Pathogenesis of testicular germ cell tumors from a developmental point of view

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Pathogenesis of Testicular Germ Cell Tumors from a Developmental Point of View

Pathogenese van testiculaire kiemceltumoren vanuit een ontwikkelingsperspectief

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Fuer meine Familie und meine Eltern

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General introduction of malignant testicular germ cell tumors

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1. Introduction

Current classification systems of human germ cell tumors (GCTs) are based on histological composition [1-3]. In the group of nonseminomas, different variants of teratoma (somatic differentiation), yolk sac tumor and choriocarcinoma (extra-embryonic differentiation), are recognized, as well as their stem cell component embryonal carcinoma. In addition, the seminomatous tumors are distinguished, subdivided into classic - and spermatocytic variants. The morphologically similar classic seminomas of the ovary are called dysgerminomas, and those of the brain germinomas. Tumors containing both a (classic) seminoma and a nonseminoma component are referred to as combined tumor according to the British classification [4], and as nonseminoma in the World Health Organisation (WHO) Classification [5]. This traditional histological description obscures the biological diversity of this type of cancer [6, 7], which hampers identification of pathogenetic mechanisms and proper comparison of the neoplastic cells to their normal counterparts. Therefore, an alternative classification was proposed, recognizing five categories (I-V) of GCTs (see Table 1), based on site of presentation, age of the patient at diagnosis, histological composition, as well as pattern of genomic imprinting, and chromosomal constitution [6]. This thesis will deal only with the type II GCTs, predominantly of the testis, and therefore the other types will not be discussed here. The testicular type II GCTs will be referred as TGCTs.

2. Epidemiology

In the male Caucasian population, TGCTs account for approximately 1% of all cancers [8, 9]. However, TGCTs represent 60% of all malignancies diagnosed in men between 20 to 40 years of age in the northern European countries [10]. Interestingly, most European countries show a significant rise in the incidence of TGCTs, as also found in the USA (Figure 1) (UK Testicular Cancer incidence statistics, info.cancerresearchuk.org) [8, 11, 12]. This rising incidence has been linked to exposure to environmental compounds, specifically those with estrogen and/or anti-androgen action, while also a genetic predisposition seems to be

involved [13-16]. Other ethnic populations, including Asian and Blacks, show a significant lower incidence, which is not influenced by migration [17, 18].

Table 1: Summary of GCT classification according to Oosterhuis and Looijenga [6], which is based on biology of these tumors. YST: yolk sac tumor.

Type	Anatomical site	Phenotype	Age	Originating cell
I	Testis/ovary/sacral region/retroperito-neum/mediastinum/neck/ midline brain/ other rare sites	(Immature) teratoma/ YST	Neonates and children	Early PGC/ Gonocytes
II	Testis	Seminoma/ non-seminoma	>15 years (median age 35 and 25 years)	PGC/ gonocyte
	Ovary	Dysgerminoma/ non-seminoma	>4 years	PGC/ gonocyte
	Dysgenetic gonad	Dysgerminoma/ non-seminoma	Congenital	PGC/ gonocyte
	Anterior mediastinum (thymus)	Seminoma/ non-seminoma	Adolescents	PGC/ gonocyte
	Midline brain (pineal gland/hypothalamus)	Germinoma/ non-seminoma	Children (median age 13 years)	PGC/ gonocyte
III	Testis	Spermatocytic seminoma	> 50 years	Spermatogo-nium/ spermatocyte
IV	Ovary	Dermoid cyst	Children/ adults	Oogonia/ oocyte
V	Placenta/uterus	Hydatiform mole	Fertile period	Empty ovum/ spermatozoa

In contrast, a role of migration has been reported for immigrants from Finland to Sweden, who have a lower initial risk for TGCTs, but they obtain the risk of the Swedish population at the second generation [19]. These observations demonstrate a significant effect of environmental factors on the incidence of TGCTs within specific ethnic subgroups. Epidemiological observations indicate also that the affected population of cells leading eventually to the invasive cancer are present only during a limited time window during fetal development [20]. Of

interest is that this window seems to be similar to experimental data due to the effect of xeno-estrogens [21], related to gonadal anomalies, as also found in DSD patients at risk for this cancer (see below).

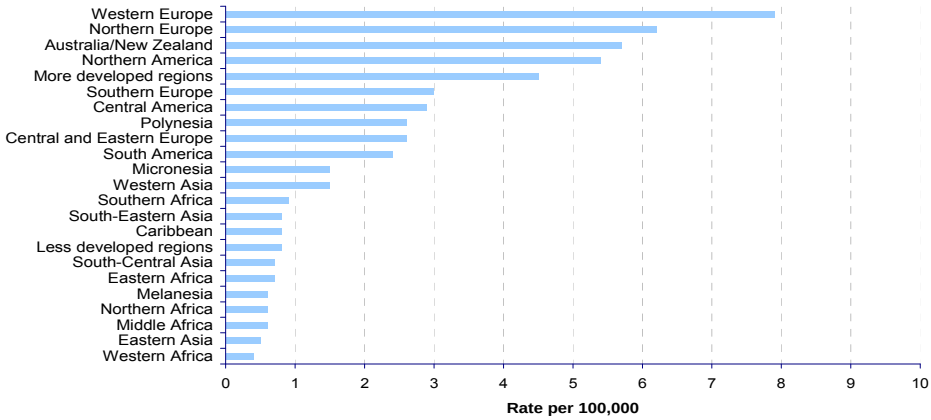

Figure 1: Age-standardized (World) incidence rates for TGCTs, world regions, 2002 estimates.

3. Normal development and differentiation of germ cells

To understand the nature of risk factors for the development of TGCTs, it is highly relevant to understand the process involved in normal gonadal development. Therefore, various aspects of mainly fetal germ cell development will be discussed in the following paragraphs.

3.1 Specification and migration of primordial germ cells in mice

Germ cells in mammals, which function to transmit genetic information to the next generation, are set aside at an early stage during embryogenesis, and are known as primordial germ cells (PGCs)[22-25]. The discrimination of the germ cell lineage from the somatic cells during early development is referred to as specification. In mice, specific transcriptional programs regulate specification of PGCs, prevent them from a continuing drift toward a somatic fate and induce their lineage-specific characteristics. Recent advances are beginning to piece together the key steps that lead to PGC specification [26].

PGCs arise in mice from the proximal epiblast around E6.5 [27] (in humans at week 5-6). These early mouse germ cells can be detected as a cluster of approximately 45 cells based on their high level of alkaline phosphatase activity at E7.25, located at the base of the developing allantois [24]. The postulated key event during germ cell specification is the repression of the somatic cell fate by the transcription factor Blimp1. Targeted deletion of Blimp1 leads to loss of PGCs shortly after specification due to differentiation [26, 28]. In contrast to Blimp1- PGCs, Blimp1+ germ cells repress expression of mesodermal genes, including Fgf8, and Snail, whereas pluripotency-associated genes such as Sox2 and Nanog, in addition to other unique genes for PGCs, such as Stella and Nanos3, are upregulated. Recent studies have shown that Blimp1 acts by binding to Prmt5, since Blimp1/Prmt5 complex was detected in PGCs. Prmt5 is an arginine-specific histone methyltransferase, which mediates symmetrical dimethylation of arginine-3 on histone H2A and/or H4 tails (H2Ame2s/H4R3me2s) [29]. Proposed function of Blimp5/Prmt5 complex is the suppression of premature differentiation and maintenance of pluripotency in PGCs. Schematic representation of a Blimp1/Prmt5 actions in mice is given in Figure 2 below.

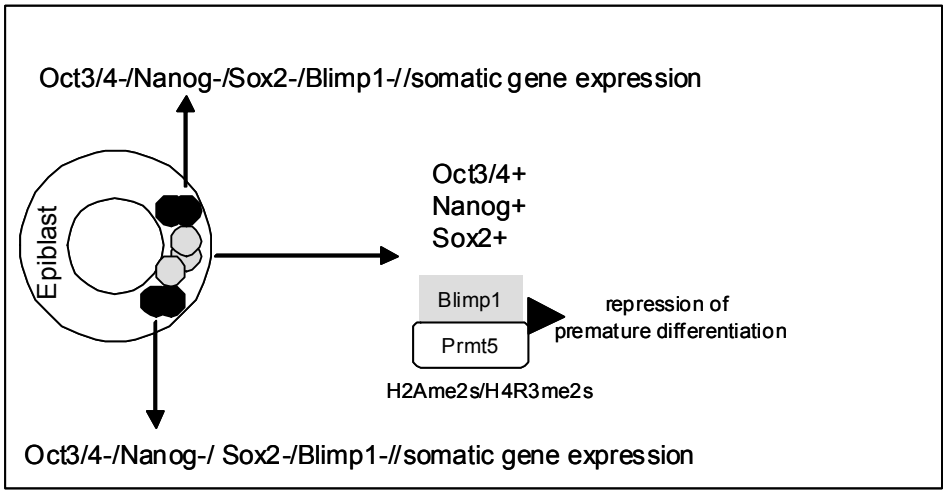

Figure 2. Schematic representation of a selection of factors involved in germ cell specification in mice (E7-E8). In the epiblast, pluripotent PGCs undergo specification by upregulation of Blimp1. Blimp1/Prmt5 complex suppresses premature differentiation by H2Ame2s/H4R3me2s.

After specification, PGCs move along the hindgut to the genital ridges, which will later develop into either ovary or testes [30, 31]. For this migratory process, the stem cell factor (SCF)–c-KIT pathway is crucial [32]. PGCs express the receptor, while the SCF is expressed in somatic cells and functions as a chemo-attractant as well as survival factor [33-35] (see below for further information).

3.2 Epigenetic changes of PGCs

PGCs undergo major changes in nuclear architecture, accompanied by extensive erasure of several histone modifications and exchange of histone variants. The histone chaperones Hira and Nap-1 (Nap111) accumulate in the nuclei of PGCs undergoing this reprogramming [36]. Histone replacement is critical for chromatin rearrangements and histone modifications, such as erasure of histone H3 at lysine 9 dimethylation (H3K9me2) and establishment of histone H3 at lysine 27 tri-methylation (H3K27me3), leading to an overall decrease level of DNA methylation [37]. This general process of reprogramming is followed by a specific demethylation of differentially methylated regions (DMRs) of imprinted genes [38-40]. The methylation pattern of imprinted genes are parental specific, and responsible for the functional difference between a maternally and paternally derived haploid set of chromosomes [41, 42]. The erasure of these imprints is a prerequisite for regeneration of a definitive parental-specific gametic methylation pattern during further germ cell development, i.e., spermatogenesis in the male and oogenesis in the female.

