

Recent Developments in Testicular Germ Cell Tumor Research

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Testicular germ cell tumors of adolescents and adults (TGCTs; the so-called type II variant) are the most frequent malignancies found in Caucasian males between 20 and 40 years of age. The incidence has increased over the last decades. TGCTs are divided into seminomas and nonseminomas, the latter consisting of the subgroups embryonal carcinoma, yolk-sac tumor, teratoma, and choriocarcinoma. The pathogenesis starts in utero, involving primordial germ cells/gonocytes that are blocked in their differentiation, and develops via the precursor lesion carcinoma in situ toward invasiveness. TGCTs are totipotent and can be considered as stem cell tumors. The developmental capacity of their cell of origin, the primordial germ cells/gonocyte, is demonstrated by the different tumor histologies of the invasive TGCTs. Seminoma represents the germ cell lineage, and embryonal carcinoma is the undifferentiated component, being the stem cell population of the nonseminomas. Somatic differentiation is seen in the teratomas (all lineages), whereas yolk-sac tumors and choriocarcinoma represent extra-embryonal differentiation. Seminomas are highly sensitive to irradiation and (DNA damaging) chemotherapy, whereas most nonseminomatous elements are less susceptible to radiation, although still sensitive to chemotherapy, with the exception of teratoma. To allow early diagnosis and follow up, appropriate markers are mandatory to discriminate between the different subgroups. In this review, a summary will be given related to several recent developments in TGCT research, especially selected because of their putative clinical impact. **Birth Defects Research (Part C) 87:96–113, 2009.** © 2009 Wiley-Liss, Inc.

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NORMAL GERM CELL DEVELOPMENT

Germ cells in mammals, which ensure transmission of genetic information to the next generation by production of mature oocytes in females and spermatozoa in males, are set aside at an early stage during embryogenesis (Witschi, 1948; Mc et al., 1953; Falin, 1969; Gins-

burg et al., 1990; Donovan, 1998). To fulfil this special task, these cells, known as primordial germ cells (PGCs), have unique and specific characteristics. These include their initial capacity to suppress induction of differentiation, their commitment to either the male or female lineage, and finally the capacity to generate highly special-

ized daughter cells that, after fertilization, regain an activated differentiation program. This means that they can form all embryonic and extra-embryonic tissues, including the germ cell population (Honecker et al., 2006). In other words, they are in fact really the totipotent stem cell population of the body, and represent the circle of life.

As indicated, PGCs are the stem cells of gametogenesis, being oogenesis in female and spermatogenesis in male. They arise early during embryogenesis at the base of the allantois (in humans in week 5–6; Witschi, 1948; Mc et al., 1953; Falin, 1969); in mice at day E7.5 (Ginsburg et al., 1990; Donovan, 1998). From here, they migrate through the embryo via the hindgut, where they subsequently exit dorsally and move laterally toward both genital ridges, where the gonads will develop (Molyneaux et al., 2001; Molyneaux et al., 2003; Molyneaux and Wylie, 2004). Once the PGCs have reached the gonadal ridges, they are called gonocytes. During these stages, the embryonic germ cells are characterized by several markers, including alkaline phosphatase, c-KIT, OCT3/4, BLIMP1, VASA, and NANOG. These are related to various biological mechanisms, such as migration (c-KIT), survival (c-KIT and OCT3/4), and

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suppression of differentiation (BLIMP1).

Depending on the micro-environment, particularly related to the chromosomal constitution, that is, XX in females and XY in males, the gonocytes will differentiate into either oogonia or prespermatogonia. In the case of female development, the oogonia will divide multiple times before they become oocytes, stop proliferating, and enter meiosis, which starts at 11 to 12 weeks of gestation (Rabinovici and Jaffe, 1990). In males, the gonocytes will gradually migrate toward the periphery of the tubules. When they are in contact with the lamina basalis, they are called prespermatogonia. During the first years after birth and until puberty, the prespermatogonia change to type A spermatogonia (Muller and Skakkebaek, 1983; Chemes, 2001; Berensztein et al., 2002). At the onset of puberty, the spermatogonia undergo further maturation and go into meiosis to produce spermatozoa.

MALIGNANT GERM CELL DEVELOPMENT

An alternative classification system for human germ cell tumors (GCTs), compared to the traditional histological subtypes as recognized by the pathologist, has recently been developed and is recognized by the WHO, (Table 1) (Woodward et al., 2004; Oosterhuis and Looijenga, 2005). The distinction into five entities is based on various parameters, including cell of origin, histology, genomic imprinting status, age at and location of clinical presentation, and chromosomal constitution. Recognition of these subtypes allows a better investigation on identification of the pathogenetic mechanisms involved, and will lead to improvement of clinical diagnosis and treatment response prediction. Moreover, it will shed light on the value of existing in vivo and in vitro model systems, and eventually generate the optimal test model, both for pathobiological and clinical studies.

Type I GCTs are the teratomas and yolk-sac tumors of neonates and infants that can present in the testis, ovary, sacral or retroperitoneal region, or other sites in the midline of the body. The type II GCTs are the seminomatous and nonseminomatous GCTs, the main focus of this review (see below). The type III GCTs are the spermatocytic seminomas that are typically seen in males more than 50 years of age (Woodward et al., 2004). The type IV GCTs are the dermoid cysts that arises from oogonia or oocytes in the ovary, whereas the type V is the hydatiform mole that develops from an empty egg and a spermatozoa.

Interestingly, the stage-specific properties of germ cells are reflected in their neoplastic derivatives. This is for example nicely illustrated for the (classic) seminomas (type II TGCT) and spermatocytic seminomas (type III TGCT) (for review see Oosterhuis and Looijenga, 2005).

TYPE II GCTS

Type II GCTs are interesting from different points of view, because they can be considered true stem cell tumors, capable of differentiating into somatic, extra-embryonal, and even the germ cell lineage itself (Honecker et al., 2006). In addition, these tumors are unique because of their overall responsiveness to DNA damaging therapy, although depending on the histological subtype (Masters and Koberle, 2003). In fact, they show a remarkably high survival rate, even in cases of metastases (see below).

The seminomatous and nonseminomatous GCTs are the most frequent malignancies in Caucasian adolescents and adults aged 20 to 40 years (Adami et al., 1994), of which more than 95% develop in the testis. The incidence of type II TGCTs, currently 6 to 11 per 100,000, has doubled in the last 40 years without a clear explanation (Giwerzman et al., 1993; Swerdlow et al., 1998; Richiardi et al., 2004; McGlynn et al., 2005; Shah et al., 2007), although various hy-

pothesis have been generated as will be discussed later.

Seminoma consists of transformed germ cells that closely resemble the PGCs/gonocytes. Apparently, these tumor cells are blocked in their normal differentiation pattern and cannot undergo normal spermatogenesis, but accumulate instead. Nonseminomas, in contrast, can be composed of different histological elements, which mirror the pluripotency of the PGC/gonocyte, normally only apparent after fertilization. Embryonal carcinomas represent the undifferentiated stem cell component, teratomas represent the somatic differentiation, yolk-sac tumors, and choriocarcinoma are the components showing extra-embryonal differentiation. In addition to the extra-embryonal and somatic differentiation, germ cell lineage differentiation in nonseminomas has been reported (Fig. 1) (Honecker et al., 2006). This demonstrates that all lines, including the germ lineage, can be formed, indicating that type II TGCTs are indeed the only known totipotent solid cancer. Often multiple of these histologies are present in a single tumor, called a mixed tumor. Even seminoma- and nonseminoma components can be intermixed in about 10% of the cases. In addition, seminomas can switch to a nonseminomatous phenotype, a phenomenon called reprogramming or activation of pluri(toti)potency (Looijenga et al., 1999; Oosterhuis et al., 2003).

