Risk Factors and Genetical Characterization

2

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Abbreviations

AFP	Alpha feta protein
CTA	Cancer testis antigens
CIS	Carcinoma in situ
CAIS	Complete androgen insensitivity
c- and a-CGH	Chromosomal- as well as array-comparative genomic hybridization
DSD	Disorders of sex development
GBY	Gonadoblastoma region of the Y chromosome
hCG	Human chorionic gonadotropin
GCTs	Human germ cell tumors
HDAC	Histone deacetylase
ISH	In situ hybridization (ISH)
IGCNU	Intratubular germ cell neoplasia unclassified
LDH	Lactate dehydrogenase
MSI	Microsatellite instability
miRNA	MicroRNA
PAIS	Partial androgen insensitivity
PGC	Primordial germ cell
SCF	Stem cell factor
SNP	Single nucleotide polymorphism
SS	Spermatocytic seminomas
TDS	Testicular dysgenesis syndrome
TIN	Testicular intratubular neoplasia
UGT	Undifferentiated gonadal tissue
XIST	X inactive specific transcript

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2.1 Introduction

The testis is a highly specialized male specific organ with in principle two main functions: generation of germ cells by a process called spermatogenesis, and formation of hormones crucial for normal male phenotypic development as well as initiation and maintenance of spermatogenesis (Grootegoed et al. 2000; Loveland et al. 2005). The final goal of the germ cells is transmitting genetic information to the next generation (Donovan 1998; McLaren 2001). Therefore, they have to be able to become pluripotent, i.e. capable of forming all differentiation lineages, both embryonal and extraembryonal upon fertilization (Cinalli et al. 2008). This requires a unique mechanism involving proliferation and maturation of germ cells as well as a germ cell-specific manner of division known as meiosis (Hunt and Hassold 2002). This results finally in generation of a haploid DNA content in highly specialized cells, called spermatozoa, able to penetrate the zona pellucida of the mature egg. The proper formation of these cells requires a delicate temporal and spatial process during embryogenesis resulting in testis formation (Wilhelm et al. 2007), as well as during and after puberty, being dependent on the interaction of many cell types, which are organized within and around the seminiferous tubules, being the functional units wherein spermatogenesis occurs (Grootegoed et al. 2000). The cell of origin of the germ cell lineage is referred to as a primordial germ cell (PGC) (Donovan 1998; McLaren 1992, 2003 Wylie 1993; Kato et al. 1999). These cells originate outside the soma and migrate to the genital ridge. Within the genital ridge they are referred to as gonocytes (to be discussed below). This system of gonadal development and gametogenesis can be disturbed in various ways, both

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during early development as well in adult life. In principle, every cell type present within the testis can undergo malignant transformation, and result in cancer, like Sertoli cell tumors, Leydig cell tumors, lymphoma's, sarcomas, etc (Woodward et al. 2004). These types of cancer will not be discussed here. This chapter will be restricted to the various human germ cell tumors (GCTs), which can occur in the human testis. When relevant, the GCTs occurring at other anatomical localizations will be referred to.

2.2 General Concept and Perspectives

The last few years, a wealth of information has become available on solid cancers, including human GCTs. This boost is due to the availability of various techniques able to generate high throughput data on (epi) genetics as well as expression profiling (both protein-encoding and noncoding genes, including microRNAs (miRNAs)). These data sets on their own are significant for the elucidation of the pathogenetic steps involved in the formation of the cancer under investigation. An integrated approach will provide an even higher level of understanding of the biology of the systems. When linked to patient characteristics, the data have been shown to be highly relevant for patient management (Swanton and Downward 2008). This approach has resulted in novel insights in the pathobiological pathways, new methods for diagnosis, prognosis, response prediction, and molecular therapies. This will benefit quality of life of the individual patient. In addition, it will allow generation of informative in vitro and in vivo models of disease. There is no doubt that patients already benefit from this endeavor in terms of increasing survival (Joensuu et al. 2001; Druker et al. 2001).

2.3 Human Germ Cell Tumors: Introduction

Human GCTs are different from other solid cancers of adults in a number of aspects, related to both biology and clinical behavior (Oosterhuis and Looijenga 2005). This is likely due to their embryonic origin, in spite of their clinical presentation in adult life as observed in most cases (to be discussed below). It is proposed that the origin of GCTs also explains their overall sensitivity to DNA damaging agents (i.e., irradiation and cisplatin-based chemotherapy) (Hong and Stambrook 2004), supported by the fact that this is influenced by the histological composition of the tumor: loss of embryonic features results in induction of treatment resistance (Masters and Koberle 2003). The recent findings on embryonic and adult stem cells in general, and cancer stem cells specifically (Zaehres and Scholer 2007; Rossant 2008; Morrison and Spradling 2008; Knoblich 2008; Jaenisch and Young 2008), are of relevance in the context of the origin and pathobiology of human GCTs (Pera 2008). The following paragraphs will focus on risk factors and genetical characterization of the various types of these tumors. Understanding the impact of these observations is also dependent on knowledge of the pathogenesis of these tumors, which requires information on normal gonadal and germ cell development. Therefore, these aspects will also be discussed where appropriate. In addition, if relevant, clinical data will be integrated in the discussion.

2.4 Classification of Human GCTs

Traditionally GCTs are classified on the basis of their histological appearance, as judged by the pathologist (Scully 1978; Mostofi and Sesterhenn 1985; Mostofi et al. 1987; Donohue 1990). Although, without any restriction this approach is relevant and informative, it underestimates the biological diversity of this type of cancer, as discussed extensively elsewhere (Oosterhuis and Looijenga 2005; Reuter 2005). More specifically, taking a different view on this seemingly heterogeneous group of cancers will likely identify novel patterns, making the pathogenesis of these cancers easier to understand, both from a developmental as well as clinical point of view. For this specific purpose, an alternative classification system was proposed in 2005, in which site of presentation of the primary tumor, age of the patient at diagnosis, histological composition, and chromosomal constitution are informative parameters. On the basis of these criteria, five categories (I–V) of GCTs are identified (Oosterhuis and Looijenga

Table 2.1 Summary of the most differentiating parameters for the type I, II, and Ill germ cell tumors

Parameters:	Type I	Type II	Type III
Histology	Teratoma/yolk sac tumor	(Non)Seminoma	Sperm. seminoma
Age	Neonates/infants	Adolescents/young adults	Elderly
Cell of origin	Embryonic germ cell	Prim. germ cell/gonocyte	Prim. spermatocyte
Genomic imprinting	Partial erased	Erased	Partial paternal
Genotype	Diploid/gain 1,12p(13),20q, Loss 1p,4,6q	Aneuploid, gain X,7,8,12p,21 Loss 1p,11,13,18	Aneuploid, gain 9
Risk factors	Unknown	Multiple related to delay germ cell maturation	Unknown
Animal model	Mouse teratocarcinomas	Unknown	Canine seminoma

2005). This has already proven to allow a more straightforward understanding of their origin, histological diversity, as well as clinical behavior. Because of the fact that within the testis predominantly the type I, II, and III GCTs are diagnosed, they will form the topic of this chapter. On the basis of the incidence as well as pathobiological and clinical aspects, emphasis will be on the type II GCTs. The different characteristics relevant to identify the major groups of GCTs of the testis, i.e., type I, II, and III, are summarized in Table 2.1. A more detailed discussion on the other types of GCTs has been made elsewhere (Oosterhuis and Looijenga 2003, 2005; Looijenga and Oosterhuis 1999; Looijenga et al. 1999).

2.5 Origin of GCTs of the Testis

To understand the nature of risk factors for the development of human GCTs, especially those of the testis, it is of relevance to have insight into normal gonadal development and the origin of GCTs. The morphological characteristics and expression profiles (see below) of the type II and III GCTs support their germ cell lineage origin (Sperger et al. 2003; Kraggerud et al. 2002; Skotheim et al. 2002; Looijenga et al. 2003a, 2006; Korkola et al. 2005; Hofer et al. 2005; Biermann et al. 2007a, b; Gashaw et al. 2007). However, this is not directly obvious for the type I GCTs, i.e., they show no characteristics mimicking germ cells in any stage of development. In this context, investigation of their pattern of genomic imprinting, defined as the germ cell-specific functional difference between a haploid set of chromosomes depending on the parental origin, is informative (Surani et al. 1990; Tycko 1994; Surani 1994). The partial erasement of the pattern of genomic imprinting supports the view that the majority of the type I GCTs are also of germ cell origin (Sievers et al. 2005a). Therefore indeed, the type I, II, and III GCTs can all be considered as GCTs truly.

The origin and migration of embryonic germ cells from the yolk sac region (proximal epiblast) to the genital ridge (Hayashi et al. 2007), provide an interesting explanation as to why the type I and II GCTs can also be found outside the gonads, i.e., along the midline of the body. In this context, the current knowledge on suppression of the somatic differentiation pathways during formation and migration of embryonic germ cells is highly relevant (see below). Still, the specific localization of GCTs in the brain is unknown on the basis of this assumption (Scotting 2006; Oosterhuis et al. 2007). However, studies on genomic anomalies support the view that they are indeed GCTs (De Bruin et al. 1994; Motzer et al. 1991; Palmer et al. 2007). Expression profiling of mRNA shows that the intracranial GCTs have a similar pattern of gene expression as those of the gonads, both testis and ovary (Looijenga et al. 2006) and Hersmus et al., submitted for publication. The question remains to be answered whether the germ cells at the extragonadal localizations have a specific function during embryogenesis and possibly later, and whether the final cancer is the result of lack of physiological apoptosis or differentiation later in life. Alternatively, the tumors can be the results of initial aberrant migration and unphysiological survival. The recent observations regarding relevant factors in the migration of PGCs, like SDF1 and its receptors CXCR4

and 7 are relevant in this context (Knaut and Schier 2008; Boldajipour et al. 2008). Although these issues are interesting, they will not be discussed here, because of the focus on GCTs of the testis. In the following two paragraphs, the type I and type III GCTs will be discussed in more detail, with emphasis on identified risk factors and genetic anomalies, including mRNA, miRNA, and protein findings. The remaining final part of the chapter will be dedicated to the type II GCTs.

2.5.1 Type I GCTs

2.5.1.1 Epidemiology and Histological Composition

The type I GCTs of the adult testis are rare (Schneider et al. 2004), and predominantly found in neonates and infants, although exceptions do occur (see below). A higher incidence in industrialized countries has been suggested, without an ethnic preference. Independent of the anatomical localization (see Table 2.1), all proven type I GCTs are composed of teratoma and/or yolk sac tumor. The teratoma can contain both immature and mature elements, possibly mixed, of all differentiation lineages, i.e., endoderm, mesoderm, and ectoderm. Overall, these tumors are clinically benign (Huddart et al. 2003). If however, other histological components are found, like seminoma, embryonal carcinoma, or choriocarcinoma, it is by definition a type II GCT (see below). Visa versa, if a tumor is composed of only a teratoma or a yolk sac tumor, or a mixture of both, diagnosed in a dysgenetic testis (see below) or in a testis after puberty, it must be demonstrated that it is not a variant of a type II GCT. This can be done on the basis of the identification of the precursor lesion or the presence of specific chromosomal anomalies (see below).

2.5.1.2 Cell of Origin

No obvious precursor cell for the type I GCTs based on morphological or immunohistochemical characteristics is identified so far. However, on analysis of mouse models, as well as determination of the pattern of genomic imprinting (see above), the cell of origin is found to be a germ cell in the majority of cases (Walt et al. 1993). A somatic origin of the (limited number of) human type I GCTs with a biparental pattern of genomic imprinting so far cannot be excluded. In general, the type I GCTs show a partial pattern of erasement, reflecting the origin of an early embryonic germ cell (Sievers et al. 2005a). Experimental data on migrating (fluorescently labeled) PGCs in Bax-deficient mice, which are therefore apoptosis disturbed, indicate that a specific subpopulation of PGCs migrate along a different route ending in the sacral region, instead of in the genital ridge (Runyan et al. 2008). This sacrococcygeal region is indeed another predominant anatomical site where type I GCTs can be found. Interestingly, this specific PGC population is larger in a female mouse compared to that in a male mouse, possibly reflecting the preferential occurrence of these tumors in baby girls compared to baby boys (Schneider et al. 2004). Although this is an interesting observation, elucidation of the cell of origin and the pathogenetic pathways involved in human type I GCTs still requires much effort. The Wnt pathway has been proposed to be involved, but mainly upon specific differentiation lineages within the tumor, and not in the initiation of the tumor itself (Fritsch et al. 2006). This is of interest because of the significant role of Wnt in stem cell biology (Walsh and Andrews 2003; Constantinescu 2003; Suda and Arai 2008) (see also below).

