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Development of malignant germ cells - the genvironmental hypothesis

LEENDERT H.J. LOOIJENGA*, TON VAN AGTHOVEN and KATHARINA BIERMANN

Department of Pathology, Erasmus MC – University Medical Center Rotterdam, Josephine Nefkens Institute, Daniel den Hoed Cancer Center, Rotterdam, The Netherlands

ABSTRACT Human germ cell tumors are of interest because of their epidemiology, clinic and pathobiology. Histologically, they are subdivided into various elements, with similarities to embryogenesis. Recent insight triggered development of a higher order division into five types of human germ cell tumors. In the context of male germ cells, only three are relevant; Type I: teratomas and yolk sac tumors of neonates and infants; Type II: seminomas and nonseminomas of (predominantly) adolescents and adults; and Type III: spermatocytic seminomas of the elderly. Various animal models, both occurring spontaneous or induced, are reported, of which their relevance is still a matter of debate. Recent multidisciplinary studies have led to a significant increase in our understanding of the parameters involved in the earliest pathogenetic steps of human germ cells tumors, particularly the seminomas and nonseminomas (Type II). This paper will discuss a number of interesting insights into the normal and aberrant regulation of germ cell development, resulting in the so-called genvironmental hypothesis. This assumes a subtle interaction between environmental- and (epi)genetic parameters, resulting in clinical/phenotypical characteristics. These influence signaling pathways and thereby developmental processes, including gonadal development, germ cell proliferation, maturation and apoptosis. In the case of a disturbed physiology, either due to the germ cell itself, or the micro-environment, embryonic germ cells, during a specific window of sensitization, might be blocked in their maturation, resulting in carcinoma *in situ* or gonadoblastoma, the precursors of seminomas and nonseminomas. The level of testicularization of the gonad determines the histological composition of the precursor. These insights will allow a better definition of individuals at risk of developing a germ cell malignancy, and allow a better selection of scientific approaches to elucidate the corresponding pathogenesis.

KEY WORDS: *testis, gonad, germ cell tumor, environment*

Introduction

To date increasing attention is given to elucidate the pathogenesis and clinical behavior of human germ cell tumors (GCT). In this context, proper classification into subgroups is relevant, particularly because various spontaneous and induced animal models are given a prominent place in the scientific scene. In spite of their potential informative status, they are not by definition representative for (all variants of) human GCT. The current interest in GCT can be explained by a number of reasons. These relate to recent ideas about the origin and progression of cancer in general, as well as long term side effect of systemic treatment on individual cancer patients. This strengthens the need for individualized and possibly tailored treatment. Indeed, human GCT show a set of unique characteristics that may shed novel light on these specific processes. In addition, and possible one of the major explanatory arguments for the increasing attention, is that GCT mimic normal embryonic development. They show regulatory processes involved in proliferation, differentiation, as well as apoptosis. It is often difficult to separate whether the observations made in GCT are intrinsic to the cell of origin and therefore representative for behavior of the different derivatives present, or related to mechanisms involved in the process of malignant transformation. In spite of this limitation, interesting findings can be obtained (Spiller *et al.*, 2012,

Abbreviations used in this paper: GCT, germ cell tumor; NGS, next generation sequencing; PGC, primordial germ cell; SNP, single nucleotide polymorphism; TSPY, Testis Specific Protein on the Y chromosome.

***Address correspondence to:** L.H.J. Looijenga. Department of Pathology, Erasmus MC-University Medical Center Rotterdam, Josephine Nefkens Institute, Building Be, Room 432, P.O. Box 2040, 3000 CA Rotterdam, the Netherlands. Tel: +31-10-704-4329; Fax: +31-10-704-4365. E-mail: l.looijenga@erasmusmc.nl

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Westerman *et al.*, 2011). The items of interest related to GCT to clinicians, scientists, and patients, deal will increasing knowledge about behavior of stem cells, i.e., embryonic-, adult- and cancer-, as well as their unique histology-related pattern of sensitivity and resistance to DNA damaging agents. Several of these aspects will also be discussed in other papers within this Special Issue. Moreover, GCT represent a wonderful model to study the putative interaction(s) between germ cells and their micro-environment, being under strict (interactive) control of genetic-, epigenetic-, and environmental parameters (Spradling *et al.*, 2011). Moreover, GCT are predominantly diagnosed in relatively young patients, i.e., neonates and infants, as well as adolescents and young adults, with major mental (short and long term), physical, social, and even economical impact. Significant numbers of patients require surgery followed by either systemic treatment (irradiation and/or chemotherapy), with proven long term effects on quality of life. Risk factors for development of specific subgroups of GCT have been identified, which are useful to identify groups within the general population with an increased risk to develop this type of cancer. These findings potentially allow selection of individuals for (targeted) screening, with potential early diagnosis and treatment, preventing long term effects of systemic treatment.

In this manuscript, an update on the current knowledge on the pathogenesis of human GCT will be given, in which relevant information about normal germ cell development and well as animal models, will be included. Some of these topics will be more extensively discussed in other papers within this Special Issue, therefore only briefly touched upon here. Finally, the most relevant hypothesis to be tested in the context of the origin of the GCT of adolescents and adults, i.e., the seminomatous and nonseminomatous tumors, according to these authors, will be presented. It includes a subtle interplay between genetic-, epigenetic-, and (micro-)environmental parameters, referred to as GENVIRONMENT, as the major mechanism in determining the embryonic germ cell stem niche, being therefore more or less susceptible for events involved in the process of malignant transformation, eventually leading to the precursor lesions and progression to full blown malignancy. Before going into more detail, the rationale to subdivide human GCT will be explained.