3.3 Postmigratory germ cells

Once the PGCs have reached the gonadal ridges they are called gonocytes. The fate of the gonocytes is dependent on the specific microenvironment of the developing genital ridge, referred to as gonadal sex, i.e., development of either testis or ovary. Based on formation of either Sertoli cells or granulosa cells, the gonocytes will mature to either pre-spermatogonia or oogonia. In 1990, the SRY gene was identified as the testis-determining factor [43, 44]. Inactivation of SRY results, both in mice and in men, in complete sex reversal, i.e., male to female [45]. The crucial gene in the SRY pathway is Sox9 [46]. This

transcription factor is a downstream target of SRY and functions in the formation and maintenance of pre- Sertoli cells, a critical step in testis formation, and subsequent generation of the male phenotype [46, 47]. In females, Wnt4 and the forkhead transcripton factor Foxl2 genes are activated and stimulate the formation of granulose cells, the female counterparts of Sertoli cells [48] (see Figure 3 for review).

Interestingly, most recent data demonstrate that induced absence of Foxl2 in a female mouse results in complete gonadal sex reversal, leading to testicular tissue, without germ cells [49], suggesting that maintenance of the ovarian phenotype throughout life is a active process sustained by Foxl2.

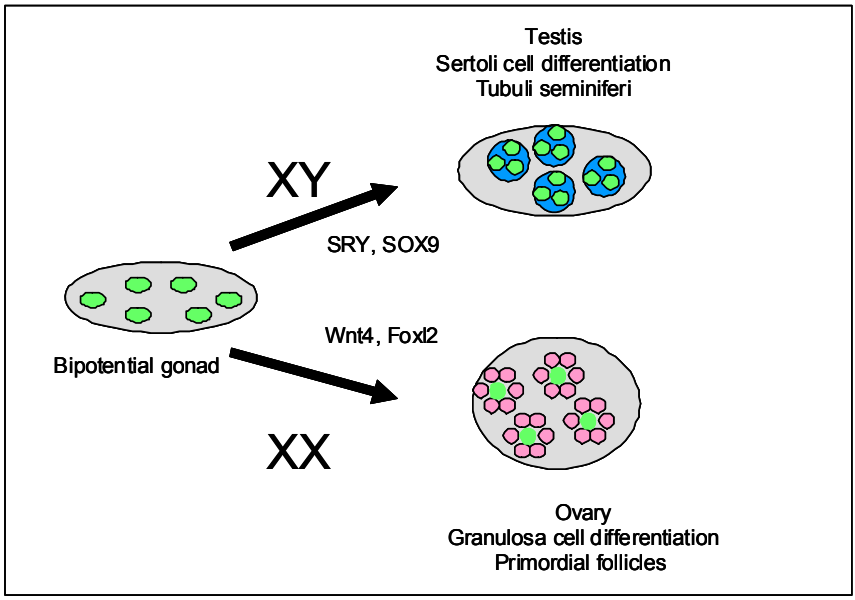

Figure 3. Schematic representation of the earliest changes in gonadal development. In male gonad, SRY upregulates Sox9, which induces differention of Sertoli cells and formation of tubuli seminiferi. WNT4 and Foxl2 are required to suppress Sox9 expression. In the absence of Sox9, granulosa cell differentiation occurs and primordial follicles develop.

During the formation of ovary/testis, both the human male and female gonocytes ondergo the process of differentiation and loose the expression of the embryonic markers, including OCT3/4 [50, 51]. This is in contrast to the mouse gonads, where a subpopulation of gonocytes, a supposed stem cell population, continues to express c-KIT and Oct3/4 [52, 53]. This implies that the knowledge about the

regulation processes in mouse germ cell differentiation can not be simply transferred to the human. Only few data are available so far about differentiation processes in normal human testis (see chapter 2 and discussion).

4. Predisposing factors and precursors of malignant germ cell tumors

4.1. Predisposing factors

While the etiology of ovarian type II GCTs is less known (except for disorders of sex development (DSD) in dysgenetic ovary, see below), risk factors for TGCTs includes history of a previous TGCT, cryptorchidism, sub- or infertility, various forms DSD and familial predisposition [54-58, 59, Chia, 2009 #12865]. Based on epidemiological observations, it has been hypothesized that TGCTs, cryptorchidism, and some cases of hypospadias and low sperm counts, comprise a testicular dysgenesis syndrome (TDS) with a common origin in fetal life [16, 60, 61].

DSD, previously referred to as intersex, is defined as a congenital condition in which development of chromosomal, gonadal, or anatomical sex is atypical [62]. This heterogeneous entity of diseases can be sub-classified further into 3 main groups:

1) Gonadal dysgenesis. This is defined as an incomplete or defective formation of the gonads, as a result of a disturbed process of migration of the germ cells and/or their correct organisation in the fetal gonadal ridge. Structural or numerical anomalies of the sex chromosomes or presumably mutations in sex determining genes underlie these disorders.

2) Hypovirilization syndromes. These may be caused by errors in testosterone biosynthesis, by testicular unresponsiveness to stimulation from the pituitary or by defects in androgen - dependent target tissues and result in an ambiguous or emale phenotype of a 46, XY individual. However, often no specific cause is found.

3) Hypervirilization syndromes. These are 46, XX individuals who are exposed to androgens (e.g due to genetic defects in enzymes involved in adrenal hormone

production) during fetal life. As a result, they show male characteristics in spite of their female karyotype.

Many different causes may lead to DSD, including mutations in genes that play a role in the different developmental programmes and cascades (SRY, Androgen receptor), chromosomal imbalances (of sex chromosomes in Turner syndrome (45,X0), and various forms of mosaicisms) and environmental influences. The risk of type II GCTs in DSD patients is only found in those belonging to category 1 and 2 [63]. Within these groups, it is specifically related to the presence of part of the Y chromosome in the karyotype, likely related to the presence of the TSPY gene [64, 65]. .

Besides DSD, various other less strong predisposing factors have been suggested, which wait confirmation, although birth weight (both low and high) seems to be relevant [66]. So far, it has not been possible to identify genes involved in familial TGCTs [67, 68]. This is likely due to the polygenetic predisposition, as well as the limited power of the families because of their small size. Recently, two genome-wide association studies revealed association of c-KIT ligand variants and TGCT susceptibility [69, 70]. This is of specific interest based on the knowledge of the involvement of c-KIT and its ligand for normal migrations, proliferation and survival of PGCs, as well as the maintenance of expression of c-KIT in the seminomatous tumors as well as the precursor lesion CIS (see chapter 2, chapter 5).

4.2 Precursors

The precursor of all TGCTs is the so-called carcinoma *in situ* of the testis (CIS) [71], also referred to as intratubular germ cell neoplasia unclassified (IGCNU) [5], or testicular intratubular neoplasia (TIN) (Figure 4A).

The hypothesis for the development of CIS is that undifferentiated germ cells with fetal characteristics, the gonocytes, persist till adulthood and transform to neoplastic germ cells. Besides morphology, CIS also show other similarities to PGCs/gonocytes, including erased pattern of genomic imprinting [72, 73], telomerase activity [74], and a hypomethylated epigenetic constitution [75], as well as their pattern of gene expression [76, 77].

CIS cells are located at the inner side of the seminiferous tubules, most frequently in a single row along the basement membrane in close connection with Sertoli cells in adult testis. CIS is often detected in the adjacent parenchyma of invasive TGCTs, especially in nonseminomas [78, 79].

The incidence of CIS in the male Caucasian population is similar to the lifetime risk of developing a TGCT, and it is therefore expected that all patients with this lesion will eventually develop an invasive TGCT [80]. In other words, no spontaneous regression occurs, and, to prevent development of an invasive cancer, clinical intervention is required in patients with CIS.

The CIS counterpart in dysgenetic gonads with a low level of virilization, i.e., no or limited testicular differentiation, is known as gonadoblastoma [81]. The neoplastic germ cells of gonadoblastoma show the same characteristics as CIS cells [63, 82-86]. Gonadoblastoma occurs almost exclusively in children or young individuals with DSD, predominantly with gonadal dysgenesis and hypovirilization (see above). The stromal cells in gonadoblastoma are similar to granulosa cells, based on expression of FOXL2, while the Sertoli cells associated with CIS indeed express SOX9 [87].

In contrast to the testis and DSD patients, the precursor lesion for the ovarian, mediastinal and intracranial type malignant GCTs have not been identified so far, although they are expected to be similar [88, and unpublished observations].

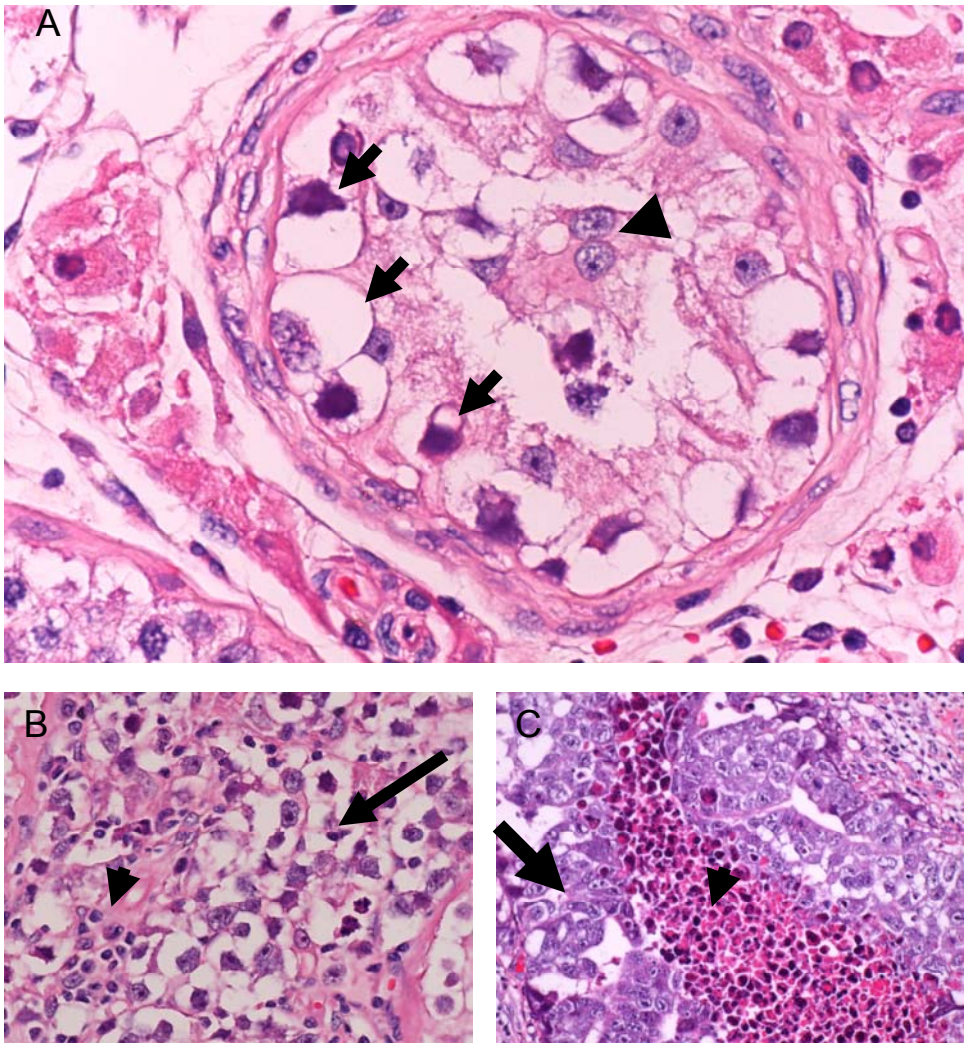

Figure 4: A, carcinoma in situ (CIS, x600), typical morphology of CIS cells, which are atypical germ cells located inside seminiferous tubules (arrow); next to Sertoli cells (arrowhead). B (x200), seminoma cells are large cells with distinct borders (arrow), separated by delicate septa and lymphoid infiltrate (arrowhead). C (x200), embryonal carcinoma sheets containing large cells with hyperchromatic nuclei (arrow), necrosis (center of the figure, arrowhead) are typical in embryonal carcinoma. H&E staining is used.