2.5.1.3 Risk Factors and Genetic Changes

No risk factors for type I GCTs have been identified so far (Malogolowkin et al. 1990); this supports an independent origin and pathogenesis from the type II GCTs (see below). A slowly increasing incidence has been noted. Interesting is the observation that teratomas are frequently observed in mice in which the function of a specific gene is disrupted in the germ cell lineage, including kras2, pten, and dnd (Looijenga et al. 2007a). So far, no indications are available that one of these genes is involved in the pathogenesis of the human type I GCTs. Dnd is of specific interest, because of its role in the function of miRNAs (see below). In the mouse, absence of this gene results in a disturbed germ cell development, resulting in infertility as well as bilateral teratomas (Youngren et al. 2005). No studies have been published on the association of a type I GCT and fertility. A rare DNA variant within the DND gene has been identified in a single type II GCT (see below) (Linger et al. 2008). Using an additional series of

18 proven type I GCTs, either teratomas or yolk sac tumors, this specific variant was not found (Looijenga, unpublished observations), and it is therefore unlikely to be a relevant pathogenetic factor. Moreover, a number of inbred mouse strains show development of testicular teratomas, which is to a certain level dependent of the genetic background used. The high tendency to form of teratomas from the germ cell lineage in these strains has been explained assuming that it reflects a rescue mechanism preventing transmission of the affected gene to the next generation (Looijenga et al. 2007a, b). The direct switch from a germ cell to a somatic cell (the stem cell of the teratoma) will generate a relatively benign tumor. Although of interest, no experimental data are available to support it yet, but the heterogeneity in function of genes of which disruption results in the phenomenon is intriguing. The heterogeneity of genes leading to mouse teratoma formation is of interest in the context of the required suppression of the somatic differentiation program in PGCs (to be discussed below). It suggests that this can be disturbed in many different ways, offering a model to study this so-called process of activation to pluripotency, also referred to as reprogramming (Silva and Smith 2008; Surani et al. 2007). This step is also of relevance in the context of type II GCTs, in which reprogramming occurs in about 50% of the tumors during the progression from the precursor lesion to the invasive cancer (see below). However, mice do not show development of type II GCTs, with possibly a single, and highly relevant, exception (to be discussed below).

Because of the lack of proven cell lines derived from type I GCTs, the data on chromosomal constitution are obtained from primary in vivo tumors, which need verification that sufficient numbers of tumor cells are included in the sample under investigation. With this possible restriction, the overall picture is consistent and as follows: no chromosomal changes are identified in teratomas, not even after microdissection, while recurrent genomic imbalances are present in the type I yolk sac tumors (Perlman et al. 1994, 1996; Mostert et al. 2000; Schneider et al. 2001, 2002; Veltman et al. 2003, 2005). These data have been obtained using conventional karyotyping, and more recently also using chromosomal- as well as array-comparative genomic hybridization (c- and a-CGH), as well as (fluorescent) in situ hybridization (ISH). The data from the different approaches are in accordance to each other. The overall pattern is summarized in Table 2.1. On the basis of genetic characteristics, it has been demonstrated that indeed the yolk sac tumor component originates from the teratoma component. This is in line with the observation that upon extensive transplantation it is also observed in mouse embryo-derived teratomas, considered as the animal model for human type I GCTs (Walt et al. 1993; Van Berlo et al. 1990a, b). Of interest is that mouse embryonic stem cells lacking functional Sox2, a regulator of pluripotency (see below), give rise to (polyploid) trophoblastic cells (i.e., reflecting extra-embryonic differentiation) (Li et al. 2007a), in which Cdx2 is a regulatory element (Deb et al. 2006). Indeed, subtle changes in the level of Sox2 regulate differentiation of embryonic stem cells (Boer et al. 2007; Kopp et al. 2008) (see also below). The aneuploidy of the human type I yolk sac tumors also parallels the observation that if mouse embryonic stem cells are tetraploidized, they form trophoblast. This serves as a rescue mechanism to allow embryonic development in gene-disrupted embryonic stem cells which lack the capacity to generate the yolk sac, which is crucial for further development. These data suggest that the processes involved in the progression from teratoma to yolk sac, both in mouse and human tumor cells, might be solely determined by evolutionary retained mechanisms, which are still operational in the type I GCT cells. The intriguing consistency of polyploidy remains unexplained (Otto 2007).

An older age of the patient, beyond the neonatal and infantile period, at clinical diagnosis does not exclude the diagnosis of a type I GCT. This is exemplified by the two Caucasian female patients of respectively 14 and 37 years of age (unpublished observations). They presented with an ovarian tumor histologically composed of pure yolk sac tumor. Because of the rareness of a pure yolk sac tumor at this age, and the knowledge that they are much more frequent at younger age, a-CGH was performed on both tumors, demonstrating the type I characteristic chromosomal imbalances, including loss of 1p, and 4 and 6q, and gain of 1q, 12p(13), and 20q (see Fig. 2.1a). The type II specific chromosomal imbalance (see below), i.e., gain of the short arm of chromosome 12, is absent (see Fig. 2.1b). The second tumor suggests the presence of a teratomatous component based on smooth muscle tissue, but it could not be confirmed. Although of interest from a pathobiological point of view, distinction between

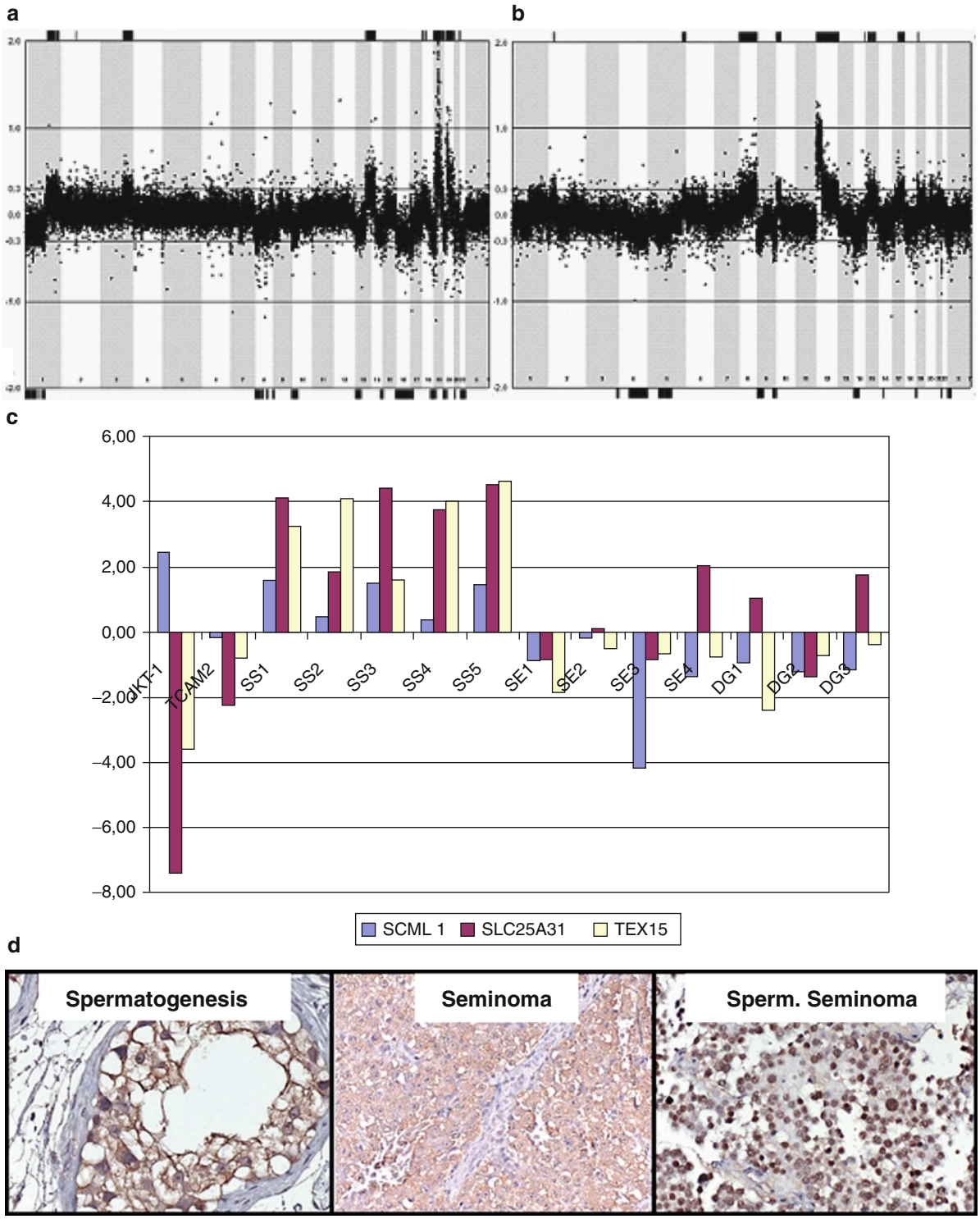


Fig. 2.1 Example of array-comparative genomic hybridization using DNA of (**a**) a yolk sac tumor of the ovary of a phenotypically normal female patient of 37 years of age. Note the presence of specific chrosomosomal imbalances, but the absence of gain of the short arm of chromosome 12; (**b**) a representative type II testicular GCT, showing the recurrent chromosomal changes, including gain of 12p; (**c**) Expression data based on Affymetrix profiling for SCML1, SLC25A31 (also known as ANT4), and TEX4. Note the specific expression in spermatocytic seminoma (SS) compared to that in seminoma (SE) and dysgerminoma (DG). The seminoma cell line TCam-2 and the nonrelated JKT-1 are included for comparison; (**d**) Represenntative examples of immunohistochemical detection of SCML1 on normal spermatogenesis (*left panel*: positive), seminoma (middle panel: negative), and SS (*right panel*: positive)

a type I and type II yolk sac tumors has no clinical implication as yet.

2.5.1.4 Concluding Points Type I GCTs

No identified risk factors
Histologically composed of either teratoma and/or yolk sac tumor
Predominantly diagnosed in neonates and infants
Early embryonic germ cell is cell of origin
Teratomas show no chromosomal anomalies
Yolk sac tumors show loss of 1p, and 4 and 6q and gain of 1q, 12p(13), and 20q
No representative cell lines available
Various representative animal models identified (i.p. mouse teratocarcinomas)

2.5.2 Type III GCTs

2.5.2.1 Epidemiology

Type III GCTs, also known as spermatocytic seminomas (SS), are rare, and preferentially found in elderly males (Muller et al. 1987; Burke and Mostofi 1993). Although they hardly metastasize, up to 30% of the patients will develop bilateral disease (Bergner et al. 1980). Although type II GCTs are significantly less frequently diagnosed in blacks, there seems to be a skewed incidence of SS. This supports the independent origin of both tumor entities (see below). In contrast to the type I and II GCTs, the SS have no counterpart in the ovary or other anatomical localizations. In other words, this tumor is specifically associated with the occurrence of spermatogenesis, which is not the case for the other GCTs, although this has been proposed otherwise (see below). In fact, the type I and II tumors are related to the presence, i.e., retention, of embryonic germ cells, with their specific characteristics (see above and below).

2.5.2.2 Histological Composition

SS have been considered as a variant of seminoma. However, the morphology and histology are in the majority of cases significantly different (Romanenko and Persidskii 1983; Dekker et al. 1992; Cummings et al. 1994; Chung et al. 2004; Talerman 1984). The less experienced pathologists may be misled by the variation of the histological appearance of SS. Generally, SS are characteristically composed of three cell types, with respectively a small, intermediate, and large nucleus, associated with a diploid, tetraploid, and hypertetraploid DNA content. No convincing haploid tumor cells have been identified so far (Looijenga et al. 2007b; Oosterhuis et al. 1989a; Kysela and Matoska 1991; Takahashi 1993). In addition, they usually lack infiltrating lymphocytes, which are characteristic for (classic) seminoma (see below). The precursor lesion is known as intratubular SS, being an accumulation of tumor cells in the luminal space of the seminiferous tubules, suggesting that the tumor cells only proliferate in the luminal compartment of the seminiferous tubule, beyond the tight junctions between the Sertoli cells (Looijenga et al. 2007a, b). This in contrast to the cell of origin of the type II GCTs (see below).