Several model systems are used to study TGCTs. Type I TGCTs of the neonates and infants are reflected in the testicular tumors of the 129SV mouse strain (Stevens and Little, 1954; Stevens, 1970; Damjanov et al., 1971; Walt et al., 1993). For type II TGCTs, no animal model has been reported, but recently, the TCam-2 cell line was confirmed to have significant similarities to seminoma (Mizuno et al., 1993; Goddard et al., 2007; de Jong et al., 2008a; Eckert et al., 2008b). Cell lines that model the nonseminomas have already been known for a much longer time (Fogh, 1978; Wang et al., 1980;

TABLE 1. Classification of the Five Different Types of Germ-Cell Tumors (Oosterhuis and Looijenga, 2005). See Text for Further Details

Type	Anatomical site	Phenotype	Age	Originating cell	Genomic imprinting	Genotype	Animal model
I	Testis/ovary/ sacral region/ retroperito- neum/ mediastinum/ neck/midline brain/other rare sites	(Immature) teratoma/yolk-sac tumor	Neonates and children	Early PGC/ gonocytes	Biparental, partially erased	Diploid (teratoma). Aneuploid (yolk-sac tumor): gain of 1q, 12(p13) and 20q, and loss of 1p,4 and 6q	Mouse teratoma
II	Testis	Seminoma/ nonseminoma	>15 years (median age 35 and 25 years)	PGC/gonocyte	Erased	Aneuploid (+/- triploid): gain of X, 7, 8, 12p, and 21; loss of Y, 1p, 11, 13 and 18 Aneuploid	-
	Ovary	Dysgerminoma/ nonseminoma	>4 years	PGC/gonocyte	Erased		-
	Dysgenetic gonad	Dysgerminoma/ nonseminoma	Congenital	PGC/gonocyte	Erased	Diploid/tetraploid	-
	Anterior mediastinum (thymus)	Seminoma/ nonseminoma	Adolescents	PGC/gonocyte	Erased	Diploid/tri- tetraploid	-
	Midline brain (pineal gland/ hypothalamus)	Germinoma/ nonseminoma	Children (median age 13 years)	PGC/gonocyte	Erased	Diploid/tri- tetraploid	-
III	Testis	Spermatocytic seminoma	Children/adults	Spermatogonium/ spermatocyte	Partially complete paternal	Aneuploid: gain of 9	Canine seminoma
IV	Ovary	Dermoid cyst	> 50 years	Oogonia/oocyte	Partially complete maternal	(Near) diploid, diploid/tetraploid, peritriploid (gain of X, 7, 12, and 15)	Mouse gynogenote
V	Placenta/uterus	Hydatiform mole	Fertile period	Empty ovum/ spermatozoa	Completely paternal	Diploid (XX and XY)	Mouse androgenote

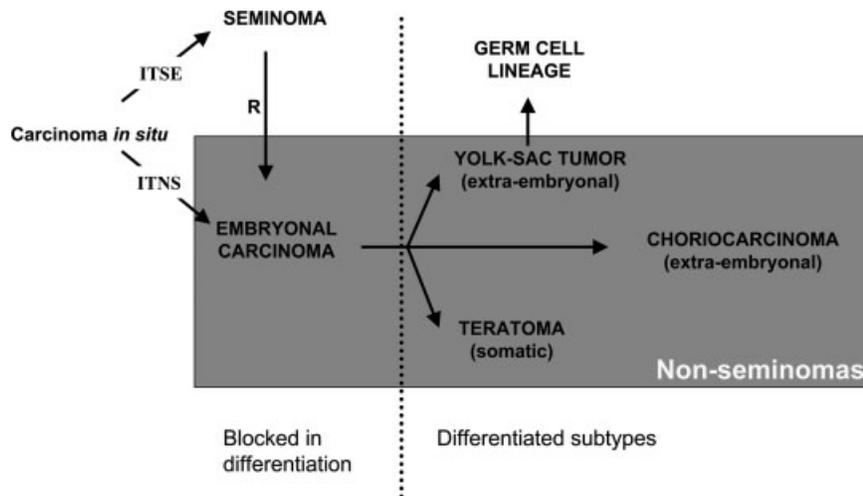


Figure 1. Overview of the different histologies from the type II TGCTs. From the precursor lesion carcinoma in situ, either a seminoma or a nonseminoma can develop via the intermediate stages of intrabulbar seminoma (ITSE) or intratubular nonseminoma (ITNS). The different histologies of nonseminomas are displayed in the gray area. Seminomas can also develop into a nonseminoma via so-called "reprogramming," indicated by R. Cells with germ cell characteristics that have been reported within the yolk-sac component of tumors.

Andrews et al., 1984; Teshima et al., 1988). Type III TGCTs, the spermatocytic seminomas, are reported to resemble canine testicular tumors (Looijenga et al., 1994).

THE PRECURSOR LESION OF TYPE II TGCTs

Both testicular seminomas and nonseminomas arise from a preinvasive lesion called intratubular germ cell neoplasia undifferentiated or carcinoma in situ (CIS) (Skakkebaek, 1972; Woodward et al., 2004). It is striking to note that the incidence of CIS and type II TGCT is similar, indicating that all cases of CIS eventually may progress to invasiveness (Giwerzman et al., 1991a). CIS cells resemble PGCs/gonocytes in many aspects: both have erased genomic imprinting (van Gurp et al., 1994), similar morphology (Rajpert-De Meyts et al., 2003) and express the same immunohistochemical markers, such as OCT3/4, PLAP, AP-2 γ , and c-KIT (Rajpert-De Meyts and Skakkebaek, 1994; Looijenga et al., 2003b; Hoei-Hansen et al., 2004; Honecker et al., 2004; Hoei-Hansen et al., 2005; Pauls et al., 2005;

Stoop et al., 2005). CIS cells are therefore assumed to be the malignant counterpart of PGCs/gonocytes. This is in line with epidemiological observations, indicating that the cells of origin of type II TGCTs are present in a defined period during embryonal development. CIS cells are most likely the result of a delayed or blocked differentiation of embryonic germ cells. CIS typically presents as a seminiferous tubule lacking the characteristic multiple-layer content of differentiating spermatogonia, spermatocytes, spermatids (Fig. 2A, B). Instead, basally located germ cells are seen mostly, under the tight junction of Sertoli cells, in contact with each other (Skakkebaek, 1972) (Fig. 2C, D). The intermediate lesion between CIS and an invasive TGCT is intratubular seminoma or intratubular nonseminoma, being predominantly embryonal carcinoma (Oosterhuis et al., 2003; Berney et al., 2004; Lau et al., 2007). In this process, CIS cells have become independent from the micro-environment (generated among others by the Sertoli cells) and fill up the lumen of the seminiferous tubule (Fig. 2E, F; indicated by arrows). This stage is but one step away

from invasiveness, when the cells spread out of the seminiferous tubule and form a seminoma (Fig. 2E, F; bottom half) or embryonal carcinoma (Fig. 2G, H).