5. Pathogenesis – Biology of malignant germ cell tumors

5.1 Histology and Classification

As indicated previously, GCTs can be classified based on various systems. The most frequently used are the nomenclature systems according to the British Classification and the WHO Classification [5]. The newer proposal (see Table 1 above) based on biology and natural history of GCTs is gaining popularity [6], and is referred to by the World Health Organization [5], and has been used by American pathologists [7].

Most testicular malignant GCTs in adults are type II tumors, above referred to as TGCTs, and only a small percentage are type III GCT, i.e. spermatocytic seminoma. Overall, about 40% of TGCTs are seminomas and 60% nonseminomas (all other histology than seminomas, with or without seminoma component) (WHO classification). Seminomas are homogenous tumors with typical morphology (Figure 4B), while nonseminomas are a heterogeneous group of tumors including embryonal carcinoma (undifferentiated nonseminoma, stem cell component, Figure 4C), teratoma (somatic differentiation, Figure 5A), yolk sac tumor (Figure 5B) and choriocarcinoma (extra-embryonic differentiation, Figure 5C), as well as the germ cell lineage [89].

Spermatocytic seminomas (type III GCTs) are, as indicated above, significantly less frequent than the classical testicular seminomas in the male Caucasian population, i.e. being about 0.2 vs. 4 per 100 000 respectively [5]. Spermatocytic seminoma shows distinctive clinicopathologic features and occurs in older men, in contrast to TGCT never arises in extratesticular sites and, with extremely rare exceptions, exhibits a benign course. Unlike other testicular tumors, it does not appear to be linked to cryptorchidism and does not share epidemiologic features with the usual forms of TGCTs [90]. Microscopically, the most distinctive feature is its cellular polymorphism, represented by three cell types (small, intermediate-sized and large) (Figure 5D). Recently, genome-wide expression profiling showed that spermatocytic seminomas are derived from spermatocytes, have a clearly specific gene expression compared to seminoma/dysgerminoma and a different pathogenesis with a DMRT1 (a male specific transcriptional

regulator) as a candidate gene [91]. A summary of the histological types of germ cell tumors and their relation to precursor lesions is given in Figure 6.

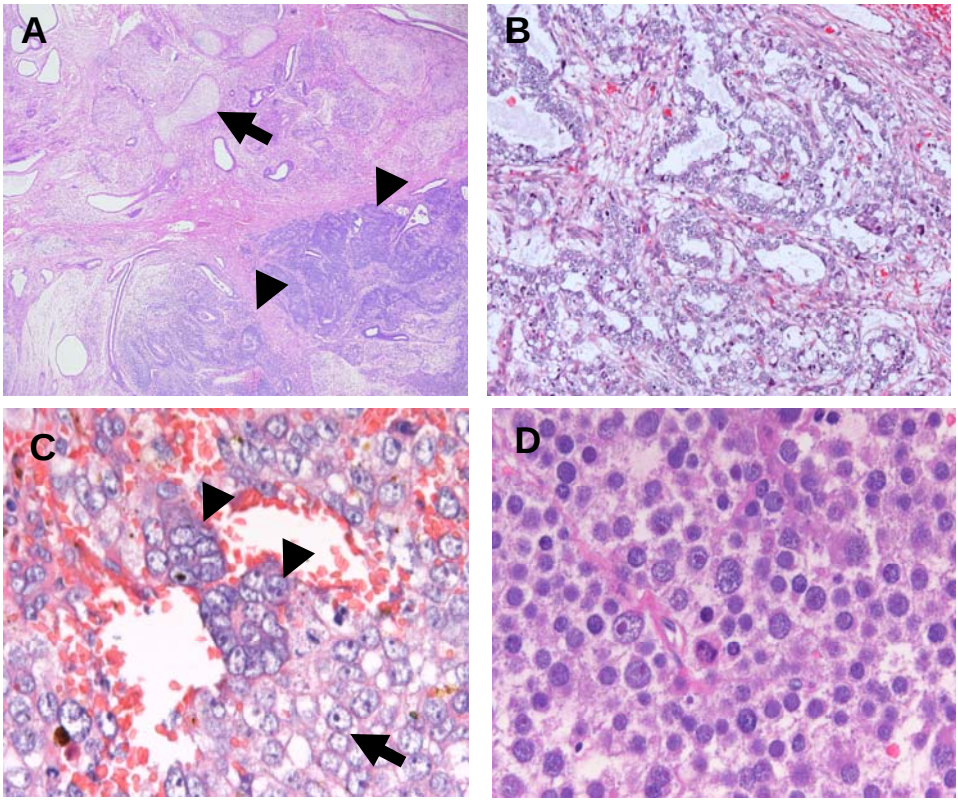

Figure 5: A, teratoma example, consisting of cartilage (arrow), immature stroma, glands, and immature neuronal tissue (arrowhead) (x100). B, example of a Yolk sac tumor consisting of glands with cuboidal cells (x200). C, choriocarcinoma. Network of cytotrophoblastic cells (arrow) and syncytiothrophoblastic cells (arrowhead) (x400). D, spermatocytic seminoma. Mixture of numerous medium sized cells and scattered giant cells with prominent nucleoli (x400).

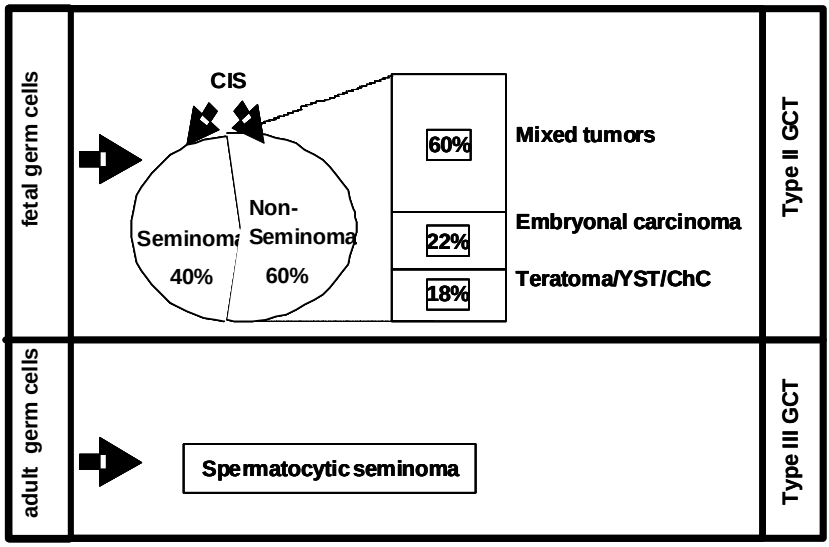

Figure 6: Summary of histological types of GCTs in the testis and their relation to precursor lesions. Both, seminomas and nonseminomas arise from CIS (carcinoma in situ). It is proposed that CIS develop from fetal germ cell, the gonocytes, which escape normal differentiation process and undergo malignant transformation. In contrast, spermatocytic seminoma develops from adult germ cells. YST: yolk sac tumor, ChC: choriocarcinoma.

5.2 Chromosomal constitution

TGCTs are highly aneuploid with specific and characteristic changes. The seminomas and CIS are hypertriploid and the nonseminomas hypotriploid [92-94]. The only recurrent structural imbalance is the gain of the short arm of chromosome 12, mostly as isochromosomes [95, 96]. Most studies indicate that gain of 12p is progression related; it occurs when the CIS cells become independent of their interaction with Sertoli cells [95, 96]. It is interesting that human embryonic stem cell cultured for an extensive period of time also show this anomaly [97-99]. In spite of many attempts, there is no single 12p-target gene identified. A number of other genes have been suggested to be relevant, including KRAS2, NANOG, STELLAR, CCND2, EKI1, BCAT1, although the actual proof is lacking so far [100-108].

The X chromosome is gained in the majority of tumors, for which a link with familial predisposition has been suggested. The presence of additional X chromosomes is relevant in the context of understanding the biology of TGCTs, including the Klinefelter syndrome patients, as well as patients with various forms

of DSD (see above). Interestingly, the supernumerical X chromosomes are inactivated in nonseminomas by methylation [109]. This is, like during normal embryogenesis, the result of the function of the non-(protein)-coding XIST gene. This unique phenomenon in males is correlated with hypomethylation of the promoter region, which can be used as molecular target for TGCTs in males [110, 111].

5.3 Epigenetic modifications

In spite of a wealth of information about the genomic make up of TGCTs, increasing knowledge on the epigenetic constitution is evolving [112-121]. Targeted – as well as genome wide studies demonstrate that overall, the CIS and seminomas show a hypomethylated DNA status, in contrast to the various histological types of nonseminomas [75].

Histone modification has also been identified as a significant regulatory element in specification of genes which will be hypermethylated upon differentiation from an undifferentiated stem cell. This is related to the histone H3 methylated at lysine 27 (H3K27) by polycomb proteins, which is a repressive mark, as well as the active mark methylated histone 3 at lysine 4 (H3K4) [122]. Interestingly, this was indeed found to be the case in cell lines derived from TGCTs, i.e., embryonal carcinoma, in which two additional repressive marks are identified. These are dimethylated histone 3 at lysine 9 (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 de-acetylase (HDAC) in these tumors [123].

5.4 Expression of embryonal stem cell markers

Transcription factor OCT3/4, encoding the POU5F1 protein, regulates whether embryonic stem cells will remain undifferentiated or start to differentiate [124-128]. Two specific variants of the protein encoding OCT3/4 are recognized, of which the A (or I) type is a nuclear protein and is related to pluripotency. The B (or II) variant is localized in the cytoplasm and is not related to regulation of pluripotency. 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 [129-132]. OCT3/4, as detected by verified antibodies regarding specificity and sensitivity, is the most informative diagnostic marker for seminoma and embryonal carcinoma, as well as CIS and gonadoblastoma [133-135]. It remains to be clarified whether OCT3/4 can be considered as an oncogenic driver, as suggested in mice [136]. 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 [137]. Expression pattern of NANOG is similar to OCT3/4 [108, 138-141]. 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 above). However, it needs to be experimentally verified whether such a relationship exist.