2.5.2.3 Cell of Origin and Markers for Diagnosis

On the basis of the various sizes of the nuclei of the SS tumor cells, it has been hypothesized that these cells undergo meiosis, generating cells with a different DNA content. This was further substantiated using immunohistochemistry for three markers, XPA, SCP1, and SSX2-4, which were indeed able to distinguish SS from (classical) seminoma (Stoop et al. 2001). Subsequently, other markers have been added on the basis of targeted analysis, including P53, CHK2, p16INK4D, and MAGE-4A (Rajpert-De Meyts et al. 2003a), indeed markers of later stages of germ cell development. The pattern of genomic imprinting of SS is highly specific for germ cell developing along the male lineage of spermatogenesis, i.e., it shows a more paternal pattern of genomic imprinting (Sievers et al. 2005a). High throughput mRNA expression profiling shows that these tumors indeed express multiple genes related to spermatogenesis, including cancer testis antigens (CTA), of which MAGE-4A is an example (Looijenga et al. 2006). This study also demonstrated that SS shows expression of genes specific for the prophase of meiosis I, i.e., TCFL5, CLGN, and LDHc. Unpublished results indicate that a number of other genes show a specific pattern of expression in SS compared to other GCTs, including (classic) seminomas. These include SCMH1 (Takada et al. 2007), SLC25A31 (ANT4) (Brower et al. 2007), and TEX15 (Yang et al. 2008) (see Fig. 2.1c). These markers are related

to different processes, like germ cell maturation, including regulation of gene expression and meiotic recombination. That the mRNA studies are informative is proven previously (Looijenga et al. 2006). It is here also demonstrated for the specific presence of SCML1 protein in SS and not in seminoma (see Fig. 2.1d, middle vs. right panel; for comparison normal testis is indicated in the left panel). Other markers are less discriminating between SS and seminoma, like PLZF and TAF4B (Dadoune 2007), which are related to spermatogenic stem cell maintenance and renewal (data not shown). Therefore, the summed data suggest that the cell of origin of SS is a later germ cell, most likely a primary spermatocyte. This is, however, difficult to reconcile with the occurrence of bilateral disease in about one-third of the cases (Burke and Mostofi 1993; Bergner et al. 1980; Eble 1994). The explanation could be that the first hit in the pathogenesis of SS in fact occurs in a migrating germ cell before it enters the genital ridge. The affected germ cell is, in spite of the initial hit, able to develop along the male germ cell lineage and the block in maturation becomes only obvious when meiosis is initiated. This hypothesis could be tested experimentally in the various spontaneous and induced animal tumors, like those in *Caenorhabditis elegans* (Subramaniam and Seydoux 2003) and the dog (Looijenga and Oosterhuis 2007; Looijenga et al. 1994). So far, no representative cell lines of SS are available.

2.5.2.4 Risk Factors and Genetic Changes

No risk factors for SS are identified yet, although as mentioned, the diagnosis of SS indicates directly that the patient has a significant increase in risk to develop a bilateral cancer. Because SS does not metastasize, orchidectomy is sufficient for cure. It will result in complete castration in some cases because of bilateral disease. The rare progression of SS towards, highly malignant, sarcomatous elements has, however, to be kept in mind (Floyd et al. 1988; True et al. 1988; Matoska and Talerman 1990). The monoclonal origin of the SS and sarcoma element has not been proven so far, for which the identified recurrent chromosomal imbalances might be informative.

Conventional karyotyping, supported by c- and a-CGH revealed that SS have a characteristic pattern of chromosomal anomalies (Looijenga et al. 2006; Oosterhuis et al. 1989a; Kysela and Matoska 1991; Takahashi 1993; Rosenberg et al. 1998; Maiolino et al. 2004; Verdorfer et al. 2004; McIntyre et al. 2007). Overall they lack translocations, duplication, and deletions, but are characterized by additional copies of chromosome 9 (see Table 2.1). Integrated analysis of both chromosomal anomalies and expression profiling demonstrated that DMRT1 is a likely candidate gene/protein to explain the gain of chromosome 9 (Looijenga et al. 2006). Although its mechanistic basis remains to be elucidated, it can be used as an informative diagnostic marker. Interestingly this protein is also found in the testicular seminomas of dogs (Looijenga et al. 2007b), one of the supposed animal models for human SS, and it is recommended to indeed reclassify these canine tumors as SS.

Recent data on expression profiling of miRNA classify SS in the group of more differentiated samples, including normal testis and teratomas (Gillis et al. 2007). Again, this supports the relatively mature stage of differentiation of the tumor cells.

On the basis of these observations, it remains to be decided whether SS are indeed a cancer, or rather a benign tumor. The unsatisfactory explanation of the high incidence of bilaterality of these tumors, often synchronously, prompts another speculation. It is conceivable that these neoplasms originate as a hyperplasia, which is common in hormonally regulated endocrine organs. This thought is supported by the fact that the canine seminomas, in fact SS, are very often multifocal and mixed with gonadal stromal tumors.

2.5.2.5 Concluding Points Type III GCTs

No identified risk factors

Histologically composed of small, intermediate, and large germ cells

Predominantly diagnosed in elderly, solely in the testis

Primary spermatocyte likely cell of origin

Gain of chromosome 9 is a recurrent anomaly

DMRT1 is a likely 9p-candidate gene

No representative cell lines available

Various representative animal models identified (*C. elegans* and dog)

Bilateral disease might be explained by early initial genetic change or hyperplasia

2.6 Type II GCTs: Introduction

Type II GCTs are the most frequent GCT of the testis, accounting for approximately 1% of all cancers in Caucasian males (Verhoeven et al. 2007; Shah et al. 2007). In contrast to most solid human cancers, type II GCTs have a peak incidence at the adolescent and young adult age, in which group they represent in fact the most frequent solid cancer. The age of presentation can be significantly younger in patients with disorders of sex development (DSD (see below)). In spite of the overall cure rate, they are the second cause of death in young adult Caucasian males (Horwich et al. 2006). In most European countries a significant rise in incidence of these cancers has been reported, although an interesting heterogeneity has been observed (Dieckmann and Pichlmeier 2004; Walsh et al. 2006). This has been linked to both genetic predisposition as well as exposure to environmental compounds, specifically those with estrogen and/or antiandrogen action (see below). A significant lower incidence of type II GCTs of the testis has been reported for other ethnic populations, including Asians and Blacks, which is not influenced by migration (Gajendran et al. 2005; McGlynn et al. 2005).

2.6.1 Histological Composition and Markers of Differentiation

The type II GCTs are subdivided into seminomas and nonseminomas, both histologically and clinically (Woodward et al. 2004; International Germ Cell Cancer Collaborative 1997). The nonseminomas are further subclassified into embryonal carcinoma, yolk sac tumor, choriocarcinoma, and teratoma (Woodward et al. 2004). In fact, all differentiation lineages as found during normal embryogenesis (both somatic and extra-embryonal) can be represented in these tumors, including the germ cell lineage (Honecker et al. 2006), making these tumors really totipotent. This is in line with the various markers suitable for diagnosis (see below). It must be kept in mind that teratomas and yolk sac tumors can therefore be both part of a type I and type II GCTs, which cannot be distinguished on histological criteria. The markers useful to distinguish the seminoma from the embryonal carcinoma of type II GCTs are summarized in Fig. 2.2a. The list is not meant to include all putative informative markers, but to indicate the overall pattern. It sheds light on the pathobiology of these tumors in general, and identifies putative interesting targets for diagnosis and possibly targeted treatment. These markers have been identified on the basis of either a hypothesis-driven approach, or using high throughput investigations. The markers AFP (for yolk sac tumor), hCG (for choriocarcinoma), and LDH1 (for tumor load) are useful as serum markers in a clinical setting, specifically related to the presence of a yolk sac or choriocarcinoma component, and tumor load, respectively (Horwich et al. 2006). It is interesting to note that most markers suitable to distinguish seminoma and embryonal carcinoma from the more differentiated nonseminoma components, and to specify seminoma from embryonal carcinoma, are known from regulation of pluripotency in (mouse and human) embryonic stem cells, like OCT3/4, NANOG, and SOX2. These and a selection of others will be discussed in more detail, clustered on the basis of their pattern of expression:

2.6.1.1 OCT3/4 (POU5F1) and NANOG

OCT3/4, encoding the POU5F1 protein was the first regulator of pluripotency identified in mouse embryonic stem cells (Nichols et al. 1998). This transcription factor regulates whether the cells will remain undifferentiated or start to differentiate (Hansis et al. 2000; Niwa et al. 2000; Pesce and Scholer 2000, 2001; Donovan 2001). The expression is at least influenced by promoter methylation, both in vivo and in vitro (Hattori et al. 2004; De Jong et al. 2007a) (see also below). On the basis of this observation, the expression of mRNA of OCT3/4 in type II GCTs was initiated. Two specific variants of the protein-encoding OCT3/4 are recognized, of which the A (or I) type is related to pluripotency. The protein is located in the nucleus. The B (or II) variant is localized in the cytoplasm. It is not related to regulation of pluripotency, and will therefore not be discussed here. Detection of OCT3/4 mRNA is not only hampered by the existence of two variants but also by the presence of a number of pseudogenes. This may result in false positive RT-PCR observations (Takeda et al. 1992; Suo et al. 2005; Liedtke et al. 2007; De Jong and Looijenga 2006). A combined approach using PCR amplification of mRNA (after DNAse treatment) and endonuclease digestion

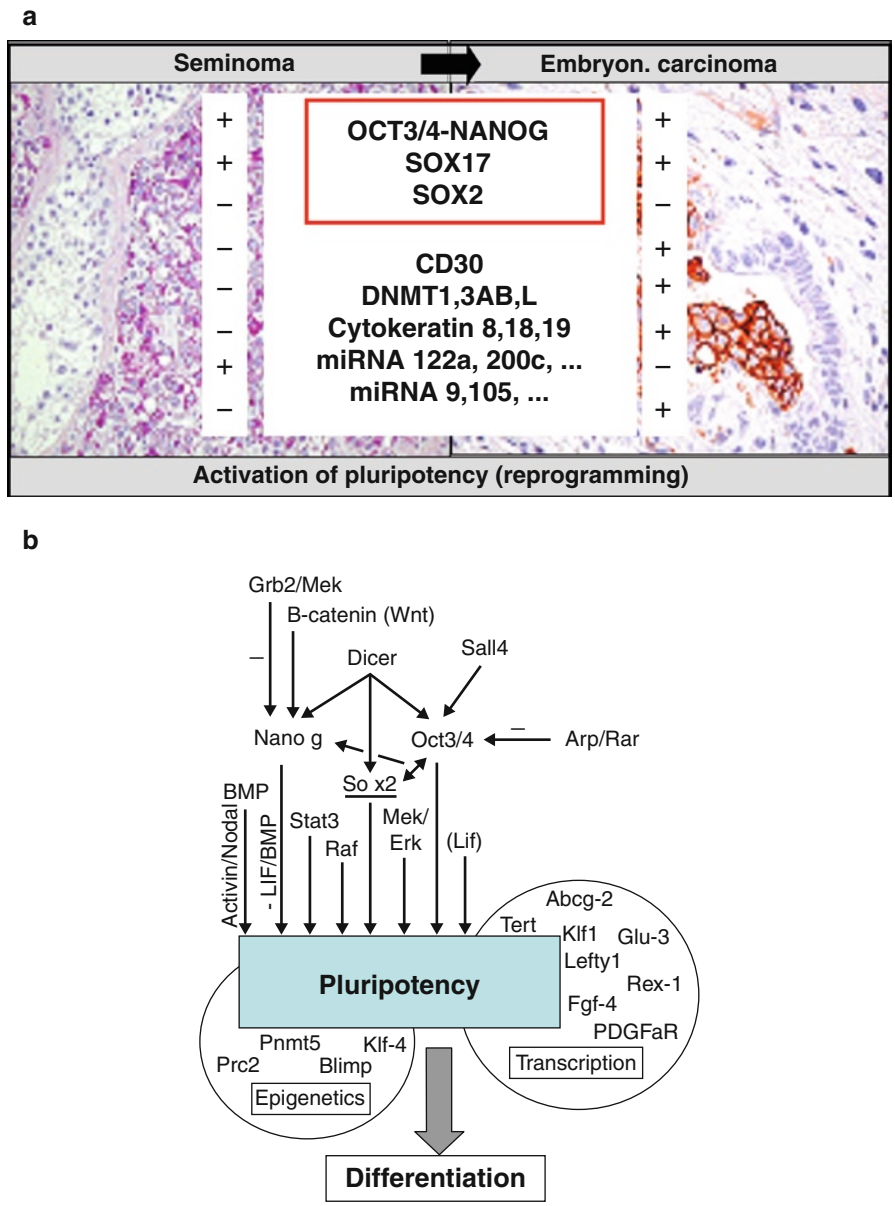


Fig. 2.2 (**a**) Summary of the different factors involved in the distinction between the human normal and malignant primordial germ cell (seminoma) stage compared to embryonic stem cell (embryonal carcinoma) stage. The *red box* set of genes, i.e., OCT3/4 (NANOG), SOX2/17 is suitable to be used in a clinical setting; (**b**) Various factors and pathways related to the undifferentiated stage of (mouse and human) embryonic stem cells. The genes used to generate pluripotent stem cells from somatic cells are underlined (Oct3/4-SOX2 and Klf-4). The difference between mouse and human embryonic stem cells regarding the need of Lif is illustrated by the use of *brackets*. The effects of these different targets/pathways are on the epigenetic status of the cells as well as their pattern of transcription; (**c**) Affymetrix expression profiling of KLF4 and c-MYC in the same samples is mentioned under Fig. 2.1c, although in addition, embryonal carcinomas (EC) are included; (**d**) Representative example of SOX9 immunohistochemistry on a normal embryonic testis. Note the staining of the Sertoli cells; (**e**) Representative example of FOXL2 immunohistochemistry on a normal adult ovary. Note the staining of the granulosa cells; (**f**) Representative immunohistochemistry for stem cell factor on carcinoma in situ cells (insert is the staining for OCT3/4); (**g**) Affymetrix expression profiling for the various DNA methyl transferases (1, 3A, 3B and 3L) on the samples is described under C; (**h**) Immunohistochemical detection of 5M-cytosine on normal spermatogenesis (positive) and CIS (negative), and the various histological elements of type II GCTs (seminoma is negative, while all nonseminomas are positive). In addition, a chemotherapy resistant seminoma, as well as the seminoma cell line TCam-2 and the embryonal carcinoma cell line (NT2), shows a high level of methylation