Heterogeneity of human germ cell tumors? Relevance of sub-classification

The traditional approach to classify human and animal GCT is based on histological characterization, preferentially done by a (n experienced) pathologist. Identification of the various lineages of differentiation is predominantly based on morphological criteria, sometimes supported by application of immunohistochemical stainings. The markers Alpha Feto Protein (AFP) and human Chorionic Gonadotrophine (hCG) can be used for immunohistochemical detection on tissue sections to identify yolk sac tumor and choriocarcinoma components, respectively. In addition, they are routinely used as serum markers. In addition, a large number of more recent markers have been identified and applied, some of them highly useful (to be discussed below). These markers are predominantly recognized as being of potential interest based on increasing knowledge derived for studying normal embryonic development, both murine and human. The majority of these markers support the expected histological composition of the lesion based on morphological evaluation, with often significant overlap. However, application of these markers in a routine diagnostic procedure allows a (more) straight-forward, relatively fast and reproducible identification/diagnosis of the histological composition present. In spite of this significant improvement, “simple” processing of the samples and characterization of the various histological elements present according to this approach is not representing optimal classification of GCT in the context of their heterogeneous origin and (related) pathogenesis. This is however required to allow proper selection of the samples for scientific and clinical studies, without comparing non-related (sub)groups and dilution potential important findings. Therefore we proposed in 2005 an alternative model for the classification of GCT, which will be presented and discussed in the next paragraph.

Patho-biological classification of GCT

Based on a defined set of criteria, five types of GCT can be distinguished. The informative parameters to distinguish these entities are anatomical site and age of clinical presentation, precursor lesion (cell of origin and intrinsic pattern of genomic imprinting), as well as chromosomal constitution. The different entities include the Type I: teratomas and yolk sac tumors of neonates and infants; Type II: seminomatous and nonseminomatous GCTs of mainly adolescents and young adults; Type III: spermatocytic seminomas of the elderly testis; Type IV: dermoid cysts, mainly the ovary; and Type V: the hydatiform mole in fertile woman. Within the context of male germ cells, only the Type I, II and III GCT are of relevance, and will therefore be discussed in more detail here. The subdivision as proposed is in line with results of detailed description of spontaneous as well as induced animal models for GCT. Before going into detail regarding the three types of human GCT, the most relevant animal models in this perspective (according to the authors) will be presented.

Animal models of human GCT; an introduction

A large and heterogeneous series of studies have been reported related to possible animal models for human GCT, spontaneously occurring and induced by laboratory manipulations. The major findings related to the current topic will be discussed here, focusing on five species: *Caenorhabditis Elegans* (nematode), *Drosophila Melanogaster* (fruit fly), zebrafish, mouse and dog. These will be presented subsequently, after which an overall interpretation on their relevance for human GCT will be given.

Caenorhabditis (C.) elegans

C. elegans is a highly informative animal model to study normal and aberrant development, including cancer, even GCT (Berry *et al.*, 1997, Subramaniam and Seydoux, 2003). PUF-8 knock outs resulted in GCT (Subramaniam and Seydoux, 2003), histologically showing similarities to spermatogonia. PUF proteins are RNA binding proteins regulating RNA stability and translation, found to be crucial in germ line stem cell self-renewal. It is demonstrated that PUF-8 is required to complete meiotic prophase. Absence will result in dedifferentiation, leading to tumors composed of mitotic undifferentiated germ cells. Another model of GCT in this species is related also to a RNA interacting component. GLD-1 has been found to be related to regulation of mitosis and meiosis (Crittenden

et al., 1994, Francis *et al*., 1995) and GCT formation (Berry *et al*., 1997). This regulates cell cycle, specifically via cyclin E and Cdk2 (Jeong *et al*., 2011). Interestingly, GLD-1 is involved in the inhibition of the translation of cyclin E mRNA towards protein (functions as a physiological si-RNA). This results in prevention of re-entry of mitosis of germ cells. In case of GLD-1 absence, cyclin E protein is formed, resulting in re-entry of the cell cycle, and a transition to a somatic lineage, resulting in teratoma, i.e., representing somatic differentiation (Biedermann *et al*., 2009). In other words, two variants of GCT can be generated in this species, one still showing similarities to the germ cell lineage, and the other showing so-called activation of pluripotency, also known as undergoing the process of reprogramming, i.e., transition to a somatic lineage.

Drosophila (D.) melanogaster

As for *C. elegans*, *D. melanogaster* has also been proven to be an informative source of relevant information related to normal and aberrant development, including germ cell behavior and gonadal formation (Decotto and Spradling, 2005, Jaglarz and Howard, 1994, Tran *et al*., 2000), as well as GCT (Gonczy *et al*., 1997, Hime *et al*., 2007). Again, cyclin E is found to be related to cell cycle regulation, and down-regulation is required for normal germ cell cycle arrest (Knoblich *et al*., 1994). Neural insulin-like peptides have been found to regulate germ line stem cells influenced by diet (Hsu *et al*., 2008). These factors specifically target the G_2 phase of the cell cycle under control of the signaling pathways PI_3K and FOXO (to be discussed in more detail later). In addition the transition related to the G_1 phase of the cell cycle is also influenced by diet. GCT in this animal, showing histological similarities of to the germ cell lineage, are still responding to this signaling pathway, suggesting that it is a germ cell intrinsic response, independent of the niche. In addition, PTEN, LKB1 and AMPK are found to regulate PGC proliferation is this species (Narbonne and Roy, 2006). Increasing information is becoming available that the supportive cells in the niche are also required to allow maturation of the stem cells to happen (Srinivasan *et al*., 2012). In other words, a subtle balance between inducing and inhibiting factors seems to determine the cell fate.

Zebrafish

Recent studies have shown that zebrafish is an informative model for embryogenesis and cancer development (Amatruda *et al*., 2002). Using a mutagenic approach, an interesting and first model of GCT in zebrafish has been generated. Forward genetic approaches showed that inactivation of Alk6, a type I Bone Morphogenic receptor, results in activation of the Smad pathway in male germ cells, and tumors composed of pre-meiotic germ cells are formed (Neumann *et al*., 2011a, Neumann *et al*., 2011b), still showing germ cell lineage characteristics.