The third pluripotency-associated gene, SOX2, is expressed in embryonal carcinoma but not in seminoma and CIS. 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 [142, 143]. SOX2 is associated with OCT3/4 as a complex in the regulation of gene expression in embryonic stem cells, both mouse and human [144-148]. In fact, OCT3/4 levels are regulated by SOX2 [148]. Interestingly, while Sox2 is found in mouse PGCs, it is absent in the human counterparts, which illustrates species specificities in pluripotency regulation [149, 150].

High throughput screening showed that SOX17 (and SOX15 to a lesser extent) is specifically expressed in seminoma and CIS, associated with OCT3/4, but not as such in the various types of nonseminoma [151]. 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 to [152]. Interestingly, SOX17 is identified as a regulatory element to distinguish embryonic from adult hematopoietic stem cells [153, 154]. This observation opens a new field of experiments linking regulation of gene expression related to pluripotency in TGCTs, especially based on the use of the various cell lines representative for this cancer (see below).

5.5 Receptor tyrosine kinase c-KIT

During normal germ cell development, the receptor tyrosine kinase c-KIT is expressed by PGCs and gonocytes [33, 155]. However, c-KIT is not restricted to germ cells, but is physiologically expressed in cells of the haematopoietic system and by mast cells, as well as by melanocytes and interstitial cells of Cajal [156]. c-KIT is normally activated by its ligand stem cell factor (SCF) which upon binding induces dimerization of receptors, activation of the receptors intrinsic tyrosine kinase activity and phosphorylation of signal transduction molecules leading to downstream signaling [157]. The phosphorylated tyrosine residues provide docking sites for signaling proteins, which leads to activation of downstream pathways such as the MAPK pathway, the PI3-kinase pathway and the Jak-Stat pathways [157]. Multiple previous immunohistochemical studies showed that c-KIT is highly up-regulated in CIS and is retained in seminomas but not expressed in non-seminomas [158, 159]. In previous studies not discriminating between unilateral and bilateral TGCT, most mutations have so far been detected in exon 17 in seminomas, with varying frequencies of up to 40% [160-162]. Furthermore, it was shown by one study that bilateral as opposed to unilateral TGCTs are highly associated with activating c-KIT mutations [163]. The role of c-KIT in the development and progression of TGCTs is not clarified so far.

5.6 Transcriptional factor AP-2gamma

Transcription factor AP-2gamma belongs to a family of five closely related genes found to be expressed mostly during embryogenesis. Members of this family display a high sequence homology and share three characteristic domains, an N-terminal transactivation domain, a central basic domain and a C-terminal dimerization domain. Basic and dimerization domain mediate DNA-binding as those transcription factors bind to various G/C-rich elements on the promoter regions of the different target genes [164, 165]. AP-2 proteins are expressed in many tissues during development and null mutants of the different factors show severe phenotypes and die during embryogenesis or shortly after birth as shown for AP-2alpha [166], AP-2beta [167] and AP-2gamma [168, 169]. Disrupting AP-2gamma results in growth retardation of the embryo at E7.5 and death around E9.5

due to a failure of the trophectoderm cells to proliferate and form a proper labyrinth layer. As a consequence, gastrulation is severely impaired and the embryo dies of malnutrition [168, 169]. In the embryo, AP-2gamma expression has beeņ described in a variety of tissues, including post migratory germ cells [170].

AP-2gamma was detected in mouse PGCs/gonocytes throughout embryogenesis and its functions were recently studied in the mouse model [171]. In a conditional mouse model, PGCs were specified but were lost around E8.0, causing a complete loss of germ cells in sterile animals, both males and females.

5.7 Mutational status

Various studies with the goal to identity pathogenetic mutations have been performed on TGCTs. These included a large number of targets, amongst others, NRAS, KRAS-2 and HRAS [172-177], and BCL10 [178, 179]. Although mutations have been identified, these seem to be limited in frequency, with the possible exceptions of c-KIT (see above), and more recently BRAF [180]. This latter proto-oncogene has been shown to be mutated in a variety of cancers, including malignant melanoma.

An overall low mutation frequency of mutations which is seen in TGCTs is rather exceptional in solid cancers [181, 182]. This is indeed not due to the pre-selection of genes under investigation, but an overall phenomenon, as supported by the results of a high throughput investigation on the mutation status of the kinome [181, 182]. This specific biology of malignant germ cells might be related to the embryonic origin of TGCTs. In fact, embryonic stem cells have a unique mechanism by which one of the two DNA strands is kept protected against any form of mutations [183]. This protects the DNA from anomalies to be transmitted to the next generation. Therefore, the power of the mutation status analysis in TGCTs is limited in elucidating the involvement of various pathogenetic mechanisms and pathways.

5.8. Available cell lines

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. NCCIT originates from a primary extragonadal type II GCT, and lacks functional P53 [184, 185]. The TCam-2 en JKT-1 cell line had been proposed to be representative for seminoma [186-188]. The seminoma cell line would be of high interest for investigation of pathogenetic mechanisms related to the development of TGCTs, i.p. the transition from a seminomatous to a nonseminomatous phenotype.

6. Therapy of malignant germ cell tumors

Nonseminomas differ from seminomas in terms of their clinical and biologic behavior, and these differences are therapeutically relevant. Seminomas are exquisitely sensitive to radiation therapy (RT) while nonseminomas are more radiation-resistant. Unlike nonseminomas, seminomas have relatively indolent growth biology and a longer natural history. As a result, the median time to relapse is longer than with nonseminomas and late relapses are more common [189]. Cisplatin-based combination chemotherapy can cure patients with disseminated type II (T)GCTs, even in the context of widespread visceral metastases, highly elevated serum tumor markers, and other adverse prognostic features. According to the International Germ Cell Consensus Classification Group (IGCCCG), several prognostic factors for seminoma and nonseminomas have been identified. In fact, three prognostic groups are identified, being good -, intermediate and poor risk [190]. The latter only includes nonseminomas. For nonseminomas the factors are mediastinal primary side, high levels of AFP, LDH and beta-HCG and presence of nonpulmonary visceral metastasis (liver, bone, brain). The latter finding is also a predominant adverse factor for a seminoma. Integration of these factors produce the three mentioned groups with different 5-year survival rate: good prognosis with a survival rate of 91%, intermediate prognosis with 79% 5-year survival rate, and only for the nonseminoimas poor prognosis with a 48% survival rate [190].

Staging of TGCTs define stage groupings from I to III and integrate the assessments of primary tumor, vascular invasion, invasive growth in epididymis, tunica albuginea and vaginalis, as well as rete testis, lymph node and distant metastasis, combined with serum tumor marker values for beta-HCG, AFP, and LDH [190, 191].

In patients with clinical stage I seminoma, an extremely high cure rate can be achieved with radical orchiectomy, whether followed by active surveillance, radiotherapy to para-aortic lymph nodes, or single agent carboplatin chemotherapy. In stage I nonseminoma, active surveillance, retroperitoneal lymph node dissection (RPLND), or an abbreviated course of adjuvant chemotherapy are all feasible options for appropriately selected patients. All three approaches are associated with a cure rate over 95% because of the ability to salvage patients who relapse [192].

For patients with stage II nonseminoma and higher, men typically undergo RPLND, which may be followed by adjuvant chemotherapy if a substantial cancer burden is confirmed pathologically. In men with seminomas and retroperitoneal lymph nodes >5 cm in diameter at initial diagnosis are usually treated with cisplatin-based chemotherapy [193].

Sensitivity to DNA-damaging agents of type II (T)GCTs is supposed to be a multifactorial mechanism and is related to the embryonic characteristics of these tumors. One of the mechanisms of chemotherapy sensitivity in ES cells is the lack the G1 arrest checkpoint due to cytoplasmatic CHK2 [183]. The apoptosis upon generation of DNA damage prevents transmission of defects to the ES progeny. In addition, ES cells have a different spectrum of mutations compared to somatic cells. In parallel to ES cells, TGCTs show an exceptionally low level of mutations of the receptor kinases [181, 194, 195]. These findings suggest a different pattern of DNA damage repair in embryonic versus adult cells. Loss of these embryonic characteristics upon the progression of nonseminomatous type II (T)GCTs is inducing resistance against chemotherapy and radiation, as found in the majority of solid cancers.

7. Aims and outline of this thesis

7.1 Aims

Overall germ cell tumors comprise a heterogeneous group of benign and malignant tumors. Based on a specific set of biological parameters, five types of GCTs can be distinghuished. Among these, the seminomatous and non-seminomatous GCTs in males and females are designated as type II GCTs, and specifically as TGCTs of the testis. This is in fact the most frequent type of solid cancer in Caucasian males between 20 and 45 years of age, with a rising incidence. In spite of various studies performed, there is a significant lack in the understanding of the pathogenesis of TGCTs. Similarities between normal embryogenesis and TGCTs are obvious. This is likely due to the model that the precursor of TGCTs, being CIS, is an embryonic germ, cell, either a PGC or gonocyte, which escapes the physiological process of maturation and differentiation, and consequently is at risk for transformation. Human fetal development and germ cell differentiation differs from the mouse, but studies in humans are rare. Thus, characterization and further understanding of human fetal germ cell development, specifically on the role of proteins involved in germ cell specification and maturation will further elucidate the mechanisms involved in the formation of CIS. Therefore, a number of studies were undertaken to shed light on the involvement of a selection of oncofetal genes/proteins in normal and malignant germ cells, of which no or limited information was available, c-KIT, AP-2gamma, BLIMP1, PRMT5, histone H2A and H4 dimethylation. Both immunohistochemical studies as well as mutation analyses (c-KIT) were performed on a defined series of embryonic male gonads of various developmental ages and representative cases of the various histological type of type II GCTs, predominantly TGCTs, including the precursor lesion CIS. Furthermore, one of the aims of this work was to establish a seminoma model to perform function studies examining the role of the genes mentioned above.

7.2 Outline

Chapter 1 gives a general introduction on TGCTs.

In **Chapter 2** the special expression pattern of a number of oncofetal genes were investigated in a series of male fetal gonads from the week 12 to the newborn period. The results indicate that two subtypes of fetal germ cells can be identified. The first category are germ cells that morphologically resemble gonocytes, and are positive for OCT3/4, c-KIT, M2A and AP2gamma. The number of this specific variant of germ cells increased till week 18/19, and subsequently decreased. After week 25, the major population was pre-spermatogonia, positive for MAGE-A4 and negative for the others.