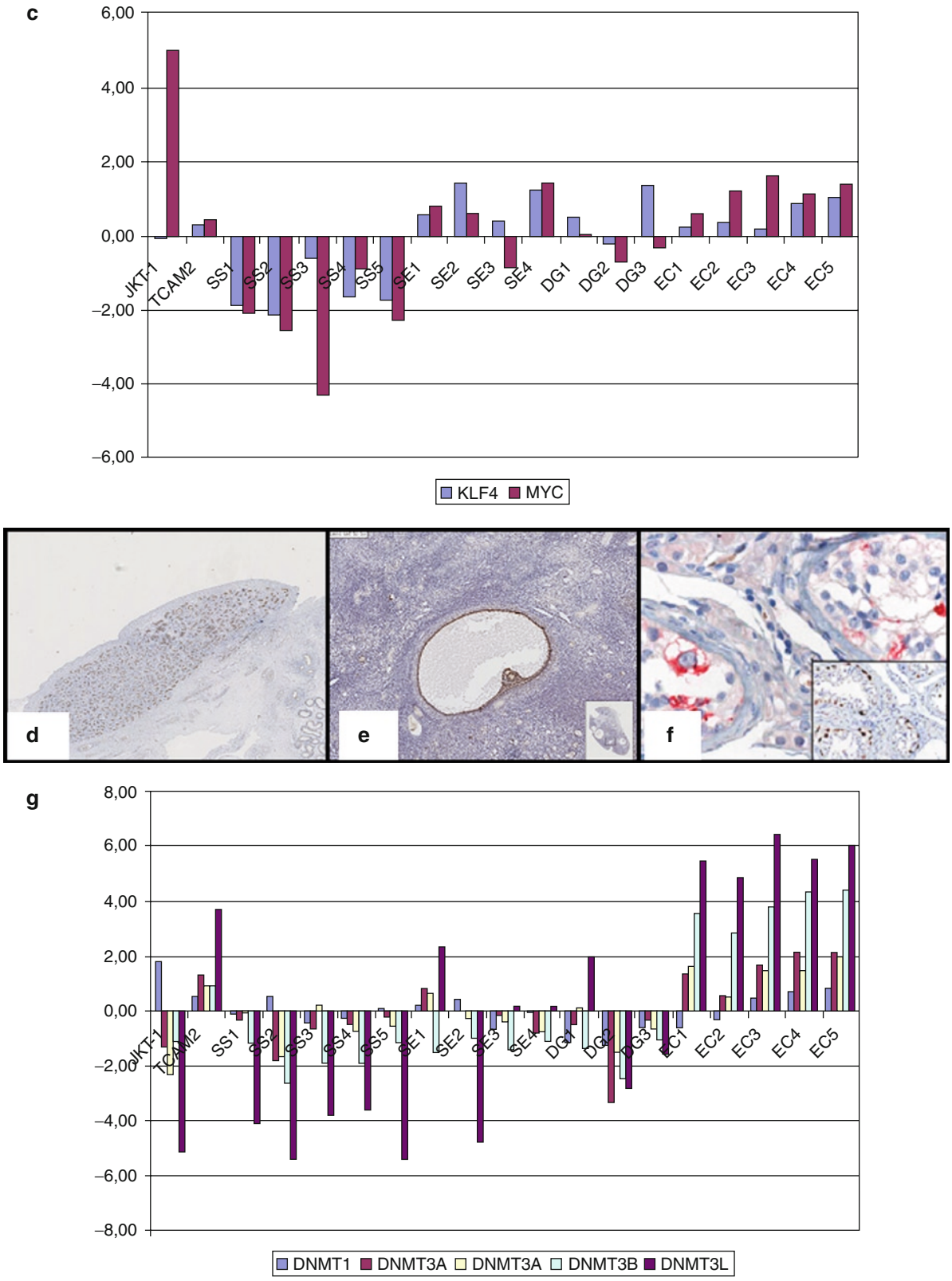
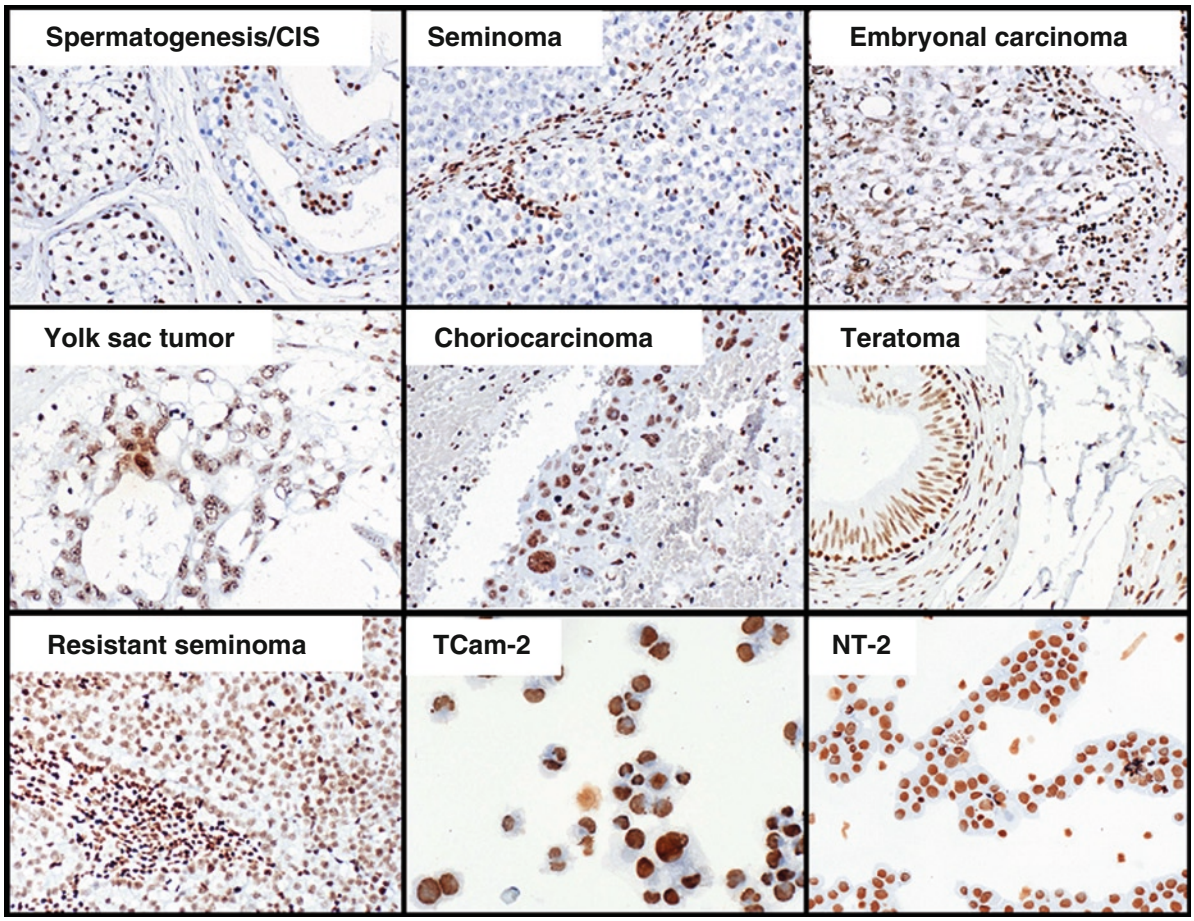


Fig. 2.2 (continued)



allows investigation of the expression of the POU5F1 encoding gene (Palumbo et al. 2002). It is expressed in seminoma and embryonal carcinoma, and not in the various differentiated components. Subsequently, this pattern has been confirmed on the protein level, using tissue microarray on several thousands of solid cancer specimens, of more than 100 different types. It shows that OCT3/4 is overall not found in other solid cancers (Looijenga et al. 2003a). In spite of this observation, many studies were initiated to investigate the presence of OCT3/4 in nongerm cell cancers (De Jong and Looijenga 2006). Predominantly on the basis of mRNA-based investigation, it has been concluded that this marker was indeed present, which would make it unsuitable as specific marker for type II GCTs. Most of these studies did not include proper protein detection, and indeed it was recently demonstrated that the results were false positive mainly because of the detection of pseudogenes. A specific primer set to detect the mRNA relating to the OCT3/4 protein has been generated, and proven to be specific (Liedtke et al. 2007; de Jong et al. 2008a). Use of this approach, as well as verified antibodies, will exclude false positive (and negative) results. In conclusion, the presence of OCT3/4 protein, detected by verified antibodies and having specificity and sensitivity, is the most informative diagnostic marker for seminoma and embryonal carcinoma (Richie 2005; de Jong et al. 2005a; Cheng et al. 2007). If applied on tissue derived from an adult testis, it is an absolute marker, but overdiagnosis is possible in infants and in the case of germ cell maturation delay (see below for further discussion). This diagnostic value is not limited to the testis, but also shown for other anatomical sites (De Jong et al. 2005b). Moreover, the pattern of staining is not influenced by metastasis or exposure to chemotherapeutic reagents. Besides these invasive components, the precursor lesions, CIS, and gonadoblastoma (see below) are also

positive. It remains to be clarified whether OCT3/4 can be considered as an oncogenic driver, as suggested on the basis of experimental data in mouse (Gidekel et al. 2003). No chromosomal anomalies have been identified supporting this model so far. The specificity of OCT3/4 for type II GCTs is in accordance to the observation that absence of this gene is not influencing the adult stem cell properties in mouse (Lengner et al. 2007). Interesting, however, is the block in differentiation and hyperplasia observed in various tissues in case of overexpression of this gene (Hochedlinger et al. 2005). The putative targets under control of regulation by OCT3/4 have been identified, which show a strong specific pattern (Boyer et al. 2005; Loh et al. 2006).

Interestingly, absence of OCT3/4 in mouse PGC does not result in differentiation, as reported in mouse and human embryonic stem cells, but induction of apoptosis (Kehler et al. 2004). This indicates that the function of OCT3/4 is cell dependent, for which so far no explanation has been provided (see also below). Although fewer studies have been published on the other regulator of pluripotency, i.e., NANOG, in type II GCTs, till now it seems that the expression pattern is similar to OCT3/4 (Clark et al. 2004; Ezeh et al. 2005; Hart et al. 2005; Hoei-Hansen et al. 2005; Korkola et al. 2006). It has been suggested that the chromosomal localization of NANOG is of specific interest, being on the short arm of chromosome 12, which is always gained in these tumors (see below). However, it needs to be experimentally verified whether such a relationship exists, because downregulation of NANOG has been reported upon differentiation of embryonal carcinoma towards other lineages (as expected, parallel to OCT3/4), but gain of 12p is still present in differentiated tumors, which indicates that still a positive selection mechanism is involved. In this context, the presence of gain of 12p in human embryonic stem cells upon extensive in vitro growth is also relevant (see below). There are, however, interesting data indicating that upregulation of NANOG is required for induction of apoptosis of mouse embryonic stem cells (Lin et al. 2005). Interestingly, expression of this gene is regulated by Oct3/4 as well as Sox2 (see below) (Loh et al. 2006; Rodda et al. 2005; Masui et al. 2007), and potentiates indeed generation of pluripotency (Silva et al. 2006; Suzuki et al. 2006). This is, among others, influenced by the Wnt pathway, via β-catenin (Takao et al. 2006). Most recently, Rex-1 has been found to distinguish within the Oct3/4 positive mouse embryonic stem cell population. The Rex-1 negative cells are related to primitive ectoderm while the positive cells represent the inner cell mass. These subpopulations are interchangeable, depending on the presence of Leukemia Inhibiting Factor (LIF) (Toyooka et al. 2008). No such subpopulations have been identified so far within type II GCTs (Kristensen et al. 2008). This might be due to the difference in LIF dependence of the mouse and human embryonic stem cells (Ginis et al. 2004). Another interesting finding is that an aberrant Oct3/4 in embryonic stem cells is related to disruption of Dicer expression, which is crucial for normal miRNA function (Cui et al. 2007) (see below).