NEWLY REPORTED MARKERS FOR TGCTs

A significant number of markers has been reported over time that can be used to discriminate CIS, seminoma, and embryonal carcinoma. The most common are OCT3/4, c-KIT, PLAP, NANOG, SOX2, AP-2 γ , and UTF1, among others (see Table 2). Several of these will be discussed in more detail here.

OCT3/4 is a well-characterized marker for PGCs. It is positive in all cases of CIS, seminoma, and embryonal carcinoma (Looijenga et al., 2003b) (Fig. 2D, F, and H), confirmed in many subsequent studies (reviewed in de Jong et al., 2005). There has been a significant amount of reports over the years that OCT3/4 is also expressed in normal adult (stem) cells and non germ cell-derived cancers. However, recent data indicate that these observations are likely related to the use of nonspecific antibodies and primers, the latter also recognizing pseudogenes (Ledford, 2007; Lengner et al., 2007; Liedtke et al., 2007; Atlasi et al., 2008). OCT3/4 is a transcription factor of the family of octamer-binding proteins (also known as the POU homeodomain proteins) and is regarded as one of the key regulators of pluripotency, (for review see Niwa et al., 2000; de Jong and Looijenga, 2006). In addition to OCT3/4, several other embryonic stem-cell-specific proteins are important for maintaining the so-called "stemness" of pluripotent cells, such as NANOG and SOX2 (Tanaka et al., 2002; Avilion et al., 2003; Adjaye et al., 2005; Boiani and Scholer, 2005; Boyer et al., 2005; Rajaraman et al., 2005; Yamaguchi et al., 2005; Yates and Chambers, 2005; Player et al., 2006; Babaie et al., 2007). SOX2 is a member of the SOX protein family, transcription factors that regulate development from

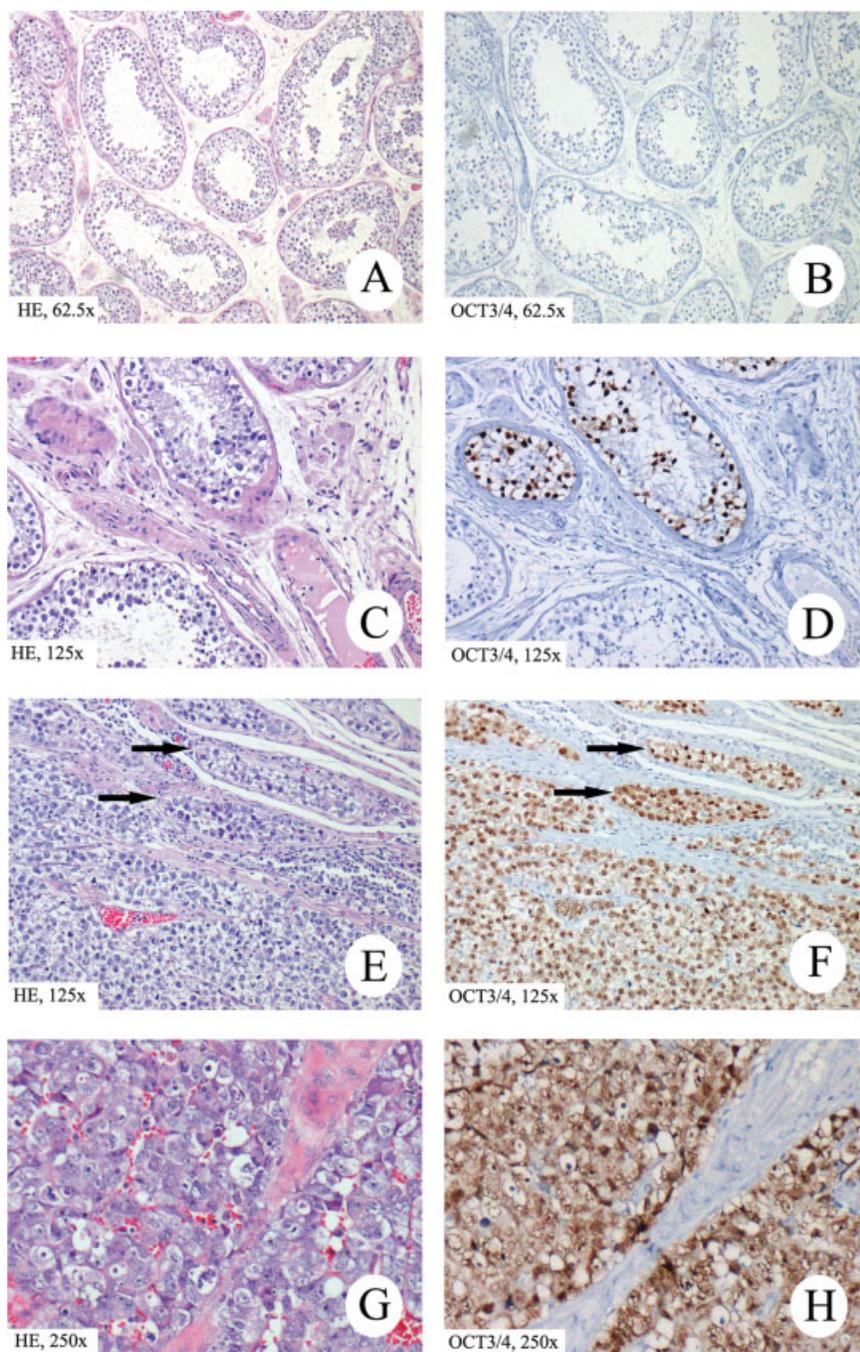


Figure 2. Representative examples of HE (A, C, E, and G) and OCT3/4 immunohistochemical stainings (B, D, F, and H) of normal and (pre) malignant testis tissue. (A) and (B): testis with normal spermatogenesis; (C) and (D): CIS; (E) and (F): intratubular seminoma (arrows) and seminoma (bottom half of pictures); (G) and (H): embryonal carcinoma.

the early embryonal stage to differentiated lineages of specialized cells. SOX proteins are known to cooperate with POU proteins. The best characterized SOX-POU cooperation is that between SOX2 and OCT3/4. SOX2 is not detected in

human germ cells regardless of their developmental age, in contrast to data in mouse embryos (de Jong et al., 2008b; Perrett et al., 2008). SOX2 is expressed in embryonal carcinoma, the undifferentiated part of nonseminomas,

but it is absent in seminomas, yolk-sac tumors, and normal spermatogenesis (de Jong et al., 2008b; Perrett et al., 2008). CIS cells are indeed negative for SOX2, although SOX2 positive Sertoli cells can be present in seminiferous tubules lacking germ cells or in the presence of CIS (de Jong et al., 2008b). Because CIS, seminoma, and embryonal carcinoma are all positive for OCT3/4, the question arises if any other SOX family members are present in CIS and seminoma to cooperate with OCT3/4. In this context, the different role of OCT3/4 in PGCs and embryonal stem cells is of interest, being suppression of apoptosis and regulation of differentiation, respectively (Nichols et al., 1998; Kehler et al., 2004). One study did not identify any redundant SOX protein in human germ cells within the group B of the SOX gene family (comprising SOX1, SOX2, SOX3, SOX14, and SOX21) (Perrett et al., 2008). Expression analysis of SOX family members in TGCTs revealed that SOX17 (a member of group F of the SOX gene family) is specifically expressed in CIS and seminoma but not in embryonal carcinoma (de Jong et al., 2008b). In addition, SOX17 maps to the chromosomal region 8p23, which is gained in seminoma (Korkola et al., 2008). This indicates that SOX17 is a candidate SOX protein for cooperation with OCT3/4 in CIS and seminoma. These data also illustrate that SOX17 is a new marker to discriminate CIS and seminoma from embryonal carcinoma. Of interest is that SOX17 distinguishes embryonic from adult hematopoietic stem cells (Kim et al., 2007). Current research focuses on the processes that may regulate the differential expression of SOX2 versus SOX17 and on the role of these SOX proteins in the different histologies of the TGCT subtypes involved. This can be relevant in the context of regulation of apoptosis and differentiation as well as sensitivity to either irradiation and/or chemotherapy.