Mouse

The initial studies on GCT in mouse were performed by Stevens, who demonstrated that the 129 strain mouse specifically has a high tendency to develop teratomas, originating from embryonic germ cells (Stevens, 1970, Stevens and Varnum, 1974, Walt *et al*., 1993). In fact, this is the model that always includes loss of germ cell characteristics, and gain of the somatic lineage profile as part of the process of tumorigenesis. These studies initiated a large series of very interesting studies. The specific susceptibility of the 129 strain for GCT development, genetically associated with Ter, is linked to a RNA modifying gene (Kedde *et al*., 2007), called Dnd (Dead End) (Youngren *et al*., 2005). Interestingly, Dnd is involved in regulation of generation of mature miRNAs (Ketting, 2007). Most recently, an interaction between Dnd and the RNA binding unit of ApoB editing complex is demonstrated (Nelson *et al*., 2012). This is regulating the actual risk for GCT development (i.e. teratomas), both in a conventional as well as transgenerational manner. In addition, it has been elegantly demonstrated that the Kit-Kitlg (Stem Cell Factor) pathway is involved in development of these types of teratomas in the 129 strain (Heaney *et al*., 2008). Loss of the soluble ligand (Kitlg) has no effect on tumor formation, while loss of the membrane bound variant increases the risk. This is particularly of importance in the context of the role of this particular pathway in the migration, proliferation and survival of mouse PGC (Runyan *et al*., 2006, Wylie, 1999). In comparison to *Drosophila*, it has been demonstrated that Igf-1 is secreted by mouse Leydig cells which is a key determinant in retaining pluripotency germ line stem cells (GSC), characterized by their alkaline phosphatase activity, as well as expression of OctT3/4, Blimp-1 and Nanog (Huang *et al*., 2009). This system functions again via the PI_3K pathway (see above). In contrast to the teratomas in mouse, also other types of GCT are reported in this species, although exceptionally. For example, a seminomatous like tumor is generated from genomic imprint-free embryonic stem cells (Holm *et al*., 2005), found to be related to aberrant activation of the TGFβ pathway. This indicates that proper erasement and establishment of a uniparental (paternal) pattern of genomic imprinting is crucial for normal germ cell maturation origin (Looijenga *et al*., 1998). Nodal signaling has been found to regulate developmental potential of mouse germ cells as well, which interestingly is reflected in human GCT as well (Spiller *et al*., 2012). This topic will be extensively discussed in a separate paper in this Special Issue (van der Zwan *et al.,* 2013, Eini et al. 2013). A seminomatous-like tumor is also generated based on overexpression of cyclin D combined with activated Ras signaling (Lee *et al*., 2009). It still remains to be shown whether these models indeed present the Type II GCT, as diagnosed in humans.

The factor cyclin E is also found to be a significant player in the field of normal mouse development, including the germ cell lineage. It is involved in regulation of endoreplication of trophoblastic cells during normal development (Parisi *et al*., 2003). Over-expression of cyclin E in spermatogonia results in reduced spermatogonial proliferation and reduced fertility (Liberal *et al*., 2010). In addition, down-regulation of cyclin E1 is specifically observed in male fetal germ cells during the 12.5-14.5 dpc period (Western *et al*., 2008). Unpublished data from our group show that upon tetraploidization of mouse ES cells, there is a significant and specific activation of the PI_3K pathway (Zanazzi and coworkers, unpublished data).

Knock out of Dmrt1 in embryonic male mouse germ cells is found to induce teratomas, in a dose specific manner (Krentz AD, 2009). Interestingly, Dmrt1 is one of the major regulators of gonadal sex determination (Matson *et al*., 2011, Matson and Zarkower, 2012), also relevant in the context of patients with Disorders of Sex Development (DSD) at risk for GCT development (see below). In fact, Dmrt1 prevents development of the ovarian lineage in the male chromosomal constitution (XY). In fact, it seems to be the male counterpart of the female Foxl2 signaling in ovarian development (Uhlenhaut *et al*., 2009, Uhlenhaut and Treier, 2006). The function of Dmnt1 is evolutionary conserved, based on the role of this

gene in *C. elegans* and *D. melanogaster* (Raymond *et al.*, 2000).

Dog

Spontaneous occurring GCT in dogs have been known for a long time (Schlotthauer, 1938, Scully and Coffin, 1961). This phenomenon has been investigated by various groups independently, using morphology alone, or including protein and mRNA expression profiles, as well as genetic constitution (Bush *et al.*, 2011, Inoue and Wada, 2000, Looijenga *et al.*, 1994, Murakami *et al.*, 2001, Yu *et al.*, 2009l). The overall conclusion is that canine GCT are relatively benign and are characterized by the presence of small, intermediate and large cells. So far, no specific signaling pathway of chromosomal anomaly has been linked to the development of this type of GCT in dogs.

Interpretation of animal models for human GCTs

The data available mainly support that most GCT in animals resulting in teratomas are resembling to the Type I GCTs of humans. Especially, data on the majority of mouse GCT support this notion, with the possible exception of the imprint-free ES derived and cyclin D overexpressing seminomatous-like tumors. Dnmt1 is of particular interest, because it has been related with DSD, and linkage has been found in the general male population with risk of type II GCT of the testis (both items to be discussed below). Knowledge on the pathogenesis of GCT has increased are thinking strategies for develop safety regulations for human stem cell therapies (Cunningham *et al.*, 2012).

The heterogeneity in genes of which disruption results in germ cell derived teratomas indicate that a transition to the somatic lineage is activated in affected undifferentiated germ cells as proposed before (Looijenga, 1999). Of specific interest is the fact that this repeatedly is found to be linked to regulation of the cell cycle. So far, no consistent genetic anomalies in type I GCT, especially teratomas have been found in humans (Linger *et al.*, 2008). This might be due to the same underlying mechanism, resulting in a rather heterogeneous spectrum of potential targets (mRNA, miRNAs, proteins though genetic or epigenetic changes) involved.