In **Chapter 3** it is demonstrated that AP-2gamma is expressed in gonocytes between week 12 and 37, while it was downregulated upon further maturation of the germ cells. In addition, AP2gamma was found to be expressed in all CIS, and seminomatous tumors, independent of stage and anatomical localization. The nonseminomatous histologies embryonal carcinomas and choriocarcinomas showed a heterogeneous pattern, while the others as well as normal testes were negative.

In **Chapter 4** the expression pattern of BLIMP1 and PRMT5 as well as the modification of histone H2A and H4 arginine dimethylation was investigated. Both, male gonocytes as well as CIS and most seminomas showed a positive staining, which was less in the embryonal carcinomas and differentiated derivatives.

In **Chapter 5** the occurrence of activating mutations of c-KIT tyrosine kinase receptor was studied in a series of bilateral and unilateral TGCTs. It demonstrated the higher incidence of this mutation in the bilateral tumors. In addition, downregulation of protein expression in seminomas compared to their precursor CIS was observed.

In **Chapter 6** two supposed TGCT-derived cell lines were investigated. The expression profiling analysis showed that TCam-2 has indeed characteristics of seminomas, being therefore the first seminoma cell line. However, the TGCT-origin of the JKT1 cell line was questioned.

In **Chapter 7** the observations made in the different chapters are summarized and discussed in detail, and integrated into the current knowledge on the pathobiology of TGCTs.

8. References

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Chapter 2

Spatial expression of germ cell markers during maturation of human fetal male germ cells: an immunohistochemical study

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Abstract:

The aim of the present study was to examine fetal male germ cells for expression of proteins associated with differentiation and maturation and to compare them with morphologically defined subpopulations. Testes of 61 fetuses from week 12 of gestation to the newborn period were selected. Immunohistochemistry was performed using antibodies to proteins associated with differentiation of germ cells (c-KIT, AP-2) or pluripotency (OCT3/4), oncofetal protein M2A and spermatogonial marker MAGE-A4. Two subtypes of fetal germ cells were detected by quantification and immunohistochemistry. Nearly all germ cells with morphological criteria of gonocytes and intermediate cells co-expressed OCT3/4, c-KIT, M2A and AP-2. Starting from week 12, their number increased up to week 18/19 and then declined continuously during further development. After week 25, pre-spermatogonia were predominant and expressed MAGE-A4 selectively. Fetal male germ cells are comprised of two major groups with distinct immunohistochemical phenotypes. Germ cells that are predominantly found before week 25 of gestation co-express oncofetal proteins OCT3/4, c-KIT, M2A and AP-2gamma. After week 25, most germ cells have lost their pluripotent potential and acquire a spermatogonial phenotype defined by expression of MAGE-A4.

Introduction:

Primordial germ cells (PGC) are pluripotent cells that migrate to the genital ridge where they are called gonocytes in male and oogonia in female gonads. In female and male murine embryos, germ cells then first undergo several rounds of mitosis before they enter a pre-meiotic stage by 12.5 days post-conception by up-regulation of meiotic genes such as Scp3 (Di Carlo et al., 2000). In male mice, block of meiosis is accompanied by Sertoli cell differentiation and the occurrence of pre-spermatogonia (McLaren, 2003). Unfortunately, the maturation process of fetal germ cells in humans is not fully understood although previous studies indicate that, in contrast to rodents, human fetal germ cells are non-homogeneous in terms of morphology and marker expression (Fukuda et al., 1975; Wartenberg, 1976; Franke et al., 2004; Gaskell et al., 2004). For example, from an examination of 25 embryonic and fetal testes, Gaskell et al. (2004) detected three different germ cell populations with different combinations of immunohistochemical markers.

Detailed knowledge of the regular differentiation process is the prerequisite for identifying pathological changes in germ cell differentiation. For example, developmentally arrested fetal germ cells are supposed to be a source of carcinoma in situ or intratubular germ cell neoplasia unclassified (IGCNU) (Skakkebaek et al., 1987). The hypothesis of the embryonic/fetal origin of neoplastic germ cells has been substantiated mainly by phenotypic similarities between neoplastic and fetal germ cells (Honecker et al., 2004a) as well as by genomic studies (Almstrup et al., 2004; Hoei-Hansen et al., 2004) and epidemiological data (Moller and Skakkebaek, 1999; Jacobsen et al., 2000). In fact, previously published studies indicate that fetal germ cells express a number of markers also found in neoplastic germ cells including placental alkaline phosphatase (PLAP), glycosylated monomeric sialoglycoprotein M2A and other proteins that are believed to be involved in the maturation process of fetal germ cells including transcription factors OCT3/4 and AP-2 and the receptor tyrosine kinase c-KIT (Franke et al., 2004; Hoei-Hansen et al., 2004b; Honecker et al., 2004b; Rajpert-De Meyts et al., 2004). Detailed studies of the expression of these

markers during fetal development have not been done until now; thus the aim of the present work was to examine the physiological maturation process of fetal germ cells during the second and third gestational trimesters by detection of c-KIT, OCT3/4, MAGE-A4, AP-2 and the oncofetal marker M2A. Therefore, we prepared tissue arrays from 61 testes of normally developed fetuses from gestational week 12 to the full-term neonate. Sertoli cells were detected by antibodies to inhibin and cytokeratin 18 (CK18). Other proteins were selected based on their involvement in regulation of the cell cycle (Ki-67) or their association with Sertoli–germ cell interaction and differentiation including neural cell adhesion molecule (NCAM) (Orth et al., 2000), E-cadherin (Di Carlo and De Felici, 2000; Honecker et al., 2004b), connexin43 (Perez-Armendariz et al., 2001) and androgen receptor (Zhou et al., 1996; Sharpe et al., 2003).

Because the expression of MAGE-A4 in IGCNU has been controversial in previous reports (Aubry et al., 2001) and in order to demonstrate whether melanoma-associated antigen (MAGE-A4) and other oncofetal proteins are co-localized in neoplastic germ cells of IGCNU, we also analysed 6 IGCNU samples from patients with invasive germ cell tumours.

Material and Methods:

Tissue samples

Fetal tissue stored at room temperature was selected from the archives of the Section of Pediatric Pathology, Department of Pathology, University of Bonn Medical Center. Use of the tissue for scientific purposes was approved by an institutional ethics committee. The tissue samples included fetuses from weeks 12 (n = 2), 13 (n = 3), 14 (n = 3), 15 (n = 3), 16 (n = 4), 17 (n = 3), 18 (n = 3), 19 (n = 3), 20 (n = 3), 21 (n = 4), 22 (n = 2), 23 (n = 3), 24 (n = 2), 25 (n = 3), 26 (n = 2), 27 (n = 2), 32 (n = 2), 33 (n = 3), 34 (n = 3), 35 (n = 2), 36 (n = 2), 37 (n = 3) of pregnancy and one from a neonate that had died within 24 h after birth (n = 61). None of the fetuses had signs of maceration or autolysis at autopsy and sufficient tissue preservation was confirmed by haematoxylin–eosin staining. Causes of death were spontaneous abortion (mainly amnion infection or placental insufficiency), neonatal death or induced legal terminations. All terminations were performed in the Gynecology and Obstetric Clinics, University of Bonn. The developmental age of the fetuses was determined by the date of the last menstrual bleeding. Weight and length measurements evaluated at the autopsy were used to assure proper gestational development.

In addition, six cases of IGCNU from patients with different germ cell tumours (one seminoma, three embryonal carcinomas, one yolk sac tumour and one choriocarcinoma) were retrieved from the tissue archives of the Department of Pathology, University of Bonn Medical Center. The ages of the patients were 33, 22, 20, 18, 17 and 19 years respectively.

Immunohistochemical staining

Testes were dissected, fixed in 4% phosphate-buffered formalin for 2 days at room temperature and processed in paraffin wax. Dewaxed, paraffin-embedded 4 µm thick tissue sections were microwave-pretreated in Tris–EDTA buffer (10 mmol/l tris base, 1 mmol/l EDTA solution, 0.05% Tween 20, pH 8.0). Primary antibodies to the following proteins were used: MAGE-A4, M2A-antigen, c-KIT, OCT3/4, AP-

2gamma, inhibin, androgen receptor, connexin43, E-cadherin, NCAM, cytokeratin 18 and Ki-67. Details of antibodies and the dilutions for immunohistochemistry are given in Table I. Single immunohistochemistry was performed using the Dako EnVision-AEC Kit and manufacturer's protocol (Dako, Hamburg, Germany). Briefly, endogenous peroxidase was blocked for 5 min in 0.03% H2O2 (diluted in distilled water). Sections washed in Tris-buffered saline (TBS; 0.05 mol/l Tris and 0.85% NaCl, pH 7.6) were incubated with primary antibodies overnight at 4°C. Thereafter, a horseradish peroxidase (HRP)-labelled polymer conjugated with a secondary antibody was applied (Dako EnVision-AEC Kit). The staining was visualized with 3-amino-9-ethyl-carbazole and counterstained with haematoxylin. Negative controls were performed using buffer instead of the primary antibody and resulted in complete absence of signal. Additional negative controls were performed by incubation with pre-immune serum from each animal species (rabbit, mouse, goat) instead of a primary antibody and also resulted in complete absence of an immunohistochemical signal.

Tissue array preparation, assessment of immunohistochemical staining and image capture

From each case, three 1.0 mm cores of testicular tissue were randomly taken to form a 20x30x10 mm recipient paraffin block. A total of 183 cores were taken and placed in two array blocks in ascending order of gestational age. Semiquantitative assessment of the immunohistochemical staining with antibodies to M2A-antigen, c-KIT, OCT3/4, AP-2gamma, and Ki-67 was performed by counting the positively stained germ cells in 5 high power fields (HPF) in each case (area of a single HPF 0.125 mm2, objective for HPF x40). The total number of germ cells was obtained from counting in slides stained with inhibin alpha, which marked Sertoli cells and omitted germ cells. Whole tissues from one testis from each of gestational weeks 14, 19, 22, 27, 33 and 37 and from the neonate were, in addition, selected for immunohistochemical detection of MAGE-A4, OCT3/4, M2A, AP-2□, c-KIT and Ki-67. This was done to ensure that results obtained from tissue microarrays are representative of the whole testis. For this purpose, 15 randomly selected high power fields were counted in stained whole tissue sections. The counting was done

independently by two different observers (K.P. and H.Z.). Non-immunofluorescence images were recorded using a Leica DMR photomicroscope (Leica, Bensheim, Germany). Fluorescence images were photographed using a Zeiss LSM Axiovert 2.05 microscope (Zeiss, Oberkochen, Germany).