Kristensen et al. (2008) studied the expression of UTF-1 and REX-1 in testes and type II TGCTs. Both

TABLE 2. Known and New Immunohistochemical Markers of Potential Diagnostic Value to Discriminate Seminomatous Histologies, Embryonal Carcinoma, and Spermatocytic Seminoma. See Text for Additional Information

Marker	NT	CIS	ITSE	SE	EC	SS	References
OCT3/4	-	+	+	+	+	-	(de Jong et al., 2005; Looijenga et al., 2003b)
c-KIT	+ ^a	+	+	+	-/+	-	(Rajpert-De Meyts and Skakkebaek, 1994; Strohmeyer et al., 1995)
SCF	+ ^a	+	+	+	-/+	-	(Bokemeyer et al., 1996; Stoop et al., 2008)
PLAP	-	+	+	+	+	N.D.	(Burke and Mostofi, 1988; Slowikowska-Hilczner et al., 2001)
NANOG	-	+	+	+	+	-	(Hart et al., 2005)
AP2-γ	-	+	+	+	+focal	-	(Hoei-Hansen et al., 2004; Pauls et al., 2005)
MZA	-	+	+	+	+focal	N.D.	(Franke et al., 2004; Marks et al., 1999)
SOX2	- (occasional Sertoli cells: +)	-	-	-	+	-	(de Jong et al., 2008b; Perrett et al., 2008)
SOX17	-	+	+	+	-	-	(de Jong et al., 2008b)
REX-1	+	+	+	+	+	+	(Kristensen et al., 2008)
UTF-1	+	+	+	+	+	+	(Kristensen et al., 2008)
BOB1	+	+	+	+	N.D.	N.D.	(Gashaw et al., 2007)
PROM1	+	+	+	+	N.D.	N.D.	(Gashaw et al., 2007)
MCFD2	- (Sertoli cells: +)	+	+	+	(nearly all)	N.D.	(Gashaw et al., 2007)

^aOnly detectable on frozen tissue, not on formalin fixed paraffin embedded material.

NT, normal adult testis; ITSE, intratubular seminoma; SE, seminoma; EC, embryonal carcinoma; SS, spermatocytic seminoma; N.D., not determined.

are transcription factors expressed in mouse embryonic carcinoma cells, regulated by OCT3/4 (Hosler et al., 1989; Okuda et al., 1998), and expressed by spermatogonia in prepubertal and adult testes. UTF-1 and REX-1 are expressed in CIS, seminomas, and nonseminomas. UTF-1 is present in all, and REX-1 is expressed in most of the type III spermatocytic seminomas that were tested, demonstrating that these markers are also expressed in the more differentiated spermatocytes. Despite the fact that these proteins are associated with pluripotency, their expression pattern differs significantly from that of OCT3/4 and SOX2, the well-characterized pluripotency proteins, which are negative in type III TGCT.

Analysis of expression patterns in microarray studies revealed additional markers, MCFD2, BOB1, and PROM1, for seminoma compared to normal testis (Gashaw et al., 2005). Studies demonstrated indeed increased expression levels of these three proteins in seminoma cells compared to normal adult testes (Gashaw et al., 2007). Because all three of these markers are also expressed at low levels in normal adult testicular tissue, their suitability as practical additional diagnostic markers remains to be proven.

SCF-c-KIT SIGNALING IN TGCT

The SCF c-KIT signaling pathway is important for proper germ cell development. This was first illustrated by mice carrying naturally occurring mutations in the *Steel* (*Sl*) and *White-spotting* (*W*) loci that involved the ligand-receptor pair SCF and c-KIT, respectively (reviewed in Loveland and Schlatt, 1997). In addition to defects in the hematopoietic system and melanogenesis, the number of PGCs decreases to about 2% of the normal level by day E12.5 in *Sl/Sl* mice and *W/W* mice. By day E14, PGCs are no longer detectable in *Sl/Sl* mice (Mintz and Russell, 1957; McCoshen and McCallion, 1975). There are differences in

expression patterns between mice and humans, but SCF and c-KIT also play an important role in human spermatogenesis, demonstrated by defects in this pathway (reviewed in Mauduit et al., 1999). c-KIT expression, similar to the markers PLAP, OCT3/4, and Ki67, disappears after birth (Honecker et al., 2004). In normal adult spermatogenesis, c-KIT is downregulated when the PGCs/gonocytes mature toward spermatogonia A, and it is not detected in the various stages of normal spermatogenesis (Rajpert-De Meyts et al., 1996). c-KIT expression can only be detected in adult testis when a sensitive detection method is used on frozen tissue (Natali et al., 1992; Rajpert-De Meyts and Skakkebaek, 1994; Strohmeyer et al., 1995; Bokemeyer et al., 1996; Stoop et al., 2008). In adult testis, SCF is expressed by Sertoli cells. The membrane bound form of SCF is the most efficient in establishing and maintaining germ cells (Dolci et al., 1991; Godin et al., 1991; Matsui et al., 1991; Marziali et al., 1993), while the soluble form of SCF activates c-KIT on Leydig cells to induce testosterone production (Yan et al., 2000). CIS and seminoma express c-KIT on their membrane as demonstrated for PGCs. Upon reprogramming of CIS to seminoma or nonseminoma, tumor cells predominantly lose c-KIT expression (Rajpert-De Meyts and Skakkebaek, 1994; Strohmeyer et al., 1995; Biermann et al., 2007). Recently, we reported that SCF immunohistochemistry is a valuable additional marker to discriminate precursor lesions of TGCTs from cells that are delayed in their maturation. This is an important distinction to make, especially in patients with germ cell maturation delay, which often show prolonged expression of PGC markers (Rajpert-De Meyts et al., 1998; Cools et al., 2005; Cools et al., 2006b). Because in these specific cases, detection of OCT3/4 was not informative per se, the use of SCF as an additional marker for malignancy is a valuable diagnostic addition (Stoop et al., 2008).

Comparative genomic hybridization experiments revealed specific amplification of region 4q12, centered around c-KIT in TGCTs (McIntyre et al., 2005a). However, in 21% of the seminomas, only the copy number of c-KIT is increased in this region and in most cases not the flanking genes *KDR* and *PDGFRA*. This indicates that c-KIT can be specifically involved in TGCT etiology. This is also supported by the clinical observation that Gleevec in a single chemotherapy-resistant seminoma resulted in complete response (Pedersini et al., 2007).