Interestingly, in *D. melanogaster*, *C. Elegans*, as well as in mouse, cyclin E has been related to normal germ cell development, as well as development of GCT. Again, a link with cell cycle regulation is becoming apparent. However, this might related to both in Type I- and Type II-GCT, requiring additional investigations. Indeed, cyclin E has also been suggested to be relevant in the pathogenesis of human (type II) GCTs, both from the testis (Datta *et al.*, 2000) and ovary (Kawauchi *et al.*, 2006). In addition, cyclin E is related to cisplatin resistance in a number of solid cancers (of epithelial origin: carcinomas), including those of the ovary (Etemadmoghadam *et al.*, 2009, Nakayama *et al.*, 2000). Moreover, we identified a chemotherapy resistant metastatic (late age metastatic Type I) GCT, composed of teratoma and yolk sac tumor, with specific amplification of the cyclin E (CCNE1) only (to be published elsewhere). The zebrafish GCT reported are more closely related to the Type II seminomas than to Type III spermatocytic seminomas. In contrast, the PUF-8 knock out GCT in *C. elegans* (Subramaniam and Seydoux, 2003) is likely an interesting model for Type III GCTs, i.e., spermatocytic seminomas, as well as those diagnosed in dogs. In this context the specific pattern of mutations found in human spermatocytic seminomas are of interest in the canine counterparts (Goriely *et al.*, 2009), as well as recent additional diagnostic markers in humans (Lim *et al.*, 2011). To further put these findings in perspective, the rest of this paper will be related to human Type II GCT, to be referred to as Germ Cell Cancer (GCC).

Epidemiology and risk factors of human GCC

GCC, predominantly diagnosed in Caucasian males, show an overall increase in incidence, as well as a birth cohort effect in multiple Western European countries (Bergström *et al.*, 1996). Males born during the second World War have a lower incidence, which has been related (partly) to maternal weight (Aschim *et al.*, 2005). This might be related to higher insulin levels, influencing the level of free estrogens (via sex hormone-binding globulin). Both a low and high birth weight is found to be related to the risk of GCC (both infants and adult) (Stephansson *et al.*, 2011). A recent meta-analysis showed that besides birth weight, cryptorchidism, inguinal hernia, twinning, and gestational age are factors influencing the risk of development of GCC (Cook *et al.*, 2009).

No link between human GCC and circulating IGF-1 (IGFBP-3) has been found in a large series of GCC patients and controls (Chia *et al.*, 2008). The main issue here might be that the window of effect, defined by us as the window of sensitization (see below) is not investigated in this study. Analysis at the proper time in development might result in a positive correlation. This is in line with the observation between birth weight and IGF-1 concentration (Joss-Moore *et al.*, 2010). Of special interest is that this has been linked to epigenetic processes, especially in the context of fetal adaptation to a changing environment.

Endocrine dysfunction has been linked to a reduced ano-genital distance (AGD). Indeed, Caucasian boys with hypospadias show this phenomenon (Hsieh *et al.*, 2012). Of interest is that also digit ratios are related to endocrine exposure (Zheng and Cohn, 2011). However, no indication for hormone disruption with this specific read out in relationship with development of GCC has been found so far (Auger and Eustache, 2011). However, such a link was found based on measurements of steroid hormone during early pregnancy (Holl *et al.*, 2009). A disturbed testicular development due to environmental influences, i.e., pro-estrogen and anti-androgen activity (xeno-estrogens), is proposed the mechanistic basis of the so-called Testicular Dysgenesis Syndrome (TDS), as developed and propagated by Niels Skakkebaek (Skakkebaek, 2003). This might result in mild – intermediate – severe forms (hypospadias, cryptorchidism, disturbed fertility, GCC). However, existence of TDS is also questioned (Akre and Richiardi, 2009), including a role for perinatal exposure to estrogens (Ramlau-Hansen *et al.*, 2009).

It is however not a discussion whether particular forms of gonadal disruption, i.e., referred to as DSD, is related to an increased risk of development of a GCC. This has been reviewed in various manuscripts (Cools *et al.*, 2006, Hersmus *et al.*, 2008, Looijenga *et al.*, 2010, Looijenga *et al.*, 2007, Pleskacova *et al.*, 2010). The main risk parameters within this specific group of patients are the following: 1) presence of a specific part of the Y chromosome, referred to as Gonadoblastoma on the Y chromosome (GBY), as originally proposed in 1987 (Page, 1987), in the karyotype of the germ cells; 2) low level of testicularization (i.e., level of testis formation); 3) delayed/blocked maturation of embryonic germ cells and 4) activation of the c-KIT-KITLG pathway (to be further

discussed in the next paragraphs). In addition, other parameters might influence the risk, like non-scrotal localization of the testis, specific risk alleles, amongst others. These issues will be discussed later as well.

Origin and pathogenesis of human GCC, diagnostic markers

A number of highly informative diagnostic markers for the earliest developmental stages of GCC pathogenesis has been identified. These predominantly based on knowledge from normal embryogenesis. These include historically alkaline phosphatase (re)activity (Millan and Manes, 1988, Roelofs *et al.*, 1999, Stoop *et al.*, 2011) and the stem cell factor receptor (c-KIT) (Biermann *et al.*, 2007, Meyts *et al.*, 1996, Rajpert-De Meyts and Skakkebæk, 1994, Strohmeyer *et al.*, 1991). Routine use of detection of c-KIT might however result in over-diagnosis as reported recently (Biermann *et al.*, 2012). Activation of the c-KIT pathway has been suggested based on mutation as well genomic amplification studies (Biermann *et al.*, 2007, Looijenga *et al.*, 2003a, McIntyre *et al.*, 2005a, McIntyre *et al.*, 2005b, Rapley *et al.*, 2004). In addition, most recently, we demonstrated that ovarian GCC without any sign of DSD have a high frequency of c-KIT mutations, while those with DSD are characterized by presence of GBY, in particularly by expression of Testis Specific Protein on the Y chromosome (TSPY, Hersmus *et al.*, 2012; see below). This dichotomy in pathogenesis between these defined subgroups of patients has been reported before (Hoei-Hansen *et al.*, 2007). This suggests that activation of the c-KIT pathway might be circumvented by the presence of TSPY (to be discussed below).