Table 1: Antibodies used in the present study

Antigen	Origin	Code	Dilution	Source
c-KIT	Rabbit	A4502	1:100	DAKO, Hamburg, Germany
OCT3/4	Goat	sc-8629	1:1000	Santa Cruz Biotechnology, CA, USA
AP-2γ	Rabbit	sc-8977	1:200	Santa Cruz Biotechnology
MAGE-A4	Mouse	MAGE-A4	1:100	Spagnoli, Basel, Switzerland
M2A	Mouse	D2-40	1:200	Abcam, Cambrigde, UK
Cytokeratin 18	Mouse	DC-10	1:100	DAKO
Inhibin α	Mouse	R1	1:100	DAKO
NCAM (CD56)	Mouse	1B6	1:50	Novocastra Laboratories, Newcastle, UK
Androgen Receptor	Mouse	AR441	1:50	DAKO
E-Cadherin	Mouse	Sc-8426	1:100	Santa Cruz Biotechnology
Connexin43	Mouse	Cx43	1:100	Biotrend, Cologne, Germany
Ki-67	Mouse	Mib-1	1:100	Dianova, Hamburg, Germany

Results

The immunohistochemical profile of germ cells was studied in normal testes of 60 fetuses and one neonate. The summary of semiquantitative assessment for each staining using antibodies to M2A, c-KIT, AP-2γ, OCT3/4, MAGE-A4 and Ki-67, the absolute numbers of germ cells and the calculated ratio of germ cells to Sertoli cells are shown in Table 2. Illustrations of representative staining obtained from single and double staining are depicted in Figure 1, Figure 2 and Figure 4, and those from double staining in Figure 3. Figure 5 shows the relative distribution of fetal germ cells expressing M2A, c-KIT, AP-2γ, OCT3/4 and MAGE-A4 during the second and third trimester.

Counting of germ cells and proliferation index

In the testes from the term neonate, germ cells were clearly distinguishable from Sertoli cells using H&E staining, since germ cells at this stage have large-sized and round nuclei compared to the cylindrical shape of Sertoli cells. In contrast, during the second and the early third trimester, germ cells varied significantly with respect to size and nuclear morphology. Therefore, immunohistochemistry with an antibody to inhibin α was employed to distinguish germ cells from Sertoli cells reliably. Similar gestational weeks were grouped for calculations (Table 2). The number of germ cells from each case was counted in 5 independent high power fields. In parallel, the number of Sertoli cells was determined and the ratio of germ to Sertoli cells was calculated (Table 2). These investigations were done mainly to estimate the distribution of germ cells positive for selected markers MAGE-A4, M2A, c-KIT, AP-2γ, Ki-67 and OCT3/4 in relation to all germ cells at the respective developmental week.

As shown in Table 2, at the $12^{th}/13^{th}$ week, an average of 46 germ cells per HPF was counted. The ratio of germ to Sertoli cells was 1:6.5 and the proliferation marker Ki-67 was expressed in 17% of germ cells. At the $18^{th}/19^{th}$ gestational week, an increase of germ cells up to 70 per HPF was accompanied by high expression of the proliferation marker Ki-67 (21%) and a higher ratio of germ cells to Sertoli cells (1:5). Thereafter, the number of germ cells per HPF decreased again and around the 36^{th} gestational week, 43 germ cells per HPF were counted.

At the same time, the ratio of germ to Sertoli cells decreased to 1:12 (week 37/neonate) and again 21% of germ cells were positive for Ki-67.

Table 2: Numbers of germ cells positive for M2A, c-KIT, AP-2γ, OCT3/4, MAGE-A4 and Ki-67 in fetal and newborn testes in relation to the total number of germ cells per high power field.

	Gestational age (weeks)										
	12/13	**14/15**	**16/17**	**18/19**	**20/21**	**22/23**	**24/25**	**26/27**	**32/33**	**34/35**	**36-nb**
N. of cases	5	6	7	6	7	5	5	4	5	5	6
Total No germ cells (per HPF)	46±5	51±3	60±6	70±5	60±7	55±3	53±4	52±2	53±3	48±4	43±5
GC/SC ratio	1:6.5	1:6	1:6	1:5	1:6	1:7	1:7	1:7	1:8	1:11.5	1:12
Ki-67	8±2 **17%**	10±2 **18%**	9±3 **15%**	15±1 **21%**	7±1 **12%**	6±2 **11%**	7±1 **13%**	10±1 **19%**	9±3 **17%**	11±2 **23%**	9±2 **21%**
M2A	42±3 **91%**	46±2 **90%**	48±3 **80%**	67±5 **96%**	41±3 **68%**	33±7 **60%**	27±5 **51%**	12±4 **23%**	5±1 **9%**	6±1 **12%**	3±1 **7%**
c-KIT	40±8 **87%**	42±5 **82%**	46±6 **77%**	65±5 **88%**	36±4 **60%**	34±8 **58%**	22±4 **41%**	10±1 **19%**	3±1 **6%**	3±2 **6%**	2±1 **5%**
AP-2γ	35±2 **76%**	38±4 **74%**	39±2 **65%**	58±5 **82%**	33±8 **55%**	30±6 **54%**	24±4 **45%**	9±5 **17%**	1±1 **2%**	1±0 **2%**	0 **0%**
OCT3/4	34±2 **74%**	36±7 **71%**	41±5 **68%**	55±5 **78%**	32±6 **53%**	28±4 **51%**	20±5 **38%**	8±1 **15%**	0 **0%**	1±2 **2%**	0 **0%**
MAGE-A4	2±3 **4%**	6±2 **11%**	8±1 **13%**	9±1 **13%**	16±3 **27%**	20±1 **36%**	26±4 **50%**	35±5 **67%**	40±2 **75%**	38±5 **79%**	40±4 **93%**

Average numbers and standard deviation were calculated from counting of positive cells in 5 independent high power magnification fields (x40, field of 0.125mm^2) Total number of germ cells was defined as inhibin α negative cells per HPF, calculated as an average number from 5 HPF. Percentages result from relation of average number of the respective marker) and total number for each gestational week GC/SC ratio results from relation of total germ cells (GC) to Sertoli cells (SC) per HPF
nb newborn

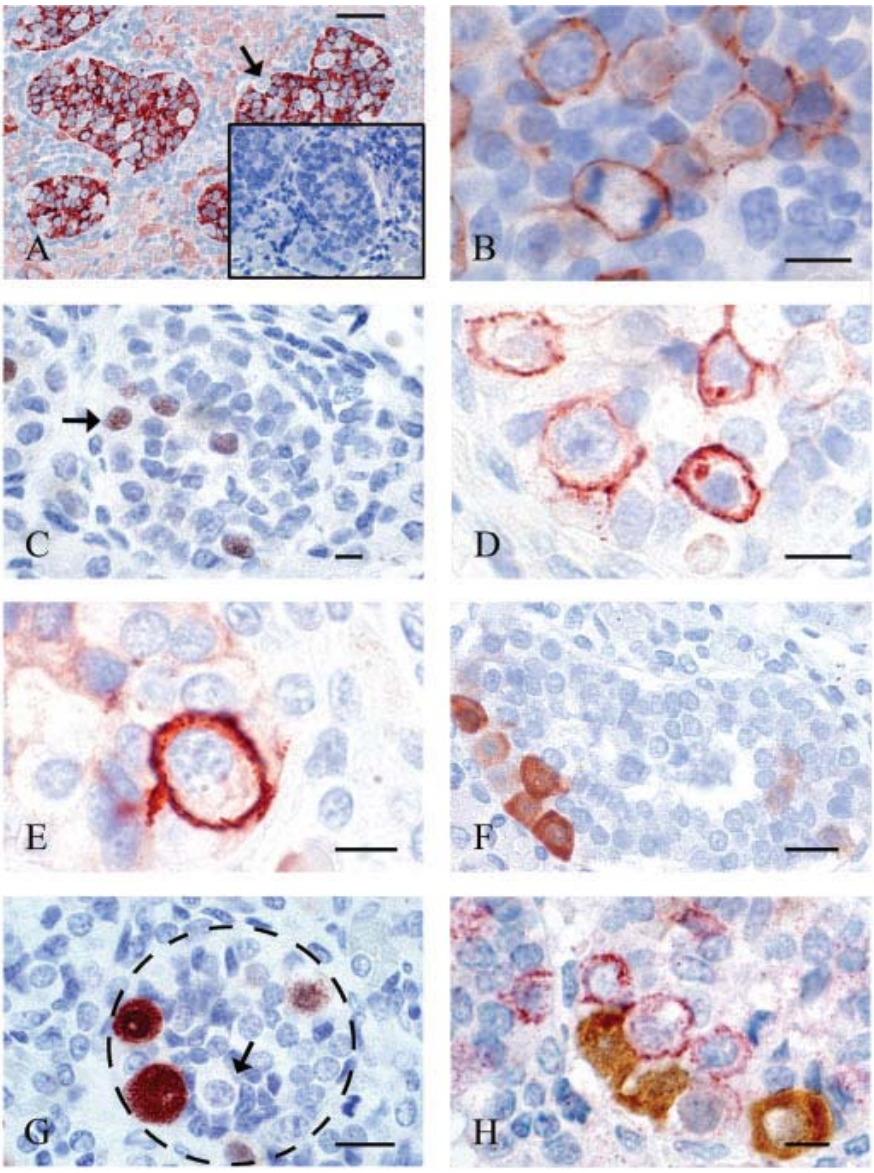
Immunohistochemical detection of c-KIT, M2A, AP-2γ, OCT3/4 and MAGE-A4 in single staining

Immunohistochemistry with c-KIT and M2A antibodies showed the expected membranous staining of germ cells. MAGE-A4 was expressed in the cytoplasm of germ cells. Antibodies to OCT3/4 and AP-2γ produced distinct and strong nuclear staining in germ cells. The number of germ cells expressing each of the proteins was estimated from single-stained immunohistochemical slides. The average value in 5 high power fields was calculated and divided by the absolute number of germ cells from the same gestational period (Table 2, Figure 5).

As seen in Table 2 and Figure 5, roughly equal numbers of fetal germ cells expressed M2A, c-KIT, OCT3/4 and AP-2γ. Around the 12^{th}/13^{th} week, most germ cells detected were positive for M2A (91%), c-KIT (87%), AP-2γ (76%) and OCT3/4 (74%). The number of positive germ cells increased further up to the 18^{th}/19^{th} weeks of gestation (M2A 96%; c-KIT 88%; AP-2γ 82%; OCT3/4 78%) and began to decrease gradually after the 19^{th} week. For example, around the 24^{th} week, the number of germ cells expressing c-KIT declined to 22/53 (41%) and decreased further at the 35^{th} gestational week (2/43, 5%). During the last weeks of the third trimester, expression of M2A, c-KIT, OCT3/4, AP-2γ was found only in loose apoptotic germ cells in the lumen of the tubules. In contrast to the decreasing number of germ cells positive for M2A, c-KIT, OCT3/4, and AP-2γ, a continuously increasing number of MAGE-A4-positive cells was observed. For example, at the 12^{th}/13^{th} week, only a few germ cells expressed MAGE-A4 (2/46, 4%). MAGE-A4 expression increased continuously during further development, reached 50% at week 24 and 93% in the testis of the neonate.