An association of a single nucleotide polymorphism (SNP) in c-KIT with low sperm counts in idiopathic male infertility was reported; however, the links to TGCT development is still unclear (Galan et al., 2006). As is the case in other malignancies such as mastocytosis, mutations in c-KIT have been reported in TGCTs. Tian et al. (1999) were the first to report codon 816 mutations in a seminoma and in a mixed ovarian dysgerminoma/yolk-sac tumor (Tian et al., 1999). Exon 17 of c-KIT, which harbors codon 816, encodes for the kinase domain of the receptor. Mutations in this area and in particular codon 816 mutations lead to constitutive activation of c-KIT and can be a transforming event in oncogenesis (Furitsu et al., 1993; Moriyama et al., 1996; Boissan et al., 2000). In follow up on the report by Tian et al. (1999), c-KIT exon 17 mutations have also been reported by others in TGCTs (Sakuma et al., 2003; Kemmer et al., 2004; Nakai et al., 2005), in mediastinal seminomas (Przygodzki et al., 2002), and in dysgerminomas (Pauls et al., 2004; Hoei-Hansen et al., 2007b). Our experiments revealed that codon 816 mutations were predominantly found in patients with bilateral TGCTs (93%), whereas they were only detected in 1.3% of patients with unilateral disease (Looijenga et al., 2003a). The mutations were only found in CIS or in invasive tumor of patients and never in adjacent normal testicular parenchyma or blood, indicating

that these are very early somatic mutations. We hypothesize that these mutations occur early in embryogenesis, before migration of the PGCs to the genital ridges. We concluded that activating codon 816 mutations could predict the development of bilateral TGCTs. Bilateral TGCTs can present synchronously, but most cases present asynchronously and the latency period can be up to 20 years (Colls et al., 1996). Therefore, analyzing material from the first TGCT of a patient for c-KIT exon 17 mutations can be used for screening patients at risk for developing bilateral disease. The presence of codon 816 mutations in bilateral TGCTs was confirmed by independent reports, although the frequency at which the mutations were found varied (Tate et al., 2005; Biermann et al., 2007). In contrast, others did not detect an increased incidence of these mutations in patients with bilateral disease (Coffey et al., 2008; Sakuma et al., 2008). This discrepancy may be explained by the different techniques that were used to detect the mutations. In the original report on the predictive value of codon, 816 mutations for bilateral disease, restriction endonuclease-mediated selective PCR, and single nucleotide primer extension were used, techniques likely to be more sensitive than regular PCR combined with sequencing (Looijenga et al., 2003a). It is also important to note that the c-KIT mutations were not always found in the invasive tumor but were present in CIS adjacent to the tumor, indicating that dependency on c-KIT may become superfluous when the CIS has progressed to invasiveness (see above). This is in line with reports that c-KIT expression is often downregulated upon progression from CIS to seminoma (Willmore-Payne et al., 2006; Biermann et al., 2007). Therefore, it is important to use sensitive detection techniques and preferably use tumor material containing CIS for mutation analysis. c-KIT mutations were not associated with an increased risk of TGCTs in familial cases, but again, a higher propor-

tion of mutations was seen in patients with bilateral disease (Rapley et al., 2004). The authors suggest that bilateral disease in the context of familial TGCT has a different pathogenesis than the sporadic bilateral cases and other somatic and susceptibility genes also play important roles.

In addition to the data on the c-KIT mutations, it is interesting to speculate whether any other mutated kinases could play a role in TGCT as well. A large-scale collaboration aimed at sequencing the protein kinase family for mutations in TGCTs revealed a remarkably low mutation frequency with only a single somatic point mutation in the *STK10* gene (Bignell et al., 2006). Therefore, there is no evidence implying any mutated protein kinase in the development of TGCTs, other than the previous reports on the role of c-KIT mutations.

EPIGENETICS OF TYPE II GCTS

Besides a wealth of information about the genomic make up of type II GCTs, increasing knowledge on the epigenetic constitution is also evolving (Peltomäki, 1991; Koul et al., 2002; Smiraglia et al., 2002; Honorio et al., 2003; Zhang et al., 2005; Kawakami et al., 2006; Lind et al., 2006; Ishii et al., 2007; Lind et al., 2007; Okamoto and Kawakami, 2007). The role of epigenetics in normal germ cell development has recently been reviewed (Biermann and Steger, 2007). Targeted as well as genome wide studies demonstrate that the seminomas show an overall hypomethylated DNA status, in contrast to the various histological types of nonseminomas. Interestingly, the supernumerical X chromosomes are inactivated in nonseminomas by methylation (Looijenga et al., 1997). This is, similar to normal embryogenesis, the result of the non(protein)-coding *XIST* gene. This unique phenomenon in males is correlated with hypomethylation of the promoter region, which can be used as molecular target for type II GCTs in males (Kawakami

et al., 2003; Kawakami et al., 2004). The difference in methylation status can be demonstrated using expression profiling for the different forms of the DNA methyltransferases (DNMT) (Looijenga, 2008). DNMT1 is required for maintenance of the methylated status during cell division, and has previously been found to be present in differentiated forms of non-seminomas (Omisano et al., 2006), while DNMT3A and DNMT3B are needed for de novo methylation (Karpf and Matsui, 2005), as happens during early embryogenesis. DNMT3L has a role in the establishment of the pattern of genomic imprinting (Oakes et al., 2007). Overall, a specific upregulation is observed in the embryonal carcinomas compared to the seminomas. Indeed, this is also reflected by immunohistochemistry using a ¹⁴C-specific antibody (Looijenga, 2008; Netto et al., 2008). Interestingly, a methylation study of the promoter region of OCT3/4 showed that in seminoma and embryonal carcinoma, the promoter region is predominantly hypomethylated, both in in vitro cell lines as well as in in vivo tumors (de Jong et al., 2007). Microdissection of embryonal carcinoma cells demonstrated complete demethylation. On differentiation of the embryonal carcinoma cells, OCT3/4 is downregulated in expression, associated with hypermethylation of the promoter region. This pattern most likely reflects the situation in most genes related to pluripotency, showing the same pattern of expression as OCT3/4, like NANOG.

Histone modification has also been identified as a significant regulatory element specifying which genes will be hypermethylated on differentiation from an undifferentiated stem cell. This is related to the histone, H3, methylated at Lysine27 (H3K27) by polycomb proteins, which is a repressive mark, as well as the active mark, methylated H3K4 (Ohm et al., 2007). Interestingly, this was indeed found to be the case in cell lines derived from type II GCTs, that is, embryonal carcinoma, in which two additional repressive marks are

identified, dimethylated H3K9 and trimethylated H3K9, both associated with DNA hypermethylation in adult cancers. This fits nicely with the observed pattern of expression of the histone de-acetylase (HDAC) in these tumors (Omisano et al., 2006). More recently, a related study investigated the expression of BLIMP-1 and PRMT-5. Expression of these factors in type II GCTs mimicks the situation of normal development (Eckert et al., 2008a). These proteins are involved in the suppression of the somatic differentiation program in PGCs/gonocytes, related to dimethylated histone H2A and H4 (Ancelin et al., 2006). Knock out of these genes results in differentiation of mouse PGCs (Ohinata et al., 2005; Hayashi et al., 2007). Indeed, these proteins and epigenetic changes are present in embryonic germ cells, as well as CIS and seminoma, including the representative cell line TCam-2. As expected, upon formation of embryonal carcinoma, these proteins are downregulated, and the dimethylated H2A and H4 are removed. Again, these studies demonstrated the close relationship between normal embryogenesis and type II GCTs. It remains a challenge to identify which of the mechanisms are reflecting normal development, and which are related to the pathogenetic process.