Finally, although interesting data have been summarized in Fig. 2.2b, still a lot of questions need to be answered. The key question is whether these transcription factors are intrinsic to the cell of origin and therefore consistently present, or whether they play a causative role in the pathogenesis of these tumors.

2.6.1.2 SOX2 and SOX17

Although OCT3/4 and NANOG are valuable markers for the study of tumor biology as well as for diagnostics, they neither distinguish seminoma/CIS from embryonal carcinoma nor PGCs from embryonic stem cells. However, the presence of cytoplasmic as well as nuclear OCT3/4 (A type) staining, especially in combination with the morphological criteria, allows identification of embryonal carcinoma to a certain extent. Of course various other informative markers have been identified for embryonal carcinoma to allow distinction from seminoma, including cytokeratin 8/18/19 and CD30 (see also Figs. 2.2a and 2.3) (Pallesen and Hamilton-Dutoit 1988; Latza et al. 1995; Herszfeld et al. 2006). From a developmental point of view, the observation that SOX2 is positive in embryonal carcinoma and negative in seminoma and CIS is highly interesting. SOX2 is associated with OCT3/4 as a complex in the regulation of gene expression in embryonic stem cells, both mouse and human (Rodda et al. 2005; Masui et al. 2007; Okumura-Nakanishi et al. 2005; Carlin et al. 2006; Nakatake et al. 2006), including NANOG (see Fig. 2.2b for summary). In fact, OCT3/4 levels are regulated by SOX2 (Masui et al. 2007). But, in contrast to OCT3/4 and NANOG, SOX2 is not specific for embryonic stem cells and their malignant counterpart, i.e., embryonal carcinoma. It is found in many different lineages of differentiation, however, always in the absence of OCT3/4 and NANOG (Avilion

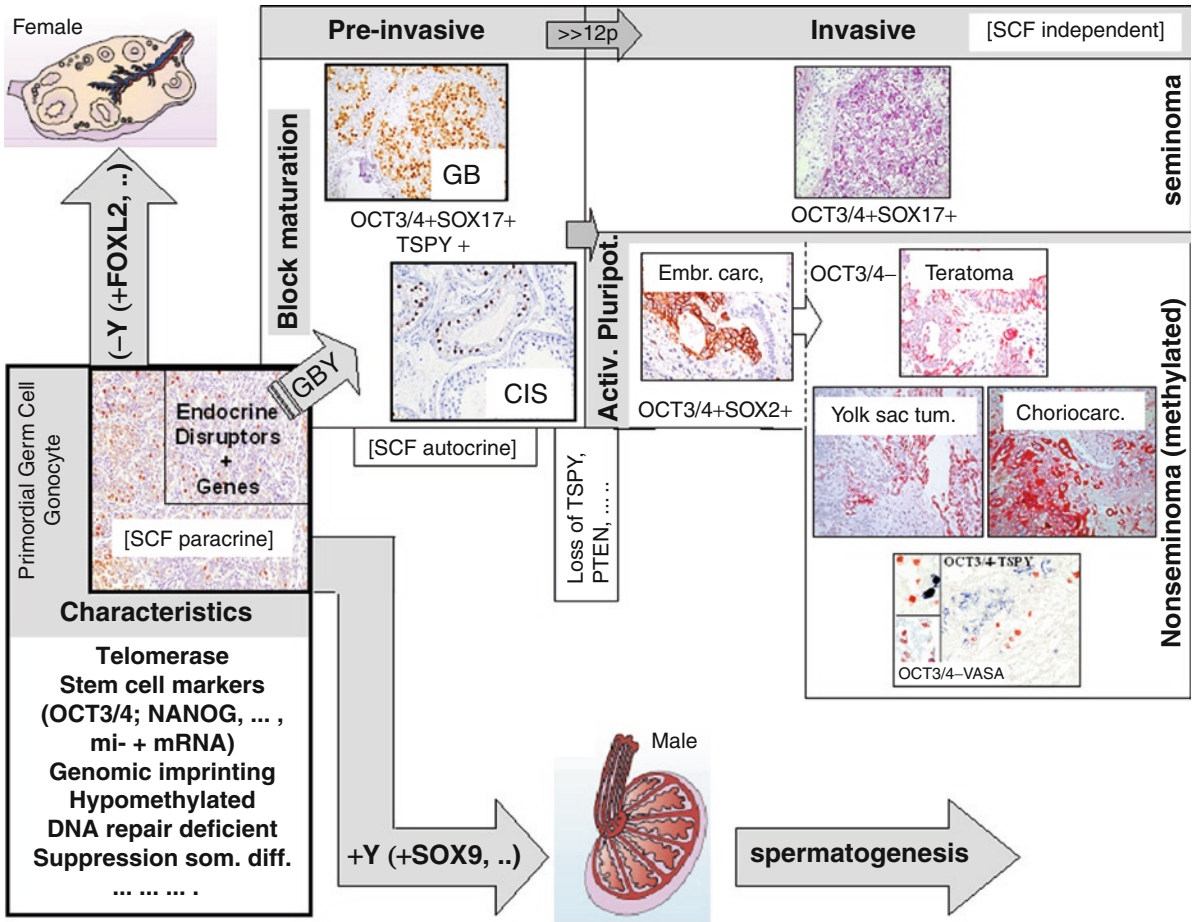


Fig. 2.3 Pathogenetic model of the initiation and progression of type II GCTs. See text for explanation

et al. 2003; Kelberman et al. 2006). This likely determines the absence of pluripotency of these cells. Interestingly, while SOX2 is found in mouse PGCs, it is absent in the human counterparts, which illustrates species specificities of regulation of pluripotency (Perrett et al. 2008; De Jong et al. 2008b). This is also demonstrated by the presence of Oct3/4 in mouse spermatogonia, in contrast to the human counterparts, which therefore makes it an informative diagnostic marker for CIS. Another intriguing observation is that human Sertoli cells associated with disrupted spermatogenesis or CIS can also show staining for SOX2, but never for OCT3/4 and NANOG. This must be kept in mind to exclude false positive intratubular diagnosis of embryonal carcinoma (De Jong et al. 2008b).

A relevant question is why OCT3/4 has a different function in PGCs and embryonic stem cells, extrapolated to seminoma/CIS and embryonal carcinoma, i.e., regulation of apoptosis vs. differentiation. It might be due to the differential presence of SOX2, which is only positive in embryonal carcinoma (see above). The next question is whether another SOX-member transcription factor is specifically expressed in PGCs, CIS, and seminoma. To get insight into this possibility, a high throughput screening was performed, which showed that SOX17 (and SOX15 to a lesser extent) is indeed specifically expressed in seminoma and CIS, confirmed at the protein level as well as in nonseminoma cell lines. Linking the genetic information to the expression data indicates that seminoma indeed shows specific gain of a region on chromosome 17, in which SOX17 is mapped (Korkola et al. 2008). Interestingly, SOX17 is identified as a regulatory element to distinguish embryonic from adult hematopoietic stem cells (Kim et al. 2007; Jang and Sharkis 2007). This observation opens a new field of experiments linking regulation of gene expression

related to pluripotency in type II GCTs, especially on the basis of the use of the various cell lines representative for human type II GCTs.

2.6.1.3 Diagnostic Expression Signature for Seminoma and Embryonal Carcinoma

The cumulated data allow now a rather simple and informative signature for seminoma/CIS and embryonal carcinoma, relevant both for diagnosis as well as investigation of the mechanism of activation of pluripotency, i.e., the transition of a seminomatous (PGC/gonocyte) cell to an embryonal carcinoma (embryonic stem) cell. This is schematically illustrated in Fig. 2.2a (in the red box). In fact, various patterns have been reported, but the most simple and straightforward is seminoma OCT3/4+/SOX17+/SOX2− and embryonal carcinoma OCT3/4+/SOX17−/SOX2+. The power of this rule of thumb is that by definition, besides OCT3/4, a positive differentiating marker is included. This is also true for the nonmalignant counterparts, i.e., human PGC/gonocyte and embryonal stem cell, which has been investigated in several studies (Gashaw et al. 2007; Hoei-Hansen et al. 2005; De Jong et al. 2008b; Rajpert-De Meyts et al. 2004; Stoop et al. 2005; Biermann et al. 2006; Kerr et al. 2008a, b; Honecker et al. 2004). In this context, it is relevant to underline that the PGC/gonocytes are in fact not pluripotent, but are equipped to transmit this capacity to the next generation. In contrast, the embryonal stem cells are capable of showing an intrinsic pluripotency, which will be lost upon derivation of adult stem cells that are committed and thereby have lost pluripotency. This is in line with the absence of OCT3/4 in adult stem cells (Ledford 2007).

2.6.1.4 Generation of Pluripotent Stem Cells Using Defined Set of Genes

It has been shown that pluripotent stem cells can be derived from somatically differentiated cells, both human and mouse, and can be generated by expressing a selected number of genes, i.e., OCT3/4, SOX2, KLF4, and c-MYC. The latter can even be omitted (Zaehres and Scholer 2007; Nakatake et al. 2006; Takahashi and Yamanaka 2006; Meissner et al. 2007; Nakagawa et al. 2007; Okita et al. 2007; Takahashi et al. 2007; Wernig et al. 2007). This however, results in a less efficient approach, and it results in the absence of malignant transformation. Interesting is also that NANOG is not required. So far, no studies specifically on KLF4 in type II GCTs have been performed, but expression profiling analysis demonstrated no differences between seminoma and embryonal carcinoma, like OCT3/4 and NANOG (see above) (Fig. 2.2c). It has been indicated that KLF4 is needed in this specific set of genes to reestablish an embryonic epigenetic pattern of the DNA and histones (see also Fig. 2.2b), which is lost upon physiological differentiation. A similar expression pattern in type II GCTs is found for c-MYC, which is indicated to be needed for proliferation induction (Fig. 2.2c). It must be kept in mind that these data on GCTs are based on mRNA levels, and confirmation on the protein level, including activity status, will be needed to get further insight in the relevance of these proteins. However, it can be concluded so far that embryonal carcinoma (and seminoma, but in the absence of SOX2) seems to express the critical genes of pluripotent stem cells.

The difference between seminoma and embryonal carcinoma might be due to the different expression, among others, of SOX17 and SOX2. In this context, the recent observation that pten and akt are involved in the generation of embryonic stem cells from mouse PGC, i.e., the so-called activation of pluripotency (or reprogramming), is highly relevant (Kimura et al. 2003, 2008). Inactivation of pten in PGCs results in generation of embryonic stem cells, related to activation of Akt. Indeed, PTEN has been found to be inactivated in the transition from CIS to embryonal carcinoma (Di Vizio et al. 2005). Of interest is that suppression of PTEN is required for allowing cellular transformation (including antiapoptotic effects) of activated RAS (Vasudevan et al. 2007) (see below). In the pten/akt mouse model, p53 was found to be a crucial downstream target (see also below). The PTEN/AKT and KRAS2 pathways seem indeed to be active in human embryonic stem cells (Humphrey et al. 2004). KRAS2 is another gene mapped to the short arm of chromosome 12, which makes it a gene of interest in the pathogenesis of type II GCTs (see below), and supportive data are available that it is indeed involved (McIntyre et al. 2008). This interesting model deserves further exploration. Experimental data on the regulatory networks of these transcription factors can be obtained using the available type II GCT cell lines

(see below). Interesting is the fact that KRAS2 is also linked to c-KIT targeting, which will be discussed in the next paragraph.