Of special interest is the finding that early malignant transformed germ cells can be distinguished from delayed matured germ cells based on specific presence of the c-KIT ligand (Stem Cell Factor SCF, also known as KITLG) based on immunohistochemistry (Stoop *et al.*, 2008). This is highly relevant based on the identification of high risk alleles in the general population for development of GCC (see below).

The main contributions to the spectrum of diagnostic markers for GCC based on knowledge of regulatory factors in stem cells is definitely the identification of OCT3/4 (POU5F1) (Looijenga *et al.*, 2003b), as well as SOX2 and SOX17 (De Jong J, 2008., Gopalan *et al.*, 2009, Korkola *et al.*, 2005). This has been supported by many independent studies. While OCT3/4 is consistently found in seminomas and embryonal carcinomas, SOX17 present in the seminomas and SOX2 in the embryonal carcinomas. This allows a straightforward set of transcription factors for the diagnosis of these histological components. This is found to be independent on stage of presentation and pre- versus post-chemotherapy status. Of notion is that SOX17 is related to the embryonic status of hematopoietic stem cells (He *et al.*, 2011, Kim *et al.*, 2007).

The precursor lesion of all seminomas and nonseminomas (embryonal carcinoma, teratoma, yolk sac tumor, and choriocarcinoma) is known as carcinoma *in situ* of the testis (CIS), as originally proposed in 1972 (Skakkebæk, 1972) and Gonadoblastoma of the dysgenetic gonad reported in 1970 (Scully, 1970). These lesions are both to be identified using OCT3/4. However, they can be distinguished from each other based on characteristics of the supportive cells, being Sertoli cells, stained by SOX9, and Granulosa cells, stained by FOXL2, respectively SCF (Hersmus R, 2008). In fact, the histological composition of the precursor lesion of GCC is determined by the level of testicularization, as defined before. The large amount of epidemiological as well as experimental data indicate that the cell of origin of GCC is a blocked PGC/gonocyte, is in line with expression profiling (Novotny *et al.*, 2012, Sonne *et al.*, 2009), epigenetic status (Eckert *et al.*, 2008, Netto *et al.*, 2008, Werman *et al.*, 2010)as well as the consistent biallelic expression of imprinted genes (Van Gurp *et al.*, 1994, Verkerk *et al.*, 1997), demonstrating their erased pattern of genomic imprinting. In contrast, Type I GCT show a more heterogeneous pattern, supporting the origin of an embryonic stem cells or early (partially erased) embryonic germ cell (Schneider *et al.*, 2001). Moreover, Type III GCT (spermatocytic seminomas) demonstrate a partial paternal pattern of genomic imprinting, in line with a latter stage of germ cell as origin (Sievers *et al.*, 2005), supported by various immunohistochemical staining studies (Lim *et al.*, 2011, Looijenga, 2011). As indicated, TSPY is the main candidate gene within the GBY region, found to be present in the precursor lesions all almost all patients with a GCC, males as well as DSD patients (males and females), with defined exceptions to this rule (see above). A number of relevant characteristics of TSPY will be presented in the next paragraph.

Unique characteristics of Testis Specific Protein on the Y chromosome (TSPY)

One of the striking observations in the pathogenesis of GB in humans is the fact that only patients with a specific part of the Y chromosome in their karyotype are at risk, referred to as GBY (Page, 1987). Although this genetic fragment contains a number of genes, the most interesting candidate in the development of GCT is TSPY (Testis Specific Protein on the Y chromosome) (Arnemann *et al.*, 1991). It is a multi-copy gene (Manz *et al.*, 1993), expressed in spermatogonia, CIS and GB (Hildenbrand *et al.*, 1999, Kersemaekers *et al.*, 2005, Ng *et al.*, 2008, Schnieders *et al.*, 1996) It is shown to regulate cell cycle progression, by interacting with cyclin B_1 (Oram *et al.*, 2006). Of interest is that the level of TSPY in the precursor lesions of GCC correlate with expression level of oncogenes on 12p (Li *et al.*, 2007). In progressed GCC however, TSPY is often lost. In addition, TSPY, besides being multi-copy in nature on the Y chromosome, has various splice variants (Dechend *et al.*, 2000, Vogel and Schmidtke, 1998). No functional mouse homologue has been identified so far (Schubert *et al.*, 2000). Therefore, a transgenetic mouse model was created, in which the human gene integrated in the mouse Y chromosome, and multiplied according to the same level as found in humans (Schubert *et al.*, 2003). Although the protein was generated at the expected time and place (in spermatogonia), the animal model showed no aberrant phenotype, and spermatogenesis was normal. Interestingly in daily clinical routine testicular tissue analysis, immunohistochemical detection of TSPY is informative to visualize presence of germ cells in clinical samples (date not shown).

Most recently it was demonstrated that TSPY might be inhibiting the androgen pathway in human germ cells during development and GCT (Akimoto *et al.*, 2010). This is of specific interest, because it might generate an androgen-insensitivity niche by keeping the androgen receptor in the cytoplasmatic compartment, independently, or on top of its role in cell cycle regulation (see above). These observations need further investigation, in which predomi-

nantly the presence of the androgen receptor during normal and aberrant germ cell development is an issue of study. However, it might elegantly link the various thoughts about TDS, DSD, c-KIT pathway activation and TSPY in the initial pathogenetic steps of CIS and GB formation, especially in the context of hypovirilization of patients at increased risk for GCC development.