THE ROLE OF MICRO-RNAS IN TGCT

Over the last years, there has been an increased interest in the role of miRNAs in development and in tumorigenesis. One of the first indications that miRNAs are also important for germ cell development came from observations of Bernstein et al. (2003), who demonstrated that knockout mice for Dicer, a protein required for the generation of mature miRNAs, also lack PGCs. In addition to this, conditional knockout mice for Dicer1 in the male germ line were infertile. The animals suffered from an early decrease in germ cell number and an impaired ability to differentiate, indicating that Dicer1 and miRNAs are important for both survival and

proper differentiation of male germ cells (Maatouk et al., 2008). A genetic screen for novel functions for miRNAs revealed that miRNAs 372 and 373 can overcome cell cycle arrest mediated by WT TP53 (Voorhoeve et al., 2006). In addition, miRNAs 372 and 373 are expressed in TGCT with WT TP53 and in several TGCT cell lines with high levels of WT TP53. In contrast, TGCT cell lines with low expression of WT TP53 or with mutant TP53 were negative for these miRNAs. MiRNAs 372 and 373 can bypass the TP53 checkpoint and allow growth of type II TGCT in the presence of WT TP53. Studies with high-throughput screening for expression of miRNAs in type II TGCTs and TGCT-derived cell lines revealed that unsupervised clustering of miRNA expression patterns can discriminate TGCT cell lines, normal testis, the type III spermatocytic seminomas, and the differentiated versus the undifferentiated type II tumor subgroups (Gillis et al., 2007). These data support the model that miRNAs are involved in regulating differentiation of stem cells and germ cells.

Another interesting link on the importance of miRNAs for germ cells and GCTs came from research on the Dead end gene (DND1). Until recently, DND1 was known to regulate germ-cell viability and to suppress the formation of germ cell tumors. This was based on work in the 129-*Ter* mouse strain where a premature stop codon in DND1 was identified (Matin and Nadeau, 2005; Youngren et al., 2005). These mice are characterized by germ-cell loss and a high frequency of (type I) TGCTs. Studies aimed at identifying susceptibility genes additional to DND1^{Ter} in the 129 mouse strain showed that all variants tested (Kit^{SUJ}, Trp53, A^Y, and 129-Chr 19^{MOLF}) lead to increased germ cell tumor frequency and in particular of bilateral disease (Lam et al., 2007). Recently Kedde et al. (2007) demonstrated that DND1 counteracts miRNA-mediated destabilization of mRNAs by binding to mRNAs and prohibiting the association of miRNAs with their target sites. This underlines the important role of miR-

NAs and regulation of miRNA expression in germ cell development. Ongoing mechanistic studies to reveal "what leads from dead end" (Matin, 2007) showed that DND1 interacts with the multifunctional protein APOBEC3, which is expressed in testes and adult germ cells (Bhattacharya et al., 2008). Linger et al. (2008) focused on the role of DND1 in humans and analyzed the presence of DND1 mutations in 263 human TGCTs. In only one case was a heterozygous variant found, which was not seen in any of the additional 842 TGCT cases analyzed (Linger et al., 2008). In addition, when analyzing eighteen type I TGCT specifically, this variation was not found (Looijenga, 2008). Based on these observations and in contrast to its role in the type I TGCTs of the 129 background mice, the role of DND1 in humans remains unclear and may indeed appear to be a dead end. However, we are still at the beginning of understanding how miRNAs are involved in regulating many cellular processes relevant for TGCTs. As an illustration of the ongoing insight into the functional roles of miRNAs, Tay et al. (2008) recently demonstrated that, in mouse, in addition to targeting the 3'UTR of mRNAs, miRNAs against NANOG, OCT3/4, and SOX2 also target the coding region of these genes, which affects embryonic stem cell differentiation (Tay et al., 2008). Analysis of these miRNAs in human TGCTs (Gillis et al., 2007) did not reveal any differences in expression between the different subgroups (unpublished observations). This underlines that the evolutionary difference in miRNA expression between mice and humans is considerable (Plasterk, 2006). Further research into the functional mechanisms of miRNAs and the role of DND in TGCT are likely to give more interesting clues.

RISK FACTORS OF TYPE II GCTS

Risk Factors: Support of a Testicular Dysgenesis Syndrome (TDS)

The nature of the risk factors for TGCTs, which are familial predispo-

sition, a history of TGCT, cryptorchidism, infertility, and various forms of disorders of sex development (DSD), support the model that the initiating step in the pathogenesis of this cancer occurs during embryonal development (Moller, 1993; Skakkebaek et al., 1998; Jacobsen et al., 2000; Raman et al., 2005; Sonke et al., 2007; Cook et al., 2008). Various other risk factors have been suggested, which await confirmation, although birth weight (both low and high) seems to be relevant (Michos et al., 2007). Seminoma is found more frequently in intra-abdominal gonads than in scrotal gonads (Ogunbiyi et al., 1996). This also likely explains the preferential occurrence of the seminomatous type in the ovary (Tewari et al., 2000). In addition, an early age of orchiopexy indeed reduces the risk for a TGCT (Pettersson et al., 2007; Walsh et al., 2007). This is likely related to the still ongoing maturation of PGC/gonocyte-like cells to prespermatogonia (see above).

So far, it has not been possible to identify the gene or genes involved in familial TGCTs (Rapley et al., 2000; Holzik et al., 2004). Overall, the genetic predisposition is difficult to investigate because of the small sizes of the affected families, (likely linked to) the relationship to subfertility, as well as the possible role of the (micro)environment. Immigrants from Finland to Sweden, who have a lower initial risk for TGCTs, obtain the risk of the Swedish population in their second generation (Hemminki et al., 2002). This demonstrates a significant effect of the environment on the incidence in a limited period of time, which is possibly overruling a genetic component. However, there are strong indications for a genetic component (for review see Krausz and Looijenga, 2008). It is of interest that most identified risk factors (are expected to) one way or another negatively affect maturation of embryonic germ cells. This led to the hypothesis of TDS (Skakkebaek et al., 2001; Skakkebaek, 2003; Rajpert-De Meyts, 2006; Sonne et al., 2008). This

syndrome integrates various elements, in which the final outcome will have a negative effect on testicular function, including sub(in) fertility, cryptorchidism, and/or an increased risk for TGCTs. The role of the supportive element, that is, Leydig cells and Sertoli cells, is of crucial importance. Indeed, this has also been concluded based on an independent approach, focused predominantly on DSD patients with an increased risk of type II GCTs (Hersmus et al., 2008a; Looijenga, 2008). In this context, a possible role of estrogen and anti-androgen function needs specific attention (Looijenga, 2008; Krausz and Looijenga, 2008), for review.

Risk Factors: Disorders of Sex Development

DSD is a group of developmental anomalies, previously referred to as intersex, which is defined as a condition of incomplete or disordered genital or gonadal development, leading to a discordance between genetic sex (i.e., determined by the chromosomal constitution, of the X and Y chromosomes), gonadal sex (the testicular or ovarian development of the gonad), and phenotypic sex (the physical appearance of the individual). Recently, a revised classification system has been proposed, with the aim to reduce uncertainties on description (Hughes et al., 2006).