2.6.1.5 c-KIT and KRAS2

c-KIT is a kinase receptor relevant for a number of crucial processes during normal development, including survival and migration of PGCs from the epiblast to the genital ridge (Wylie 1993; Godin et al. 1991; Runyan et al. 2006). Disturbances in the function of the c-KIT pathway, dependent on the ligand stem cell factor (SCF) result in various anomalies, including sub- or infertility (Lennartsson et al. 2005). In normal development of germ cells, c-KIT is downregulated upon arrival of the PGCs in the genital ridge (Wylie 1993; Godin et al. 1991), although it can still be detected at a relatively low level in human spermatogonia (Stoop et al. 2008). In contrast, it remains high in expression in the mouse counterparts, as also reported for Oct3/4 (see above). c-KIT is also present at a high level in CIS and gonadoblastoma, the precursor lesions of type II GCTs (see below) and is overall downregulated upon invasive growth, although still some c-KIT can be found in invasive tumors (Strohmeyer et al. 1991; Izquierdo et al. 1995; De Meyts et al. 1996; Sakuma et al. 2003; Miettinen and Lasota 2005; Nikolaou et al. 2007). Activating mutations, leading to an SCF independent active receptor, have been found predominantly in bilateral type II GCTs. The sensitivity of the mutation detection leads to seemingly conflicting data (Sakuma et al. 2003; Looijenga et al. 2003b; Kemmer et al. 2004; Tate et al. 2005; Biermann et al. 2007c; Rapley et al. 2008). Some studies predominantly find the activating mutations in primary unilateral seminomas. That indeed c-KIT has an important role in the pathogenesis of type II GCTs is also supported by the observation that this gene can be overexpressed because of a highly restricted genomic amplification only including this gene (McIntire et al. 2005). That particular tumor indeed showed a high and consistent staining at the protein level using immunohistochemistry. The c-KIT signaling pathway has been linked to PI_3K (De Miguel et al. 2002; Shivakrupa et al. 2003), both in mouse PGCs as well as type II GCTs. This is of course relevant in the context of the previously described link to PTEN (see above). Moreover, it is of interest because of the observations that activating KRAS2 mutations are also found, in a mutually exclusive manner (Goddard et al. 2007). Activation of a mutated KRAS2 results in an increased in vitro survival of seminoma cells (Olie et al. 1994, 1995a, b), which are normally not able to survive outside the patient, as well as an earlier age at clinical presentation of the tumor.

2.6.2 Risk Factors

A number of risk factors have been identified for type II GCTs, including cryptorchidism, in(sub)fertility, familial predisposition, birth weight, and birth order, as well as various forms of DSD (Moller 1993; Skakkebaek et al. 1998; Jacobsen et al. 2000; McGlynn et al. 2003; Pamenter et al. 2003; Raman et al. 2005; Costabile 2007; Sonke et al. 2007; Walsh et al. 2007; Cook et al. 2008). In spite of the overall consistency of the role of these risk factors, various others have been identified, with variable impact. Interestingly, some of them seem to be specific for either seminoma or non-seminoma. In spite of much effort, it has not been possible to identify the gene or genes involved in familial type II GCTs yet (Rapley et al. 2000; Holzik et al. 2004). The link to the long arm of the X chromosome is likely related to the occurrence of cryptorchidism, and thereby indirectly to the development of the cancer. Overall, the genetic predisposition is difficult to investigate because of the small sizes of the affected families, the relationship to subfertility, as well as the possible role of the (micro)environment. Because of their weakness as risk factors, it is often not possible to divide the impact of both parameters within a single family. The likely multigenetic basis of the predisposition makes the identification of genes even more complex (Lutke Holzik et al. 2006). It is noteworthy in this context that immigrants from Finland to Sweden, who have a lower initial risk for type II GCTs, obtain the risk of the Swedish population at the second generation (Hemminki et al. 2002). This demonstrates a significant effect of the environment on the incidence for a limited period of time, and possibly overruling a genetic component, if present. Recent studies of transgenerational effects of exposure to certain chemicals, including endocrine disruptors, are of specific interest (Anway et al. 2005, 2006; Chang et al. 2006; Crews et al. 2007; Skinner 2007a, b). The link to epigenetic regulation is intriguing and might explain for the

so-called testicular dysgenesis syndrome (TDS) (see below). It is of interest that the other identified risk factors commonly, in one way or the other, affect the maturation of embryonic germ cells negatively. These factors have been brought together into TDS (Skakkebæk et al. 2001; Fisher et al. 2003; Skakkebaek 2003; Rajpert-De Meyts 2006; Sonne et al. 2008). This model 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 development of a type II GCT. In this model, the role of the supportive element, i.e., the Leydig–Sertoli cells is crucial. Within the subgroup of sub(in)fertility, it has recently been identified that the presence of bilateral microlithiasis is an informative parameter to identify males with a high risk (up to 20%) of CIS (De Gouveia Brazao et al. 2004), which is in line with the observation of a high incidence of these microcalcifications in patients with a unilateral type II testicular GCT, and contralateral CIS (Holm et al. 2003). This finding can be of value for screening purposes (Costabile 2007).

A recent meta-analysis demonstrated that both a low and a high birth weight increase the risk (Michos et al. 2007). In addition, trisomy 21 patients have an increased risk and indeed, a delayed maturation of germ cells has been identified (Cools et al. 2006a). It remains to be elucidated whether the extra chromosome 21 in the germ cells or the supportive cells in the testis results in an increased risk for type II GCTs. Although this was suggested, because of the gain of chromosome 21 in the tumor cells, this seems to be unlikely, because this has not been found for women. Therefore, it is more likely that the suboptimal microenvironment of the testis due to the trisomy status of the individual results in a delayed maturation of germ cells and thereby a higher risk for malignant transformation. In this context, the observation that Klinefelter patients (47,XXY) have no increased risk of type II GCTs of the testis, but rather of the mediastinum, is of interest (Isurugi et al. 1977; Lee and Stephens 1987; Nichols et al. 1987; Hasle et al. 1992, 1995; Volkl et al. 2006). Most recently, such an association has also been suggested for the intracranial GCTs (unpublished observations). The absence of testicular type II GCTs in Klinefelter patients is likely due to induction of apoptosis of germ cells related to an improper microenvironment (Aksglaede et al. 2006). The resulting pituitary/gonadal overstimulation may play a role in the increased risk of mediastinal GCTs. A similar phenomenon has been reported for germ cells in the ovary of Turner syndrome patients (45XO) (Modi et al. 2003), as well as patients with complete androgen insensitivity (CAIS) (Cools et al. 2005) (see below).

The mechanistic basis of the increased risk of the various conditions remains to be elucidated, but the possible role of estrogen and antiandrogen functions, being the basis of the TDS model, is worth investigating in more detail. This hypothesis is supported by multiple observations. The higher level of testosterone in blacks might indeed explain the lower incidence of this type of cancer (Henderson et al. 1988). This is supposedly related to the role of testosterone during embryonal development in pushing the PGCs along their maturation pathway to spermatogonia, which thereby lose their characteristics of PGCs/gonocytes, and therefore their ability to form CIS (see above). The higher risk of the first child in birth order is in line with a role of a higher level of estrogen exposure at early embryonal developmental age (Weir et al. 2000). Although type II GCTs are rather specific for the Caucasian population, the Maori are an interesting exception (Wilkinson et al. 1992). Men of this ethnic group show a similar incidence as Caucasians, possibly again related to an increased level of estrogen. The intrinsically higher level of testesterone in blacks, already during embryonal development, might be related to their lower risk for type II GCTs. This possibly prevents delayed maturation of PGC/gonocytes into the stage of spermatogonia. A number of studies also indicate that polymorphisms in enzymes which increase the level estrogen are related to a higher risk of type II GCTs (Starr et al. 2005). Moreover, the differences between the people of Denmark and Finland are associated with different exposures to chemicals that have estrogen or antiandrogen activity (Toppari et al. 1996; Rajpert-De Meyts et al. 2003b). A counterargument on the role of increased estrogen is that during the early development the level of estrogen is high, but it could be that a critical window is relevant in this context. Of specific interest is that an animal model for disrupted testicular development, used as model for endocrine disruptors in the generation of TDS, indicates the same window (Welsh et al. 2008). The possible role of androgens in the pathogenesis of type II GCTs is also suggested on the basis of the various types of patients with DSD, with a higher or lower risk of a type II GCT. These will be discussed below.

2.6.3 Disorders of Sex Development

This group of developmental anomalies, previously referred to intersex, is defined as conditions 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). Because of the topic of this review, a number of relevant issues in the context of type II GCTs will be discussed here. Indeed, as expected, these patients have no increased risk for the type I and III GCTs.

2.6.3.1 Parameters Related to Tumor Risk

DSD patients with either hypovirilization or gonadal dysgenesis can show an increased risk for the development of type II GCTs. A number of recent reviews have been published recently (Cools et al. 2006b; Looijenga et al. 2007c). The most important issues will be summarized here. The precursor can indeed be CIS or gonadoblastoma, related to the level of virilization of the gonad. This can nicely be demonstrated by the use of protein detection by immunohistochemistry for SOX9 (indicative for SRY function and Sertoli cell differentiation), and FOXL2 (granulosa cell differentiation) (Hersmus et al. 2008a). In contrast to the link between ovarian differentiation and FOXL2 and that between testicular differentiation and SOX9, 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. It is suggested that in fact CIS and gonadoblastoma are the same type of lesion (Hersmus et al. 2008b), of which the histological context is determined by the level of virilization.

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 GCTs of the testis (Batata et al. 1980; Muller et al. 1984; Giwercman et al. 1987; Abratt et al. 1992). Interestingly, it has been demonstrated that a seminoma is more frequently found in intraabdominal gonads than in gonads localized in the scrotum (Ogunbiyi et al. 1996). This likely also explains the preferential occurrence of dysgerminomas in the ovary, which are always abdominal (Susnerwala et al. 1991; Dietl et al. 1993; Chow et al. 1996; Cusido et al. 1998; Tewari et al. 2000). In addition, it has been shown that an early age of orchiopexy indeed reduces the risk for a type II GCT of the testis (Walsh et al. 2007; Jones et al. 1991; Engeler et al. 2000). This is likely related to the still ongoing maturation of PGC/gonocyte like cells to spermatogonia. Moreover, complete absence or very low level of testosterone also diminishes the risk of a type II GCT. This is nicely illustrated by patients with hypogonadotropic hypogonadism, who always have cryptorchid testis, but never will develop a GCT. In addition, patients with CAIS have a significantly lower risk compared to patients with the partial form of this disorder (Cools et al. 2005, 2006b; Hannema et al. 2006). Most likely this is related to the induction of apoptosis of the germ cells in the testis of CAIS patients, as observed in Klinefelter patients (see above).

Development of type II GCTs in DSD patients seems to be related to formation of specific histological structures. In patients with a certain level of virilization and therefore testis formation, it results in the characteristic lesion of CIS, as also found in men without any sign of DSD, but related possibly to TDS (see above). In contrast, DSD patients lacking such a level of virilization will develop gonadoblastoma, which may in rare cases be combined with seminiferous tubules with CIS. The precursor lesion of gonadoblastoma is known as undifferentiated gonadal tissue (UGT), which allows a better diagnosis at early developmental stages (Cools et al. 2006c). Interestingly, the various genetic anomalies related to an increased risk for type II GCTs indicate that it has a link to the function of Sertoli cells (Hersmus et al. 2008b). This might be the missing link between TDS and DSD (Hutchison et al. 2008). The structures, being the precursors of invasive type II GCTs, contain double positive cells for OCT3/4 and TSPY. The latter is the most interesting candidate gene for the involvement of the Y chromosome, which will be discussed in the next paragraph.

2.6.3.2 Involvement of the Y Chromosome; TSPY as Candidate Gene

The risk of development of a type II GCTs in DSD patients is directly related to 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 indeed supported by the clinical observation of patients with a translocation of the SRY gene to an X chromosome or an autosome, resulting in 46,XX men, who have no increased risk of this type of cancer. Although SRY is not the gene of interest in this context, knowledge of its function is relevant. The first downstream target of SRY is the transcription factor SOX9, which in the testis is Sertoli cell specific (see Fig. 2.1d). It has been assumed that simply the absence of this pathway results in formation of an ovary, which has been recently challenged by a number of observations, including identification of FOXL2 (Baron et al. 2005; Uhlenhaut and Treier 2006; Ottolenghi et al. 2007) as the gene required for granulosa cell formation (see Fig. 2.2e). A recent study reports that SOX9 and FOXL2 are indeed highly informative to identify the testicular and ovarian differentiation lineages in gonads of patients with DSD (Hersmus et al. 2008a). The presence of SOX9 is associated with CIS and FOXL2 with gonadoblastoma.

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, which is in fact a multicopy gene (Vogel and Schmidtke 1998). It has similarities to the DEK/CAN family of proteins; it interacts with cyclin B1 and is therefore supposed to be involved in cell cycle regulation. Various splice variants have been reported, which indeed can be present in type II GCTs. Protein expression analysis demonstrate that 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 unknown so far (Lau 1999; Schnieders et al. 1996; Hildenbrand et al. 1999; Kersemaekers et al. 2005; Delbridge et al. 2004; Li et al. 2007b). The consistent aneuploidy of type II GCTs might be related to this. In fact, the increased level of this protein is used as supportive parameter to distinguish a malignant germ cell from a germ cell showing delayed maturation. Upon invasive growth, expression of the gene is mostly lost, associated with subsequent absence of the protein, although the Y chromosome can still be retained. Therefore, this is due to downregulation of expression. Transfection expression analysis 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 a number of the upregulated genes in the TSPY transfected cells map to the short arms of chromosome 12. In fact, a correlation between the level of TSPY and expression of these genes, including KRAS2 and NANOG, was found only in the precursor lesion CIS, and not in the invasive tumors (Li et al. 2007c). This observation nicely fits with the downregulation of TSPY upon progression of the tumor towards invasiveness.