To confirm the results obtained from immunohistochemical staining of tissue arrays, whole testes from the 14^{th}, 19^{th}, 22^{nd}, 27^{th}, 33^{rd} and 37^{th} week and from a neonate were stained with antibodies to OCT3/4, M2A, c-KIT, MAGE-A4, Ap-2γ and Ki-67. The numbers of positive germ cells assessed in whole testes were identical with the results obtained from tissue arrays.

Figure 1: Immunohistochemical analyses of germ cells at the 20th week of gestation



(A): Inhibin α positive Sertoli cells strongly contrast to negative germ cells (arrow) (weakly stained cells between tubules are Leydig cells). (B-D) Germ cells morphologically typical of gonocytes stained by antibodies to c-KIT (B), OCT4 (C, arrow) and M2A (D). (E) Intermediate germ cells also stained by M2A antibody. (F+G) The spermatogonial marker MAGE-A4 was expressed only in basally located pre-spermatogonia but not in intermediate cells (arrow in G). (H) In co-localization studies, MAGE-A4 (brown) and M2A (red) were never co-expressed in germ cells. (A-E) and (G-H) 20th gestational week, (F) 25th gestational week. Scale bars: (A) = 50µm, (B-E, H) = 10µm, (F,G) = 20µm.

Histological and immunohistochemical correlation analyses and co-expression studies of c-KIT, M2A, AP-2γ, OCT3/4, Ki-67 and MAGE-A4

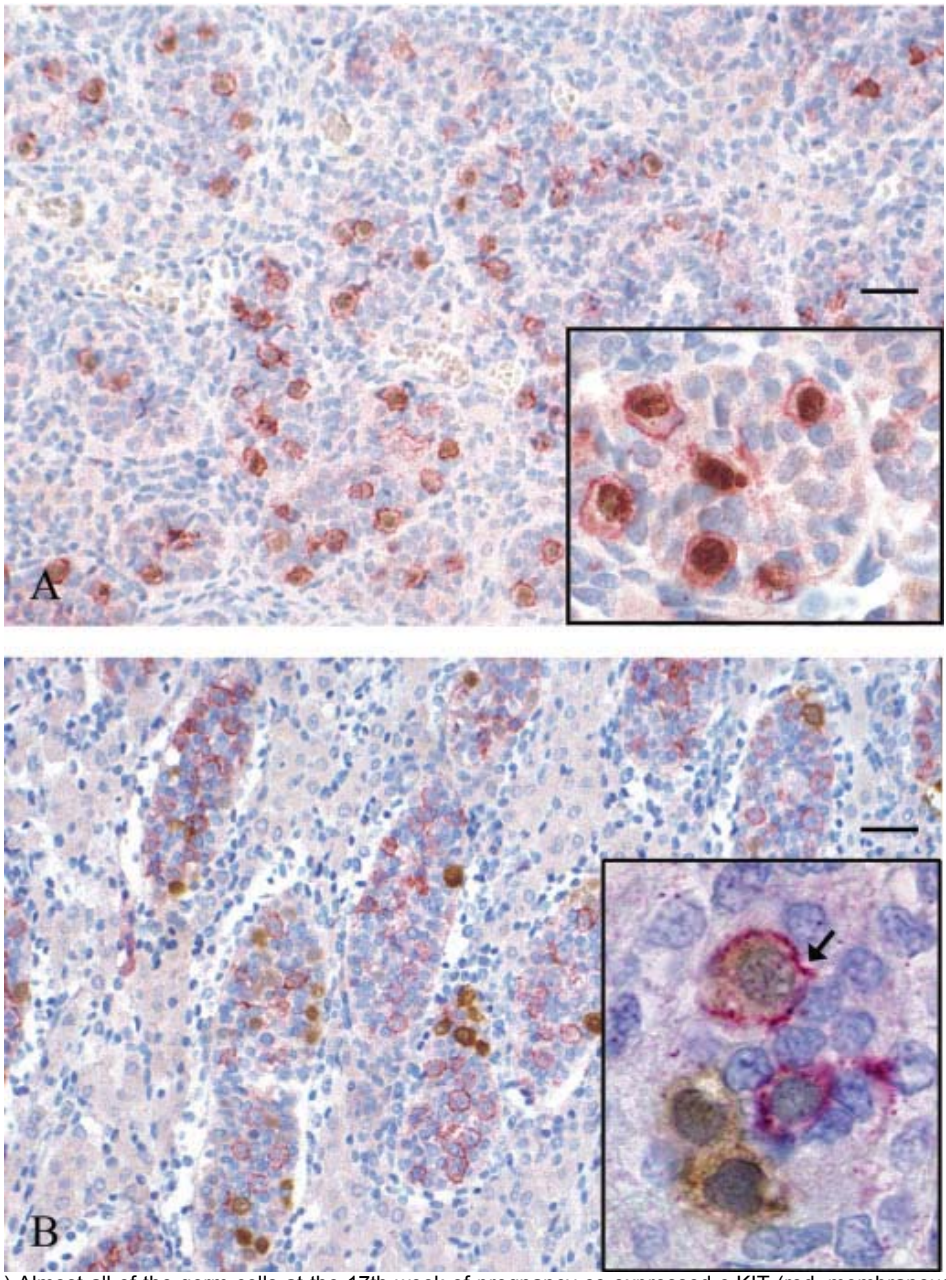
Expression of the markers described above was correlated with conventionally defined types of germ cells as established by Fukuda and Hedinger (Fukuda *et al.*, 1975). We found that expression of M2A, AP-2gamma, c-KIT and OCT3/4 was mainly associated with gonocytes, which were identified by their round central nuclei, high nuclear to cytoplasmic ratio and often prominent nucleoli (Fig. 1A-D). All intermediate cells histologically defined by oval shape, enlarged cytoplasm and a round but slightly excentric nucleus were also positive for these markers (Fig. 1E). Gonocytes and intermediate cells were frequently located in the outer or middle layer of the tubule. In contrast, germ cells corresponding to pre-spermatogonia (Fukuda *et al.*, 1975) expressed MAGE-A only (Fig. 1F-H, Figure 2-3).

Co-localization studies showed that most gonocytes and intermediate cells co-expressed M2A, c-KIT, AP-2gamma and OCT3/4 but not MAGE-A4 at every stage of testicular development. In particular, this co-expression was confirmed by immunohistochemistry using antibody pairs c-KIT and OCT3/4; M2A and c-KIT; c-KIT and AP-2γ, M2A and MAGE-A4, c-KIT and MAGE-A4, AP-2γ and MAGE-A4, OCT3/4 and MAGE-A4 and confirmed by immunofluorescence using antibody pairs c-KIT and M2A (Fig. 2A, Fig. 3A/B). However, very few intermediate cells coexpressed also MAGE-A4 (Fig. 2B, inset). We also observed few germ cells that were positive for c-KIT or M2A but negative for AP-2gamma and OCT3/4. Some of these cells had the appearance of typical gonocytes, but others had the form of intermediate cells. Furthermore, through examination of the antibodies used in our study, we found that M2A staining was extremely robust to different staining conditions and tissue preservation. Therefore, the highest yield of positively stained gonocytes and intermediate cells was obtained from staining with M2A antibody (compare Table 2, Figure 5).

In contrast, all MAGE-A4-positive pre-spermatogonia did not express markers found in gonocytes/intermediate cells (c-KIT, M2A, AP-2γ and OCT3/4) (Fig. 2B, Fig. 3C/D). In the fetal testes from the second trimester, MAGE-A4 positive germ cells were mostly located at the basement membrane and less frequently in the

inner tubular layer. At the end of the third trimester and in the neonate, MAGE-A4-positive germ cells were found within the tubules in basal location.

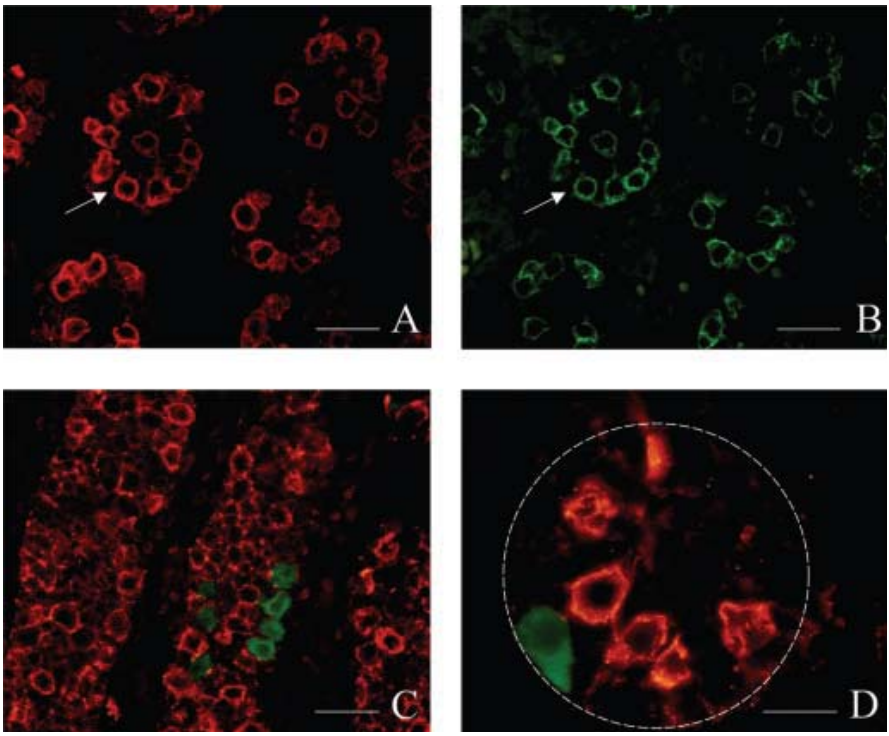
Figure 2: Co-expression analysis of germ cells



(A) Almost all of the germ cells at the 17th week of pregnancy co-expressed c-KIT (red, membranous), AP-2gamma (brown, nuclear) and OCT3/4 (brown, nuclear, inset). (B) At the 22nd gestational week, germ cells phenotypically corresponded to pre-spermatogonia (MAGE-A4-positive, brown) and gonocytes (c-KIT-positive, red). Very few intermediate cells co-expressed MAGE-A4 and c-KIT (arrow in inset). Scale bars (A-B) = 50µm.

Co-localization studies with antibodies to Ki-67 and c-KIT revealed that most cells positive for Ki-67 expressed c-KIT on their surface. However, some MAGE-A4 positive germ cells were also positive for Ki-67 in their nucleus (not shown).