As mentioned, DSD patients with either hypovirilization or gonadal dysgenesis specifically show an increased risk for the development of type II GCTs (for review see Cools et al., 2006a; Looijenga et al., 2007). In dysgenetic gonads, the precursor lesion is called a gonadoblastoma. It is morphologically different from CIS, and precedes the development of invasive tumors (Scully, 1970; Robboy et al., 1982; Savage and Lowe, 1990). In DSD cases, the precursor can be either CIS or gonadoblastoma or a combination of both (Li et al., 2007b), related to the level of virilization of the gonad. This can be nicely demon-

strated by the use of immunohistochemistry for SOX9 (read-out of SRY function and Sertoli cell differentiation), and FOXL2 (granulosa cell differentiation) (Hersmus et al., 2008b). These results indicate that CIS and gonadoblastoma are part of a histological continuum, in which the development into either a Sertoli cell or a granulosa cell determines the histological context of the premalignant cells (Hersmus et al., 2008a), elegantly linking TDS and DSD.

Multiple findings support that at least a certain amount of testosterone is needed for the precursor lesion to progress to an invasive disease. Patients with complete androgen insensitivity have a significantly lower risk compared to patients with the partial form of this disorder (Cools et al., 2005; Cools et al., 2006a; Hannema et al., 2006). Most likely, this is related to the induction of apoptosis of germ cells in the testis of patients with complete androgen insensitivity, as observed in Klinefelter patients (Wikstrom et al., 2006). Moreover, complete absence or a very low level of testosterone also diminishes the risk of a type II GCT. This is nicely illustrated by patients with hypogonadotropic hypogonadism, which can present with cryptorchid testis, but are not reported to develop TGCTs.

Parameters Related to Tumor Risk

In contrast to the link between ovarian differentiation and FOXL2 and testicular differentiation and SOX9 (see above), the correlation between the presence of the Y chromosome and testicular development is less obvious (Cools et al., 2007). In fact, no correlation between the Y chromosome and testis development has been identified in patients with sex chromosomal mosaicisms, for which no explanation is available so far.

The anatomical position of the gonad also seems to be significantly related to the risk of malignant transformation. This is in line with the fact that cryptorchidism is indeed one of the strongest risk

factors for type II TGCTs (Batata et al., 1980; Muller et al., 1984; Giwercman et al., 1987; Abratt et al., 1992).

The risk of development of type II GCTs in DSD patients is directly related to the presence of a specific part of the Y chromosome, known as the gonadoblastoma region of the Y chromosome (GBY) (Page, 1987). This area maps around the centromeric region, and excludes the SRY gene as candidate. This is supported by the clinical observation that patients with a translocation of the SRY gene to an X chromosome or an autosome, which results in 46XX males, have no increased risk for this type of cancer. Several candidate genes map within the GBY region, of which TSPY is one of the most interesting ones. It stands for testis-specific protein on the Y chromosome and is a multicopy gene (Vogel and Schmidtke, 1998). It has similarities to the DEK/CAN family of proteins, and it interacts with cyclin B1, thereby supposed to be involved in cell cycle regulation. Various splice variants have been reported, which indeed can be present in type II TGCTs. The corresponding protein is present in spermatogonia during normal development. The level of protein is increased in CIS and gonadoblastoma, for which the mechanistic basis is still unknown (Schnieders et al., 1996; Hildenbrand et al., 1999; Lau, 1999; Delbridge et al., 2004; Kersemaekers et al., 2005; Li et al., 2007b). The fact that type II TGCTs are consistently aneuploid might be related to this. The increased level of TSPY protein is used as a supportive parameter to distinguish a malignant germ cell from a germ cell with delayed maturation. Upon invasive growth, although the Y chromosome can still be retained, expression of the gene and, subsequently, the protein, is mostly lost. Therefore, the loss of expression is due to downregulation. Transfection experiments demonstrated that induction of TSPY in human cells lacking this protein results in an increase in proliferation, both in vitro and in vivo. In fact, the cells show a

shorter G2 phase of the cell cycle (Oram et al., 2006). Interestingly, a subsequent study shows that many of the upregulated genes in the TSPY transfected cells map to the short arms of chromosome 12. A correlation between the level of TSPY and expression of these genes, including KRAS2 and NANOG, was only found in the precursor lesion CIS, and not in invasive tumors (Li et al., 2007a). This observation nicely fits with the downregulation of TSPY upon progression of the tumor toward invasiveness.

Mice lack TSPY. Transgenic animals containing a complete structural human TSPY gene interestingly show integration in the Y chromosome, in a tandem repeat organization, like the organization in the human genome (Schubert et al., 2003). This is intriguing but unexplained so far. However, no GCTs were identified, not at younger or older age. In other words, the simple overexpression of TSPY in OCT3/4 positive cells is not enough to generate a type II GCT in the mouse.

NONINVASIVE AND INVASIVE DIAGNOSIS OF CIS: SEMEN ANALYSIS, MICROLITHIASIS, AND TESTICULAR BIOPSIES

Given the similar incidence of CIS and type II TGCT, all cases of CIS are expected to progress to invasiveness (Giwercman et al., 1991a). CIS can be cured with low-dose irradiation. This results in infertility in the affected testis, but hormonal function is preserved in the majority of cases (Giwercman et al., 1991b). In contrast, development of an invasive TGCT requires (hemi-) castration and depending on the stage and type of TGCT, irradiation or chemotherapy. Therefore, early diagnosis and treatment of CIS can prevent development of TGCT, consecutive treatment, and therapy-related serious diseases such as secondary malignancies or cardiovascular diseases (van den Belt-Dusebout et al., 2007). As CIS is asymptomatic

in most cases, it is usually not detected before development of invasiveness. Currently, the only way to diagnose CIS is by looking at the morphology and expression of markers in a testicular biopsy, taken by a surgically invasive procedure (Schmoll et al., 2004). Because of the low incidence of TGCT and the invasiveness of the procedure, with possible side effects, a testicular biopsy in the general population is not the preferred screening tool.

Over the years, there have been several reports on noninvasive methods to detect CIS in semen samples. Detection of CIS cells in semen has been reported first based on cytology (Czaplicki et al., 1987; Howard et al., 1989). Giwercman et al. (1988a, 1988b, 1988c) demonstrated CIS-like cells in semen by using immunohistochemistry for the marker protein, M2A, or by looking for aneuploid cells in semen. The use of placental-like alkaline phosphatase in semen was reported not to be specific due to cross-reacting germ cell alkaline phosphatase (Brackenbury et al., 1993). Unfortunately, none of these studies resulted in an assay suitable for clinical screening purposes, mainly due to frequent false positive or false negative findings. Recently, there have been new attempts on this issue using immunohistochemical detection of (some relative new) TGCT markers. Høei-Hansen et al. (2007a) tested the suitability of immunohistochemical detection of several fetal germ cell-specific markers (AP2 γ and OCT3/4) in the semen of 503 men including 294 infertile patients and 209 patients with TGCT or other diseases. AP-2 γ positive cells were detected in 50% of the cases with CIS, with similar results for OCT3/4. Notably, a young subfertile man who was included as a control subject was detected with CIS by the AP-2 γ -based method. The authors conclude that immunohistochemical detection of CIS cells in semen has additional diagnostic value, but a negative result does not exclude the presence of a TGCT (Høei-Hansen et al., 2007a). van Casteren

et al. (2008b) looked at the presence of OCT3/4 positive cells in the semen of 41 men judged to be at risk for CIS based on a testicular ultrasound and 15 controls. OCT3/4 positive cells were detectable by immunohistochemistry in semen in the majority of CIS patients tested. Follow-up studies including larger patient cohorts are crucial, especially because the number of CIS-only cases (which would be the target patient population for this screening tool) tested so far is very low (1 and 3 cases in the reports by Hoei-Hansen and van Casteren, respectively). However, these reports indicate in principle a possibility to use this technique to screen for CIS specifically in patients at risk for TGCT. The non-invasive screening could be of use in selecting patients for surgical biopsies, thereby reducing unnecessary surgeries. This will especially be of interest in populations with an increased risk of development of a TGCT, such as infertility, bilateral microlithiasis, and a previous unilateral tumor.