Mice lack TSPY. Transgenic animals containing a human TSPY genomic fragment 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.

2.6.4 Cell of Origin and Markers of Diagnosis

The presence of the different markers in the precursor cells of type II GCTs of the testis, known as carcinoma in situ (CIS) (Skakkebæk 1972), testicular intratubular neoplasia (TIN) (Loy and Dieckmann 1990), and intratubular germ cell neoplasia unclassified (IGCNU) (Woodward et al. 2004), is supportive to an embryonic origin. As indicated, the counterpart in dysgenetic gonads, with a low level of virilization, is known as gonadoblastoma (Scully 1970). These lesions also contain germ cells showing the same characteristics as CIS cells. The nonmalignant counterpart is most likely a PGC or gonocyte. The difference between these two is only that a gonocyte is a PGC that has arrived in the genital ridge, after migration from the yolk sac region. In other words, they only differ in anatomical localization. The consistent biallelic expression of imprinted genes in invasive type II GCTs, as well as CIS, is in

agreement with this origin (Van Gurp et al. 1994; Szabo and Mann 1995; Rachmilewitz et al. 1996; Verkerk et al. 1997; Looijenga et al. 1998; Ross et al. 1999; Sievers et al. 2005b; Kawakami et al. 2006; Lind et al. 2007). Interestingly, induction of erasement of imprinting in mouse embryonic stem cells results in development of a number of hematopoietic and solid cancers, including a single testicular seminoma (Holm et al. 2005). Although this is a single observation, and the histology of the tumor is not confirmed independently, its existence is highly relevant, because it indicates that an animal model can possibly be generated, and that an erased pattern of genomic imprinting is required.

During this migratory and early gonadal stage of germ cell development, the cells are positive for c-KIT, PLAP, OCT3/4, NANOG, etc., the markers of which are also found to be expressed in CIS and gonadoblastoma, as well as seminoma (Oosterhuis and Looijenga 2005; Rajpert-De Meyts 2006). Normally, upon maturation from the gonocytes to spermatogonia, these markers are downregulated, and others, including MAGE-4A, are initiated (Gashaw et al. 2007; Biermann et al. 2006). In addition, high throughput expression profiling shows that CIS cells shows strong overlap with embryonic stem cells regarding expression profile (Almstrup et al. 2005). This supports the model of an embryonic origin of type II GCTs, which is in line with the epidemiological observation of the dip in the incidence of this type of cancer in men who were conceived during World War II (Moller 1993; Moller and Skakkebæk 1996), as well as other risk factors. This clearly distinguishes this population of cells from the adult stem cell identified of the spermatogenetic lineage (Hofmann et al. 2005; Chen et al. 2005; Kanatsu-Shinohara et al. 2006). The alternative model in which type II GCTs originate from a pachytene spermatocyte is in disagreement with these observations (Chaganti and Bosl 1995). Possibly, the most convincing argument against this latter model is the fact that patients with various forms of DSD, most of whom will never develop proper spermatogenesis, not even spermatogonia, have an increased risk for this type of cancer. Therefore, it can be concluded that the cell of origin of type II GCTs is a germ cell blocked in their PGC/gonocyte stage. This also explains why similar tumors can be found in the ovary, as well as extragonadal sites. The occurrence of mediastinal type II GCTs in Klinefelter patients also strongly argues against a pachytene spermatocyte as cell of origin, as these patients have no spermatogenesis.

That indeed OCT3/4 has additional diagnostic value for the detection of CIS is demonstrated by a recent study. This is a retrospective analysis on testicular biopsies of men for fertility related problems. None of these was initially diagnosed as having CIS, although they all in time developed an invasive tumor. Expert pathology review identified in 50% of the cases the malignant cells, which were identified in 70% using immunohistochemistry for OCT3/4 (unpublished observations). The rule of thumb is that every marker showing a specific pattern of expression in embryonic germ cells and which becomes negative upon differentiation will be informative for the diagnosis of CIS and gonadoblastoma, as well as seminoma. This was recently confirmed for newly identified markers. Because of the consistency and specificity of OCT3/4 in staining in the adult testis, there is no need for identification of additional markers from the diagnostic point of view. However, they will be useful in dissecting the biology of these tumors.

There are two exceptions in which OCT3/4 is not as informative as would be needed for the diagnosis of the precursor of type II GCTs. That is in the case of tissue obtained during the first year of life, and in case of gonads showing germ cell maturation delay. Under these conditions, OCT3/4 staining can still be present in germ cells which have not undergone malignant transformation. These cells are also positive for TSPY, as well as SOX17. On the basis of the morphology, as well as additional criteria, supportive arguments can be obtained to diagnose or rule out. These criteria are not easy to apply in routine pathology, and they are not without any restriction (Cools et al. 2005). For this purpose, availability of a more informative marker would be of great clinical diagnostic value in these patients. A possible marker fulfilling these criteria will be discussed in the next paragraph.

2.6.5 SCF as Marker for Early Malignant Germ Cells

As mentioned, SCF is the ligand of c-KIT. It is crucial for proper migration and survival of PGCs. Experiments in vitro support this model, and indicate that SCF prevents induction of apoptosis by, among others, activation of the PI_3K pathway (De Miguel et al. 2002; Shivakrupa et al. 2003). Two variants of SCF can be

generated by Sertoli cells by alternative exon usage. One is membrane bound and is highly effective in supporting survival of PGCs (Lev et al. 1994; Yan et al. 2000). The soluble form is related to activation of the Leydig cells present in the stromal compartment of the testis. Under normal physiological conditions, both embryonic and adult, no SCF can be identified in human gonads by immunohistochemistry using a specific antibody (Stoop et al. 2008). However, it is consistently present in testis with CIS, but not in case of the presence of germ cells showing delayed maturation. This is in contrast to OCT3/4 (NANOG and c-KIT) being present under all these conditions. Upon invasive growth of the tumor cells, SCF, like c-KIT (Biermann et al. 2006), is predominantly downregulated although it can be present heterogeneously in various histological elements. It could be demonstrated that SCF has a significant additional value to detect the earliest malignant changes in germ cells (see Fig. 2.2f). No specific upregulation of gene expression could be identified using Q-RT-PCR, although mRNA ISH data indicate that the gene is predominantly expressed in CIS cells. This suggests the presence of an autocrine loop, which is in line with the observation of both c-KIT and SCF in a subpopulation of cells of the embryonal carcinoma cell lines, while it is found in all cells of the seminoma cell line TCam-2. Also the effect of inhibition of c-KIT supports an autocrine loop (Goddard et al. 2007). This observation is both biologically and diagnostically relevant. It suggests that during the early stages in the pathogenesis of type II GCTs, a switch occurs between a paracrine to an autocrine loop of the SCF and c-KIT pathway. Upon development of an invasive tumor, either seminoma or nonseminoma, this mechanism is overruled, and not under selective pressure anymore (see Fig. 2.3).

2.6.6 Possibilities for Early (Noninvasive Diagnosis)

Various attempts have been undertaken to develop a method for early diagnosis of preferentially the precursor lesions of type II GCTs. This has been on the basis of their aneuploidy (see below), as well as their protein expression profiling (Giwercman et al. 1990a, b; Giwercman 1992; Meng et al. 1998; Hoei-Hansen et al. 2007). Overall, most studies show rather disappointing results. This is likely related to the heterogeneous expression of the markers used, as well as the selection of patients for screening. A recent study shows that if OCT3/4 is used as marker for diagnosis, the majority of patients known to have CIS (80%) can be identified on the basis of the presence of positive cells in semen (Dieckmann 2009; van Casteren et al. 2008). Although various questions have to be answered before this protocol will be applicable in a clinical setting, it was proven that in principle the approach can be informative, using the optimal marker, protocol, and selected patient cohort. This will especially be of interest in populations with an increased risk of development of testicular type II GCTs, such as those with infertility, bilateral microlithiasis, and a previous unilateral tumor. A prospective study will be needed to show the power of the method compared to that of the surgical biopsy, which is considered as the gold standard. In addition, the presence of activating c-KIT mutations in bilateral type II GCTs can also be an interesting target for clinical implementation although, as mentioned, the sensitivity of the detection system might be a limiting factor.

2.6.7 Chromosomal Constitution

Many studies have been performed to investigate the chromosomal constitution of type II GCTs, including its precursor lesion (Kraggerud et al. 2002; Oosterhuis et al. 1989a; Castedo et al. 1989; Samaniego et al. 1990; Skotheim et al. 2001; Summersgill et al. 2001; von Eyben 2004). In fact, this started with total DNA content analysis using flow cytometric studies, followed by conventional karyotyping and targeted ISH, and more recently c- and a-CGH as well as single nucleotide polymorphism (SNP) arrays. Overall, the different approaches showed the same results; type II GCTs are highly aneuploid with specific and characteristic changes. The seminomas and CIS are hypertriploid and the nonseminomas hypotriploid. Specific chromosomal gains and losses are identified, some of which are suggested to be histology related. The only recurrent structural imbalance is the gain of the short arm of chromosome 12, mostly as isochromosomes (see Table 2.1). Most studies indicate that gain of 12p is progression related; it occurs when the CIS cells

become independent for their interaction with Sertoli cells (Summersgill et al. 2001; Rosenberg et al. 2000). The presence of additional copies of 12p is independent from the histological constitution, as well as anatomical localization. It is interesting that human embryonic stem cells cultured for an extensive period of time also show this anomaly (Draper et al. 2003; Cowan et al. 2004; Li et al. 2006). In spite of many attempts, there is no single 12p-target gene identified. A number of genes have been suggested to be relevant, including KRAS2, NANOG, although the actual proof is lacking so far.

The X chromosome is gained in the majority of tumors, for which a link with familial predisposition has been suggested (see above). The presence of additional X chromosomes is relevant in the context of understanding the biology of type II GCTs, including in the Klinefelter syndrome patients, as well as in patients with various forms of DSD. Moreover, it has a molecular diagnostic value (see below).

SNP analysis in type II GCTs demonstrated the presence of so-called uniparental disomies (Lu et al. 2005). These have been more frequently detected in nonseminomas than in seminomas. The proposed explanation is that the latter originates from fusion of a haploid (postmeiotic) germ cell with a diploid germ cell, also explaining their consistent peritriploid DNA content (Oosterhuis et al. 1989b). This hypothesis is highly unlikely, because these tumors can develop without the presence of spermatogenesis, as discussed before. In addition, this pattern of uniparental disomy has also been found in an ovarian type II GCT (unpublished observations). The most likely explanation is that the tumor cells undergo significant mitotic recombination.

Of interest is that currently an integrated analysis of expression of genes and proteins as well as DNA copy changes is initiated (Skotheim et al. 2002, 2005, 2003a, b; Korkola et al. 2005, 2006, 2008; McIntyre et al. 2004, 2007). Overall, the data suggest a close correlation between the two, in which the expression drives the chromosomal imbalances or vice versa. For example, gain of a specific region of chromosome 17 is found to be overrepresented in seminoma, which includes the SOX17 gene (Korkola et al. 2008), which is characteristic for seminoma (see above). These models are highly relevant to explain the chromosomal changes as observed in solid tumors, which likely will also have clinical impact.

2.6.8 Epigenetic Modification

In spite of a wealth of information about the genomic make up of type II GCTs, increasing knowledge on the epigenetic constitution is evolving (Kawakami et al. 2006; Lind et al. 2006, 2007; Peltomäki 1991; Ishii et al. 2007; Zhang et al. 2005; Honorio et al. 2003; Smiraglia et al. 2002; Koul et al. 2002; Okamoto and Kawakami 2007). The role of epigenetics in germ cell development has been reviewed recently (Biermann and Steger 2007). Targeted – as well as genome wide studies demonstrate that overall the seminomas show a 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, like during normal embryogenesis, the result of the function of the non(protein)-coding XIST gene. This unique phenomenon in men is correlated with hypomethylation of the promoter region, which can be used as molecular target for type II GCTs in men (Kawakami et al. 2003, 2004). The difference in methylation status can indeed be demonstrated using expression profiling for the different forms of the DNA methyltransferases (see Fig. 2.2g). The DNMT1 is required for maintenance of the methylated status during cell division, and previously found to be present in differentiated form of nonseminomas (Omisanjo et al. 2007), while DNMT3A and B 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 ^{M}C-specific antibody. Representative examples are shown in Fig. 2.2g. This pattern of methylation is in accordance to the expected pattern based on observations during embryogenesis, i.e., PGCs are hypomethylated and differentiated derivatives (locally) hypermethylated. In this context, it is relevant to remember that in vitro culturing may induce specific changes in DNA methylation, which may bias the findings made in type II GCT-derived cell lines. Interestingly, indeed, the TCam-2 cell line, representative for seminoma, being hypomethylated, shows a hypermethylated pattern based on immunohistochemistry. Therefore, the observations made in cell lines must always be verified in in vitro tumors.