High throughput approaches (SNP, NGS, methylation)

A major break through in further understanding the pathogenesis of human GCC came from the two high throughput risk single nucleotide polymorphism (SNP) analyses, one from the USA and one from the UK (Kanetsky *et al.*, 2009, Rapley *et al.*, 2009). A selected number of risk SNPS, preferred to be regarded as susceptibility alleles, were identified, suggested to relate to a common pathway. This included KITLG, DMRT1, SPRY4, and BAK1, all involved in early gonadal development and germ cell survival. Of interest is that Bak1 knock out mice, being a crucial determinant in regulation of germ cell apoptosis, allows visualization of extragonadal, fluorescently tagged PGC (Runyan, 2008). The risk SNPs are in fact the most frequent alleles in the general Caucasian population, although less frequent in distribution in the general Asian and Black populations, as expected related to the incidence of GCC. Additional studies have been published (Turnbull *et al.*, 2010), including description of a single risk SNP within the DMRT1 locus (Kanetsky *et al.*, 2011). These risk SNPS have been found to be independent of the role of cryptorchidism, familial predisposition (Kratz *et al.*, 2011a, Kratz *et al.*, 2011b), as well as spermatogenic function (Ferlin *et al.*, 2012). In addition, gene variants within sex hormone pathways showed significant changes (Kristiansen *et al.*, 2012). Subsequent analysis demonstrated that patients with TDS show specific linkage to defined SNPs (Dalgaard *et al.*, 2012), specifically related to TGFBR3 and BMP7.

miRNAs in the development of germ cells and GCC

A defined set of non-protein encoding RNAs, referred to microRNAs (miR), are found to be crucial for normal embryonic development and maintenance of an adult individual. In fact, this was originally demonstrated to be relevant in *E. elegans* (Lee *et al.*, 1993). Moreover, a significant role in development of cancer has been proven in a large number of cases (Esquela-Kerscher and Slack, 2006). miR regulate the transition from mRNA to protein, in which it is expected that around 30% of all protein encoding genes are under influence of miRs. Profound presence of miR has been reported in embryonic stem cells (Rosa and Brivanlou, 2011). In addition, we reported specific patterns and functions of miRs in GCTs, including both Type II and Type III (Gillis *et al.*, 2007), in line with studies of others, also including Type I (Palmer *et al.*, 2007). The tumor components could be distinguished from each other based on their differentiation status (Gillis *et al.*, 2007). A functional proof for the miR-371-373 cluster was shown in the context of inhibition of down stream target LATS2 of the P53 pathway (Voorhoeve *et al.*, 2006). Interestingly, Lats2 has also been found to be of importance to overrule cellular senescence. A positive feedback loop between p53 and Lats2 is involved in preventing tetraploidization (Aylon *et al.*, 2006). The fact that it regulates Cdc2 activity in *D. melanogaster* might be of specific interest (Tao *et al.*, 1999). Most recently, an elegant miR screen has been performed on the precursor lesion of GCC, i.e. CIS, showing similar findings (Novotny *et al.*, 2012). In addition, it is suggested that specific miR are involved in the maturation of mouse gonocytes to pre-spermatogonia (McIver *et al.*, 2012a,b). Most recently, detection of miR in serum of GCC (adult and pediatric) patients has been reported from two independent groups (Belge *et al.*, 2012, Murray and Coleman, 2012, Palmer *et al.*, 2010). This opens novel possibilities for the diagnosis and follow-up of GCT patients. The impact of miR in the early development of GCT, and their putative clinical implications will be further discussed in the paper by Eini *et al.* (2013).

Treatment sensitivity and resistance of GCTs and embryonic and adult stem cells

Overall, GCT, including the Type I and II, can be highly effectively treated using a combination of surgery, irradiation and/or (cisplatin-based) chemotherapy (Horwich *et al.*, 2006). These approaches result in survival of the majority of patients (>90%), even in case of presence of extensive metastatic disease. However, GCC is the most frequent reason of cancer related death in young Caucasian males. In spite of the overall sensitivity of GCC for the applied treatment strategies, it has become evident that exposure to either irradiation or chemotherapy at young age results in significant side effects at later life, affecting quality of life. These (long term) side effects can include secondary cancers, fatigue, metabolic syndrome, heart and vascular damage and reduced fertility (Hooning *et al.*, 2007, Travis *et al.*, 1997, van den Belt-Dusebout *et al.*, 2007, Van Leeuwen *et al.*, 1993). This strengthens the need for further insights into the sensitivity of GCC and possible resistance. In line with knowledge of undifferentiated verses differentiated cells, the teratomas (both as Type I and II) show the highest resistance to DNA damaging agents. This has been linked to various mechanisms, including DNA repair, export pumps, cell cycle control as well as specific genetic changes, including P53, micro-satellite instability and BRAF (Honecker *et al.*, 2003, Kersemaekers *et al.*, 2002, Koberle *et al.*, 1997, Koberle *et al.*, 1999, Koberle *et al.*, 1996, Koster *et al.*, 2010, Mayer *et al.*, 2002a, Mayer *et al.*, 2003a, Mayer *et al.*, 2002b, Mayer *et al.*, 2001, Mayer *et al.*, 2003b, Spierings *et al.*, 2004). In this context it is interesting that a high proliferation rate is required for cell reprogramming and maintenance of embryonic stem identity (Ruiz *et al.*, 2011). In addition, regulation of G_1 arrest determines the sensitivity of embryonic stem cells for DNA damage (induced by irradiation) (Hong and Stambrook, 2004). More relevant aspects in this context are discussed in the papers by Van der Zwan *et al.* (2013) and Eini *et al.* (2013).

Window of sensitization (masculinization) of GCC; the genvironment

The overview provided so far, indicate that there are a number of defined parameters of interest in the early stage development of human GCT, particular GCC. These are related to a window of sensitivity. This matched nicely with the so-called window of masculinization as defined by Philip Sharpe (Welsh *et al.*, 2008). It can be influenced by various other parameters mentioned, like cell cycle regulation (by TSPY or miRNAs), IGF-1 (related to body weight), estrogen-androgen exposure (xeno-estrogens, DSD, TDS), high risk SNPs (affecting regulatory pathways for early gonadal development), and cell signaling by PTEN-PI_3K (pluripotency).