Within the subgroup of sub(in)fertility, bilateral microlithiasis has been identified as a characteristic of males with a high risk (up to 20%) for CIS (De Gouveia Brazao et al., 2004). This is in accordance with the high incidence of microcalcifications in patients with a unilateral TGCT, and contralateral CIS (Holm et al., 2003). This finding can be of value for screening purposes. A proposal on how to deal with testicular microlithiasis in a Urological/Andrological Department is reported elsewhere (van Casteren et al., in press).

When a unilateral TGCT is removed, it is standard practice in Germany and Denmark to take a biopsy from the contra lateral testis to screen for the presence of CIS. This is however not the case in most other countries. Studies by Dieckmann et al. (2007a, 2007b) reported significant discordant findings between double biopsies taken from contralateral testes in TGCT patients in 5.4% of the cases when spermatogenesis was evaluated and in 31.1% of the cases when scored for the presence of

CIS. Discordance was significantly associated with poor spermatogenesis and atrophy when spermatogenesis was the endpoint and discordance was predominantly seen in normal-sized testicles of fertile individuals when screening for CIS. It was therefore suggested to take two-site biopsies for fertility evaluation and CIS diagnosis. Double contralateral biopsies in selected patients with large testes, irregular ultrasonography results, or microlithiasis may yield extra information. Before applying double contralateral biopsies to all TGCT patients, additional evaluation is warranted with respect to the more invasive procedure and the risk of inducing additional damage (Hoei-Hansen et al., 2007c).

Careful evaluation is mandatory when a testicular biopsy is taken. van Casteren et al. (2008a) and de Jong et al. studied material from 20 TGCT patients who, before development of the tumor, had undergone testicular biopsy where no malignancy was found. The biopsies were reviewed blind by an experienced pathologist using morphology (HE staining) and by immunohistochemistry for c-KIT, PLAP, and OCT3/4. The use of immunohistochemistry over morphology alone allowed identification of four additional cases of CIS, an addition of 20%. This illustrates how crucial the proper choice of immunohistochemical markers is when reviewing a testicular biopsy for the presence of CIS.

TREATMENT SENSITIVITY AND RESISTANCE

It is proposed that the origin of type II GCTs also explains their overall sensitivity to DNA damaging agents (i.e., irradiation and cisplatin-based chemotherapy) (Hong and Stambrook, 2004). This is supported by the fact that it is influenced by the histological composition of the tumor: loss of embryonic features results in induction of treatment resistance (Masters and Koberle, 2003). Selective eradication of embryonic components leaves treatment resistant residual teratoma (Oosterhuis, 1983). In

fact, because of exquisite sensitivity to cisplatin-based chemotherapy, up to 80% of patients with metastatic disease can be cured by a combination of chemotherapy and tumor resection in case of residual disease (Einhorn, 2002). However, even with modern treatment, type II GCTs are a deadly disease in a minority of cases (approximately 5% of all patients), and the biology underlying treatment failure is poorly understood (Mayer et al., 2003). Various findings point toward a key role for DNA damage response in the exceptional cure rates in GCTs achieved by DNA damaging therapy (Bartkova et al., 2007). The role of mismatch repair (MMR) deficiency and microsatellite instability (MSI) has previously been investigated, yet results have been controversial (Mayer et al., 2002; Olsz et al., 2005; Velasco et al., 2008). Patients with tumors showing MSI had a higher rate of relapse (Velasco et al., 2004), and cancer specific death was associated with MSI and absent or low expression of hMLH1 (Velasco et al., 2008). However, the incidence of MSI in the subgroup of patients with refractory disease was not reported in this analysis. We demonstrated a significantly higher incidence of MSI in a small series of 11 patients with refractory disease, compared to 100 unselected cases of GCT, used as a control group (Mayer et al., 2002). In sporadic colorectal cancer, MSI is strongly associated with an activating *BRAF* mutation, leading to a V600E substitution (Yuen et al., 2002; Deng et al., 2004). In one study including 62 stage I TGCTs, *BRAF* mutation V600E was found in three of 32 nonseminomas (9%) (Sommerer et al., 2005), although this was not correlated with clinical data. Other studies could not demonstrate mutated *BRAF* in GCT tumor samples or cell lines (Davies et al., 2002; McIntyre et al., 2005b). Extending our earlier analysis, we investigated a series of 35 clinically well documented resistant GCTs and the same control group of 100 unselected GCTs to clarify the role of MMR deficiency, MSI,

and *BRAF* mutation status in treatment resistance. The resistant tumors had more MSI affecting two or more loci than controls. There was a significantly higher incidence of *BRAF* V600E mutation in resistant tumors compared to controls. *BRAF* mutations highly correlated with MSI, and MSI and mutated *BRAF* correlated with weak or absent immunohistochemical staining for hMLH1. Low levels or absence of hMLH1 correlated with promoter hypermethylation. The percentage of lack of either hMLH1 or MSH6 was significantly higher in the resistant GCTs compared to controls. This is the first observation of a correlation between a gene mutation, *BRAF* V600E, and cisplatin resistance in type II GCTs (Honecker et al., in press). These results hold promises for the future use of targeted therapy in type II GCTs, as recently reported for a single metastatic seminoma (Pedersini et al., 2007). Multi-kinase inhibitors targeting *BRAF*, like sorafenib, are clinically well established (Hiles and Kolesar, 2008). In addition to improving treatment, these findings should encourage steps toward screening and monitoring of treatment in type II GCTs in the future.

CONCLUSIONS AND FUTURE PERSPECTIVES

TGCTs are a fascinating group of malignancies for several reasons. As discussed in this review, they can show differentiation in all lineages: somatic, extra-embryonal, and even the germ cell lineage. This indicates that these tumors represent the true stem cell population of the human body. The "stemness" of the cells making up the TGCTs is the reason for their second unique characteristic, their exquisite sensitivity to treatment. The less differentiated the tumor cells are, the better they will respond to DNA damaging therapy, as illustrated by the better responses of seminomas versus nonseminomas, and by the frequent recurrence of mature teratomas in residual treatment-resistant tumors. For proper diagnosis of

the different histological subgroups, immunohistochemistry is required using a panel of suitable markers, including OCT3/4, SOX2, and SOX17. Recent developments such as the discovery of the role of miRNAs in oncogenesis also revealed highly interesting features of TGCTs. Specific miRNAs were shown to be involved in bypassing the WT p53 pathway, which is another characteristic of TGCTs. Further research into the role of miRNAs is likely to give more useful insights in the biology of TGCTs, as well as that of stem cells. The fact that the incidence of TGCTs has been increasing over the last decades and that the remarkable differences in incidence between (adjacent) countries, for which no explanations are yet reported, calls for further studies. More insight into the pathogenesis of TGCTs is likely to contribute not only to better treatment of these tumors but also to a better understanding of stem cells and oncogenesis in general.

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