That possibly the hypermethylated pattern of this seminoma cell might have a biological function is suggested by the hypermethylation of chemotherapy resistant seminomas (see Fig. 2.2h) (unpublished observation). It remains to be investigated whether this relates to specific genetic changes in this cell line (see below). Interestingly, a methylation study was recently done for the promoter region of OCT3/4. In seminoma and embryonal carcinoma, the promoter region is predominantly hypomethylated, in in vitro cell lines as well as in vivo tumors (De Jong et al. 2007a). Microdissection of the embryonal carcinoma cells even demonstrated a complete demethylated pattern. Upon differentiation of the embryonal carcinoma cells, OCT3/4 is downregulated in expression, associated with hypermethylation of the promoter region. Most likely, this pattern is reflecting 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 in specification of which genes will be hypermethylated upon differentiation from an undifferentiated stem cell. This is related to the histone H3 methylated at lysine 27 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, i.e., embryonal carcinomas, in which two additional repressive marks are identified, being dimethylated H3K9 and trimethylated H3K9, both associated with DNA hypermethylation in adult cancers. This is nicely fitting with the observed pattern of expression of the histone deacetylase (HDAC) in these tumors (Omisanjo et al. 2007). More recently, a related study investigated the expression of BLIMP-1 and PRMT-5 (unpublished observations). 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 (Hayashi et al. 2007; Ohinata et al. 2005). 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. For this purpose, investigation of genetic anomalies affecting genes or pathways might be highly informative. Therefore, the next section will be related to this topic.

2.6.9 Mutational Status

Various studies with the goal to identity pathogenetic mutations have been performed on type II GCTs. These included a large number of targets, among others, NRAS, KRAS-2, and HRAS (Goddard et al. 2007; Mulder et al. 1989; Ganguly et al. 1990; Ridanpaa et al. 1993; Przygodzki et al. 1996; Oosterhuis et al. 1997), and BCL10 (van Schothorst et al. 1999; Kakinuma et al. 2001). Although mutations have been identified, these seem to be limited in frequency, with the possible exceptions of c-KIT and KRAS-2 (see above), and more recently BRAF. This latter proto-oncogene has been shown to be mutated in a variety of cancers, including melanoma. Interestingly, the affected pathway is the MEK-pathway, in which RAS also act. Activating mutations of KRAS and BRAF are mutually exclusive in type II GCTs. A correlation between BRAF mutation and hypermethylation of the promoter of hMLH1 has been reported (Imai 2007). hMLH1 is involved in mismatch repair, and improper function of this protein. Absence of or mutations in this gene result in microsatellite instability (MSI). Indeed, MSI instability has been reported to be related to treatment resistance (i.p. cisplatin-based) in multiple studies (Mayer et al. 2002; Devouassoux-Shisheboran et al. 2001; Velasco et al. 2004, 2007). However, the exact link between BRAF status, MSI, and treatment sensitivity of type II GCTs has to be clarified. For this approach, the TCam-2 cell line might be a suitable tool.

An overall low mutation frequency is rather exceptional for solid cancers, although it seems to be the rule for type II GCTs. That this is indeed not due to the preselection of genes under investigation, but an overall phenomenon is supported by the results of a high throughput investigation on the mutation status of the kinome (Bignell et al. 2006; Greenman et al. 2007). This might again be related to the embryonic origin of

the tumors. In fact, embryonic stem cells have a unique mechanism in which one of the two DNA strands is kept protected against any form of mutations (Hong and Stambrook 2004). This protects the DNA from anomalies to be transmitted to the next generation. The activation of pluripotency of the germ cell after disruption of the integrity of the genome, in type I GCTs (see above), might be related to loss of pluripotency of the immortal strand. Therefore, the power of the mutation status analysis in type II GCTs is limited in elucidating the involvement of various pathogenetic mechanisms and pathways. However, on the basis of the observations made, a number of interesting conclusions can be drawn, especially when different platforms of data are combined. Besides the already mentioned role of KRAS2 and c-KIT, this also accounts for the role of the TP53 in the pathogenesis of type II GCTs.

2.6.9.1 TP53 and MicroRNAs

One of the intriguing observations is that also TP53 is hardly mutated in type II GCTs (Guillou et al. 1996; Moore et al. 2001; Kersemaekers et al. 2002a, b; Mayer et al. 2003; Emanuel et al. 2006). It is however, interesting that TP53 target genes have been found to be frequently hypermethylated in type II GCTs (Christoph et al. 2007). The absence of TP53 mutations has been a matter of much discussion, especially because the observations in the supposed mouse model are counterindicative. The absence of low level of P53 mutations in type II GCTs is a rare phenomenon among solid cancers. The mutations found in TP53 in type II GCTs are predominantly detected in so-called nongerm cell malignancies (Houldsworth et al. 1998). These are somatic cancers formed as a result of progression of a teratomatous element. In fact, these mimic the mutational status of solid cancer in adults, including the mutational status of TP53. The reason for the presence of wild type TP53 remained elusive for a long period of time. The selective pressure on TP53 to be inactivated in many solid cancers is related to its function in overruling cellular senescence upon for example mutation of a proto-oncogene (Lundberg et al. 2000; Yeager et al. 1998). Thereby the organism is protected from the formation of cancers due to single mutations. The explanation for the wild type TP53 status in type II GCTs was obtained as a result of the expression analysis of certain miRNAs. MiRNAs are a subgroup of nonprotein-encoding RNA, which interacts with mRNAs to block translation (Dalmay and Edwards 2006; Mattick and Makunin 2005; Hall and Russell 2005; Sontheimer and Carthew 2005). A close link between miRNA and genetics (Calin and Croce 2006) and epigenetics (Chuang and Jones 2007) has been indicated. Several thousands of miRNAs are expected to exist within the mammalian genome, which underwent an increase in evolution in the human genome (Wienholds and Plasterk 2005). It is assumed that about one-third of the protein-encoding mRNAs are also regulated by miRNAs. In type II GCTs, a specific pattern of expression of miRNA has been observed using a high throughput approach (Gillis et al. 2007). In fact, the tumors were classified into undifferentiated and differentiated components, which indeed support the model that shows that miRNA are involved in regulation of differentiation. The miRNA cluster 371–373 (mapped to chromosome 19) is specifically expressed in the seminomas and embryonal carcinomas. As expected, this set of miRNAs is also expressed in human embryonic stem cells (Suh et al. 2004). This cluster of miRNAs was previously found to be able to mimic the presence of a mutated TP53 in overruling cellular senescence in a high throughput in vitro model system (Voorhoeve et al. 2006). Using a unique series of type II GCTs and cell lines, a good correlation between the level of expression of these miRNAs and the mutational status of TP53 was identified. The miRNAs interact with the 3′ UTR of the mRNA encoding the tumor suppressor gene protein LATS-2, which is involved in the regulation of G1–S transition in the cell cycle. LATS-2 is indeed a downstream target of TP53, and inactivation of TP53 results in absence of LATS-2 protein, thereby overruling cellular senescence. A role of LATS-2 in polyploidization has also been suggested (Aylon et al. 2006). Intriguingly, DND is recently identified to be a regulatory element is this process. In brief, the miRNAs 371–373 mimic the effect of mutated TP53 regarding the interaction with LATS-2. However, this does not influence the function of TP53 in the DNA damage response. That miRNAs have a significant role in the causation and possibly also in differentiation of type II GCTs is supported by the observation of the discrepancy between mRNA and protein of E2F1, which is regulated by the miRNA 17–92 cluster (Novotny et al. 2007). In addition, two miRNAs (miR-145 and

324-5p) are highly expressed in seminoma and the cell line TCam-2, and not in the other histologies, including cell lines (De Jong et al. 2008b). These miRNAs are predicted to interact with the mRNA of SOX2, which is indeed specifically expressed in embryonal carcinoma and cell lines but not in seminoma (see above). It is highly interesting to investigate the involvement of these miRNA in the transition of CIS/seminoma to embryonal carcinoma, likely related to the process of activation of pluripotency (reprogramming), which also occurs during normal development (see Figs. 2.2a and 2.3). In addition, it can be related to the mechanism of suppression of the somatic expression program (omnipotency), which is essential for germ cells.

The role of miRNAs in the pathogenesis of type II GCTs opens an exciting area of research, in which indeed the interactive analysis of miRNA and mRNA expression, DNA copy number changes, and protein expression will be highly informative and also useful for understanding treatment sensitivity (Duale et al. 2007). Currently, a number of histological subtype-specific miRNAs have been, besides the above mentioned examples. These miRNA may give insight into the regulatory elements involved in the pluripotency of type II GCTs, and may be of diagnostic and therapeutic relevance. To facilitate the selection of in vitro and/or in vivo models for type II (and also I and III) GCTs, the following paragraph gives an update on the existing models.

2.6.10 Available Cell Lines and Models

Till recently, only cell lines representative for nonseminomas, i.p. embryonal carcinomas, were available. These have been proven to be of value for many different studies. The most frequently used cell lines are NT2, Tera-1, 833KE, NCCIT, and 2102Ep. It must be kept in mind that NCCIT originates from a primary extragonadal type II GCT, and lacks a functional P53 (Voorhoeve et al. 2006; Damjanov et al. 1993). The JKT-1 cell line proposed to be representative for seminoma has been proven to be unrelated and therefore not informative in the context of type II GCTs (Jo et al. 1999; Kinugawa et al. 1998; Eckert et al. 2007; de Jong et al. 2007b). Therefore, the data published are not relevant for GCTs. In contrast, the TCam-2 cell line is of interest. This cell line has indeed most characteristics of seminoma (Goddard et al. 2007; Eckert et al. 2007; de Jong et al. 2007b), although some nonseminomatous features also are found (to be published elsewhere). One of the intriguing observations is that this cell line has a mutated BRAF, which is rare in type II GCTs (see above). This probably explains the success in generating this cell line. One of the other type II-derived cell lines, i.e., 833KE, contains a KRAS2 mutation. In spite of this possible limitation, for sure the TCam-2 cell line will be valuable for investigation of pathogenetic mechanisms related to the development of type II GCTs, i.p. transition from a seminomatous to a nonseminomatous phenotype. It has to be kept in mind that cell lines have a high incidence of mutated proto-oncogenes compared to an unselected series of type II GCTs of patients. This might be due to an enhanced in vitro survival caused by these mutations.

2.6.11 Pathogenetic Model

On the basis of the different levels of information described, a comprehensive model for the pathogenesis of type II GCTs can be proposed (Fig. 2.3). For sure it does not contain all available information, but it reflects the most interesting observations, and demonstrates the close link with mechanisms involved in normal development.

2.6.11.1 Concluding Points Type II GCTs

PGC/gonocyte origin

Various identified risk factors, mostly related to germ cell maturation delay

Histologically composed of seminoma or nonseminoma

Nonseminoma are omnipotent

OCT3/4 is informative diagnostic marker in adult testis

Seminoma is characterized by OCT3/4+, SOX2−, SOX17 +

Embryonal carcinoma is characterized by OCT3/4+, SOX2±, SOX17−

Predominantly diagnosed in adolescent and young adults

Consistently aneuploid with multiple structural anomalies

Gain of short arm of chromosome 12 is characteristic

Mutations are rarely found

TSPY is a candidate gene for the Y-involvement

Multiple representative cell lines available, including a seminoma cell line

Possible model for suppression of somatic differentiation program

No representative animal model identified

2.7 Overall Conclusions

Different types of human GCT can be recognized; the subclassification proposed here allows a better understanding of the pathogenesis of this type of cancer, regarding cell of origin as well as mechanisms of progression. Overall, GCTs mimic normal germ cell development to a certain extent, which explains both the biology and clinical behavior of the subtypes. Specific markers for diagnosis for the various histological elements have been identified, on the basis of targeted- as well as high throughput approaches. These give insight into the fundamental mechanisms involved in proliferation, differentiation, and apoptosis, also during normal development. An integrated analysis of the different data sets will allow a high level of understanding of the processes involved. On the basis of these observations, novel approaches are under development, in the field of early (noninvasive) diagnosis, treatment, and generation of informative in vitro and in vivo model systems.

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