The data on risk factors for GCC support the model that a particular time and place window is crucial in the earliest development of this type of cancer. Interestingly, this has similarities to the hematopoietic system, in which also KITLG (SCF) seems to be a major player in the field (Perry and Li, 2012). To support this model in more detail, some aspects related to signaling pathways in embryonic germ cells will be discussed in the next paragraph.

Signaling pathways in PGCs and gonocytes

Identification of the relevant pathways in embryonic germ cells (PGC and gonocytes) is of specific relevance to shed light on the putative disturbance of it in the precursor lesions of human GCC. A number of highly relevant findings has been made during the last years. In all these studies it remains to be shown whether the observations are cross-species in nature. In other words, it remains to be determined whether the data can be interpreted as informative for all models, as well as humans. Mouse PGC (day 11.5-12.5 pc) are found to express both the estrogen receptor α and β, and estrogen exposure results in phosphorylation of Akt, Erk and Src, as well as Kit (La Sala *et al.*, 2010). This could be inhibited specifically, which indicates that estrogens act via the non-genomic signaling pathway. Proper balance of PI_3K/Akt is crucial for development of the mouse germ line, preventing dedifferentiation (Kimura *et al.*, 2008). Overactivation results in formation of tumors (teratomas) and embryonic germ cell derivation efficiency. This acts via the p53 pathway, being downstream of PI_3K/Akt. In addition, it is shown that loss of Pten is required for the formation of teratomas (Kimura and Nakano, 2011). The sensitivity window of the Akt signaling is highly restricted, and non-sensitive in the germ cells in mitotic arrest and beginning of meiosis defined (Kimura *et al.*, 2008). Akt activation resulted in phosphorylation of Gsk3, resulting in stabilization of Mdm2, and inhibition of P53, the latter found to be the crucial downstream target.

One of the most intriguing observations is the fact that excess of estrogens during *in vitro* growth of mouse genital ridges results in induced PGC proliferation (Moe-Behrens *et al.*, 2003). This is due to upregulation of the Kitlg in the somatic cell compartment, resulting in activation of the Kit-pathway via Akt/Pten. The combined estrogen activation, Pten PGC down-regulation and addition of Lif, results in high frequency of transformation of the germ cell population. Bisphenol A (BPA), which has estrogenic activity has been shown to induce proliferation in mouse spermatogonia cell line (GC-1), via activation of Pkg, Egfr/Erk/c-fos (Sheng and Zhu, 2011), associated with ER-α phosphorylation. Besides the role of ER-α, also GPR30 was found to be involved.

Knock out of PTEN in human ES resulted in increased self-renewal, cell survival, and proliferation (Alva *et al.*, 2011). These affected human ES were disrupted in their capacity for differentiation, due to retained OCT3/4 and NANOG expression levels. Interestingly, Pten and P53 have been reported to suppress Nanog expression independently in mouse spermatogonial stem cells (Kuijk *et al.*, 2010). In other words, loss of these markers will result in overexpression of Nanog, and support of undifferentiated stem cells. Interestingly, loss of PTEN protein has been reported consistently in the progression from the pre-invasive to the invasive stage of GCC (Di Vizio *et al.*, 2005). This study also demonstrated the genetic loss as well as inactivation of the PTEN

