the most specific marker for both GCNIS and GB to date is POU5F1 (Fig. 2) (110). Prolonged POU5F1 expression is thought to be crucial in GCC development as it allows embryonic germ cells to migrate, proliferate and survive (120). It is important to recognize, that in patients with cryptorchidism or DSD, often delayed maturation of the germ cells occurs. In these patients the germ cells still express POU5F1 after the age of 1 year, possibly leading to misinterpretation of germ cell maturation delay as GCNIS and thus over-diagnosis. KITLG has been found to be informative in distinguishing delayed mature germ cells from GCNIS: the marker being positive in GCNIS/GB cells and absent in germ cells having maturation delay (Fig. 3) (111). This is of interest in the context of the putative role of the KITLG P53 binding SNP in testicular GCC development (see above). Also the distribution and position within the seminiferous tubules of the germ cells is helpful to differentiate between the two conditions (121). Another important player in GCC development is the aforementioned *TSPY* gene. The physiological role of TSPY is not fully understood, but in normal testis it is thought to control mitotic proliferation of the spermatogonia. TSPY overexpression in germ cells may therefore, together with prolonged expression of POU5F1, result in the survival and proliferation of germ cells in an unfavorable milieu. Strikingly, development of GB occurs only in DSD patients with presence of the GBY region in their karyotype. This region contains several genes, but *TSPY* is the most likely candidate. In the fetal gonad TSPY is expressed at a stable level, but it becomes more abundant in GCNIS, GB and sometimes seminoma (122, 123).

In addition to the aberrant expression of genes and transcription factors associated with pluripotency, which are similar in malignant and normal fetal germ cells, the epigenetic pattern of GCNIS is, like in PGCs, associated with an open chromatin structure. GCNIS cells show hypomethylation, as evidenced by immunohistochemical staining, as does the invasive GCC component seminoma (108, 109). Histone modification marks in GCNIS indicate that these cells are arrested in a methylation state comparable to mouse PGCs between E7.5 and E8.25 (124). It has been found that *DPPA3* (*STELLA*), a maternal effect gene expressed in testicular GCC and PGCs, is hypomethylated in PGCs and testicular GCC, indicating that expression in testicular GCC is linked to a retained methylation status from the PGC progenitor (125). Interestingly, analyses of 14 of the SNPs related to testicular GCC identified in the GWAS in a cohort of 52 postpubertal 46,XY DSD patients with androgen insensitivity syndrome (AIS), showed that the SNPs near *KITLG* (rs995030) and *ATFZIP* (rs2900333) distinguish between patients with and without (pre-)GCNIS (101). This implies that genetic susceptibility plays a role in GCC at least in AIS, but possibly also in other forms

of DSD. It remains to be clarified whether differences exist dependent on the pathogenetic stage of the disease regarding the impact of the SNPs.

Concluding remarks and relevant questions for further investigation(s)

In summary, based on our interpretation of the current data, the driving factor behind the development of GCNIS or GB in individuals with a DSD is the inappropriate or defective microenvironment in which the germ cells reside. The absence of a proper niche can result in apoptosis, or an arrested development of the germ cells, leading to infertility, or alternatively these cells can subsequently give rise to the different precursor lesions. Whether GCNIS or GB develops depends on the level of testicularization of the gonad, as evidenced by the expression of, respectively, SOX9 or FOXL2 in the supporting cells. The expression of embryonic markers, and epigenetics found in these precursor lesions is most likely not a causal mechanism, but a reflection of the developmental stage at which the cells are blocked. Whether these precursors will develop later in life into a full blown GCC likely depends on additional factors like a changed endocrine milieu in a susceptible individual. This susceptibility might be conferred by the presence of the risk SNPs identified in the different GWAS in men with a testicular GCC. In this respect, the finding that a specific SNP in a p53 responsive element of *KITLG* can upregulate its expression is of particular interest. A remaining question is if the identified SNPs also play a role in GCC development/risk in DSD. And if so, whether they can be used as a diagnostic tool in GCC risk stratification of DSD patients, next to the existing parameters. To summarize, a schematic representation of the pathobiology of GCC in DSD is given in Fig. 4. To be able to answer these questions and draw any meaningful conclusion, international collaboration is necessary to reach a sufficient case load of individuals with DSD in order to robustly analyze the distribution of the risk SNPs.

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