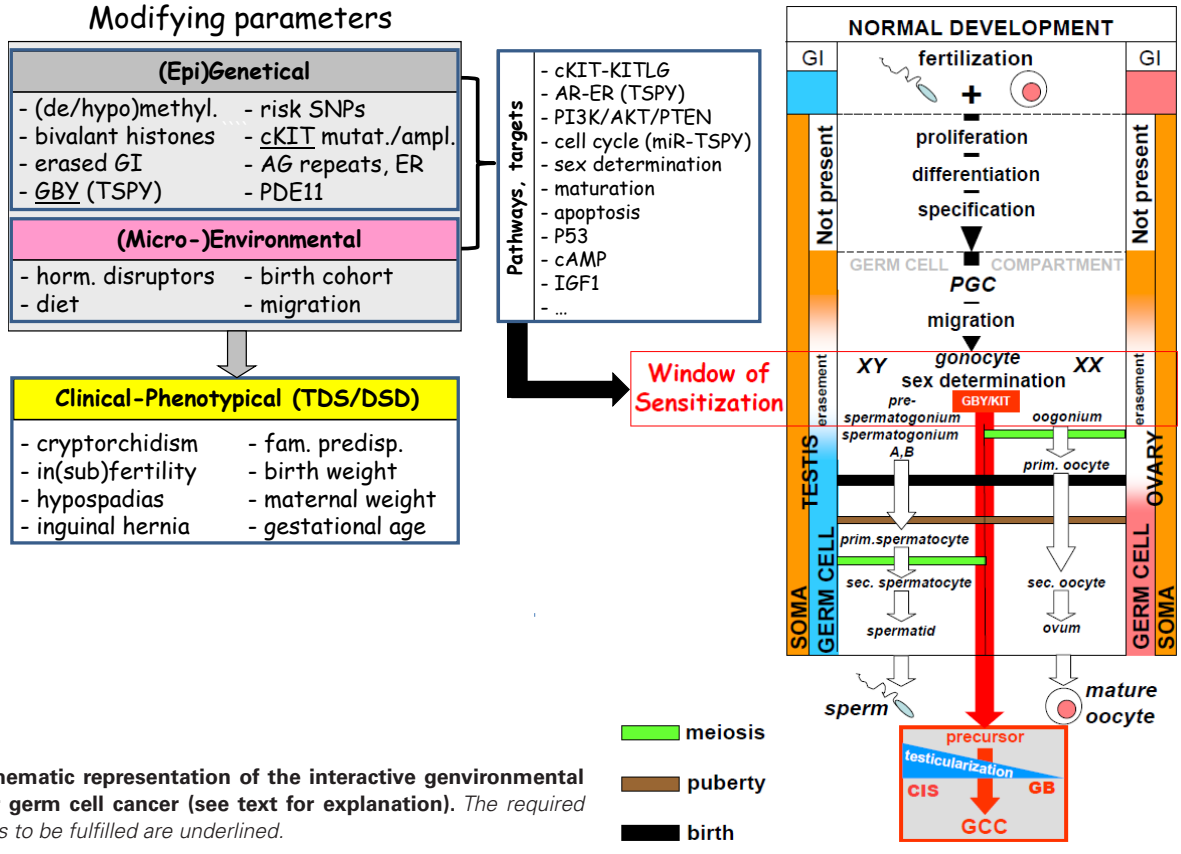

Fig. 1. Schematic representation of the interactive genvironmental model for germ cell cancer (see text for explanation). *The required parameters to be fulfilled are underlined.*

gene in these tumors. Inactivation of PTEN in GCC is supported by an independent analysis (Teng *et al.*, 1997). In addition, this pathway (including $p27^{kip1}$) is crucial for the proliferative activity of NT2 cells, being representative for embryonal carcinoma. In line with this finding AKT signaling has been found in nonseminomatous GCTs (Hennenlotter *et al.*, 2011).

IGF and insulin has been discussed related to the risk of cancer development in general (Gallagher and LeRoith, 2011). IGF-1 receptor is expressed during early human gonadal development, which might be in line with a role of this pathway in this process (Coppola *et al.*, 2009).

To translate these findings to GCC, recent relevant findings of endocrine signaling in GCC will be discussed in the next paragraph.

Endocrine signaling pathways in GCC

GPR30 has been reported to be present in GCC (Franco *et al.*, 2011). This transmembrane estrogen receptor, which acts parallel to the traditional estrogen pathway, was found by immunohistochemistry to be high in CIS, SE and EC. In addition expression was also found in TCam-2, being the only representative SE cell line so far. However, it must be kept in mind that it is derived from a primary GCC composed of both a SE and EC component. BPA is shown to induce proliferation of TCam-2 cells via the GPER/GPR30 pathway (Chevalier *et al.*, 2012). Human germ cells only express ER-β, while reduced in CIS, SE and EC, but high in TE (Esposito *et al.*, 2011). Interestingly, PATZ1 interacts with ER-β in normal germ cells, and delocalization is found in SE (with low ER-β). This is regulated by cAMP levels, related to an increased level of nuclear ER-β, to be inhibited by specific anti-estrogen inhibitors. In addition, an association between specific genetic variants of PDE11 and development GCC has been reported, linked to cAMP signaling via endocrine steroidogenic pathways (Greene *et al.*, 2010, Horvath *et al.*, 2009)

Presentation of the ultimate hypothesis; the model to be tested

Based on the review of a selected number of observations, an interactive model for the earliest pathogenetic steps in GCC formation is proposed. This relate to a specific window of sensitivity, in parallel with the window of masculinization, being the period of initial maturation of gonocytes to either pre-spermatogonia (in males) and oogonia (in females). Interestingly, this links to the period we refer to as Testicularization. The involved parameters include in principle two levels, being (epi)genetics, including (de)methylation, histone modification, pattern genomic imprinting, GBY, cKIT, SNPs, etc, and (micro)environmental, including hormone disrupters, diet, birth cohort and migration. This will determine clinical-phenotypical (TDS/DSD) characteristics, including cryptorchidism, in(sub)fertility, hypospadias, inguinal hernia etc. These levels interact with each other, and might influence a number of signaling pathways, and processes, including c-KIT-AR-PI3K/AKT/PTEN-P53-cAMP-IGF1, affecting cell cycle control ad sex determination, again organized in a higher order structure of interaction. The bottom line is that subtle changes (pro- and con-) might determine the actual risk for a final blocked maturation of a gonocyte, leading to formation of either CIS of GB at an individual level. The requirements to be fulfilled (underlined in the Fig. 1) include presence of GBY (i.e., TSPY), c-KIT activation, and absence of physiological maturation of a gonocyte. An intriguing possible mechanism might be the recently described specific and targeted phosphorylation of the OCT3/4 protein (at position threonine 235), redirecting its function to pluripotency (by interaction with other pluripotency proteins, including NANOG) and loss of its inhibitory effect on the *AKT1* promoter, resulting in enhanced resistance to apoptosis (Lin *et al.*, 2012). In addition, it also remains to be demonstrated whether this model is specific for GCC of adolescents and adults, or is also relevant for the (Type I) GCT diagnosed at pediatric age. This interactive genvironmental model directly demonstrates the complexity for proof in a (spontaneous or induced) animal model, or correlation studies in humans. The presence of the potential parameters involved the decision tree for the ultimate transition from a normal to a malignant germ cell must be elucidated in proper clinical and pre-clinical models and studies.

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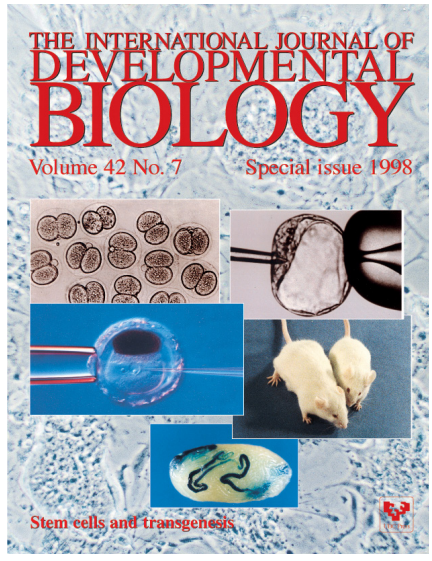

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