Figure 3: Co-expression analysis of germ cells by immunofluorescence



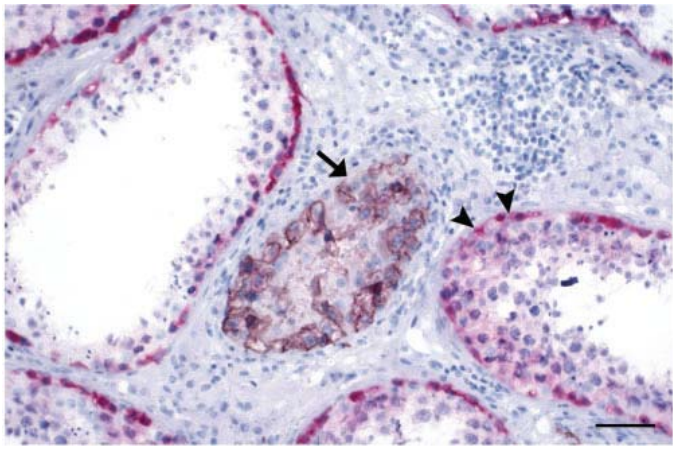
(A-B). Immunofluorescence visualized co-expression of M2A (A) and c-KIT (B) within the same population of germ cells (arrow). (C-D) MAGE-A4 (green staining) was expressed in germ cells in the tubule periphery and did not cross-react with gonocytes positive for M2A (red staining, C) and c-KIT (red staining, D). 25th gestational week. Scale bars (A-C) = 50µm, (D) = 20µm.

In addition, double staining of 6 adult testes with normal spermatogenesis and IGCNU was performed using antibodies to c-KIT and MAGE-A4, M2A and MAGE-A4. This was done to address the issue of whether neoplastic germ cells express markers of pre-spermatogonia. In each case, normal spermatogonia were consistently positive for MAGE-A4. In contrast, no staining could be demostrated in neoplastic germ cells, which were marked by c-KIT and M2A (Fig. 4).

Immunohistochemical detection of Ki-67, E-cadherin, NCAM (CD56), cytokeratin 18, connexin 43 and androgen receptor in fetal testes

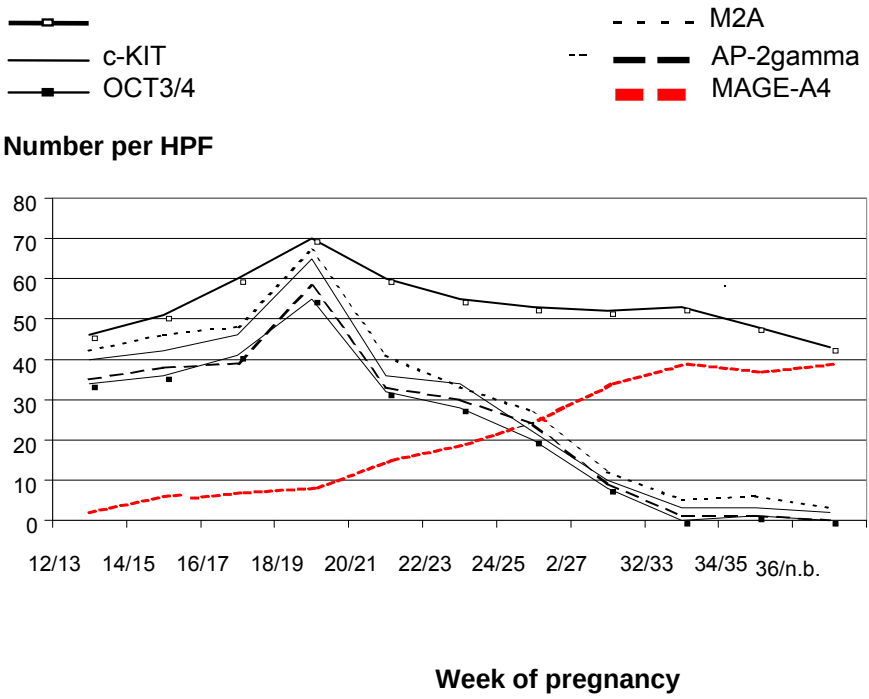
No expression of E-cadherin or androgen receptor was found in fetal testicular cords. Diffuse cytoplasmic staining for connexin 43 was observed in Leydig cells at all gestational weeks examined but not in fetal testicular cords. NCAM and cytokeratin 18 expression was present in Sertoli cells in the testis in weeks 12 to 19 but not in later stages of testicular development (data not shown).

Figure 4



Co-localization study of an adult testis with normal tubules (double arrowhead) and intratubular germ cell neoplasia (IGCNU, arrow) using antibodies against MAGE-A4 (red) and M2A (brown). Scale bar 50µm.

Figure 5



Distribution of germ cells per high power field (HPF): germ cells expressing M2A, c-KIT, AP-2γ, OCT3/4, and MAGE-A4 in comparison with total number of germ cells during the second and third trimester of pregnancy**.** The Y-axis gives the average numbers of germ cells calculated from counting positive cells in 5 independent high power magnification fields (HPF), the X-axis the gestational week (nb newborn). Standard deviations were given in table 2.

Discussion

The aim of the present study was to examine the expression of proteins associated with differentiation and maturation of germ cells in human fetal testes. We detected oncofetal markers including transcription factors OCT3/4 and AP-2γ, stem cell factor receptor c-KIT and antigen M2A in fetal male germ cells predominantly between the 12th and 26th gestational weeks. These results are in accordance with previous studies, which examined expression of respective markers during fetal development in normal male gonads (Aubry *et al.*, 2001; Franke *et al.*, 2004; Hoei-Hansen *et al.*, 2004; Honecker *et al.*, 2004; Jorgensen *et al.*, 1995; Rajpert-De Meyts *et al.*, 2004; Robinson *et al.*, 2001). Furthermore, we found that two populations of fetal germ cells exist during the second and third trimester. One of these populations is comprised of germ cells consisting of gonocytes and intermediate cells, according to morphological characteristics established by Fukuda, and co-express c-KIT, M2A, OCT3/4 and AP-2γ (Fukuda *et al.*, 1975). The second population of germ cells matches the morphological criteria of pre-spermatogonia (Fukuda *et al.*, 1975) and expresses melanoma associated antigen MAGE-A4, which is a specific marker for normal premeiotic germ cells (Aubry *et al.*, 2001).

Our findings partially confirm the recent qualitative study published by Gaskell, who detected distinct expression of OCT3/4 together with c-KIT in a gonocytic population and MAGE-A4 in the population of pre-spermatogonia (Gaskell *et al.*, 2004). Gaskell also proposed the existence of a third germ cell population with the morphology of intermediate cells, which is devoid of c-KIT and MAGE-A4 expression. A similar third group was not found in our series. Differences between our study and previous reports might be the result of different tissue fixation (Bouin fluid versus formalin), different antigen retrieval techniques and application of highly sensitive immunohistochemical detection method in the present study. According to our results, all intermediate germ cells belong to the immunohistochemical group with the expression of c-KIT, AP-2γ, OCT3/4, and M2A. However, very few intermediate cells showed also expression of MAGE-A4. We believe that they represent a transition stage from gonocytes to pre-

spermatogonia. Furthermore, compared to other markers used (c-KIT, AP-2γ, OCT3/4), D2-40 antibody always resulted in sensitive and distinct staining independent of fixation artefacts or autolysis. We believe that the robustness of D2-40 was also the reason why slightly more germ cells were positive for M2A compared to c-KIT, AP-2γ and OCT3/4 in our study (Figure 5, Table 2).

Furthermore, we found that the number of germ cells co-expressing oncofetal markers c-KIT, M2A, AP-2gamma and OCT3/4 varied significantly depending on the developmental stage. In particular, their expression was strongly increased in the $18^{th}/19^{th}$ week (Figure 5). We believe that this peak reflects a burst of proliferation activity of gonocytes as we found an increase in both Ki-67 expression and the overall germ cell number, while there was a transient decrease in the ratio of Sertoli cells to germ cells at this point of development. In general, the number of gonocytes and intermediate cells decreased continuously during the second and third trimester, while the number of MAGE-A4 positive germ cells increased to the same degree. The differentiation process obviously progressed after the 25^{th} week, when over 50% of germ cells expressed MAGE-A4 (Figure 5).

Understanding the process of pre-spermatogenesis and the role of proteins involved in germ cell differentiation may help to elucidate the pathogenesis of IGCNU and germ cell tumors as it is believed that malignant transformation of germ cells occurs in early fetal life (Rajpert-De Meyts *et al.*, 2004; Skakkebaek *et al.*, 1987). Expression of transcription factor Ap-2γ has recently been detected in IGCNU and various germ cell tumors (Hoei-Hansen *et al.*, 2004; Pauls *et al.*, 2005). Its cellular functions, particularly in germ cells, are not known in detail yet. Mice overexpressing AP-2γ in the mammary gland exhibited hyperproliferation and impaired differentiation of lactiferous ductules, suggesting a role of Ap-2γ in the proliferation and maintenance of an undifferentiated state (Jager *et al.*, 2003). Receptor tyrosine kinase c-KIT is crucial for germ cell migration, survival and proliferation in rodents and it is likely to be of the same importance in humans (Robinson *et al.*, 2001; Yamamoto *et al.*, 1993; Yasuda *et al.*, 1993). KIT has been described as a target gene of AP-2 transcription factors (Yamamoto *et al.*, 1993;

Yasuda *et al.*, 1993). Thus, prolonged AP-2gamma expression and permanent activation of c-KIT might have effects upon malignant transformation by an increase in the survival of immature germ cells and arrest of germ cell differentiation. Another potential transforming mechanism involves OCT3/4, which is a POU-domain class 5 transcription factor and one of the candidate regulators in pluripotent and germline cells (Niwa *et al.*, 2000). Disturbed regulation of OCT3/4 may also cause arrest of gonocytes at the pluripotent stage, consequently providing the basis for malignant germ cell transformation. We do not know the real biological pathway of the malignant transformation of germ cells, but we show here that putative factors involved in this process including OCT3/4, AP-2γ and c-KIT are strictly regulated in fetal germ cells. All of them are co-expressed during fetal development in the same germ cell population with morphological characteristics of gonocytes or intermediate cells. Before germ cells transit to pre-spermatogonia, as marked by expression of MAGE-A4, downregulation of OCT3/4, AP-2γ and c-KIT occurs. We further show that fetal pre-spermatogonia are phenotypically close to mature spermatogonia because they share the expression of MAGE-A4, which has been found in pre-meiotic spermatogonia of adults in the present and in previous studies (Yakirevich *et al.*, 2003). In contrast, neoplastic germ cells of IGCNU were devoid of MAGE-A4 in our series. In conclusion, our findings further strengthen the hypothesis of the embryonic/fetal origin of germ cell neoplasia, although the latter have some phenotypic overlap with adult germ cells as has been documented by previous studies using *VASA* gene product and Y-encoded testis-specific protein (TSPY) (Arnemann *et al.*, 1991; Castrillon *et al.*, 2000; Honecker *et al.*, 2004; Zeeman *et al.*, 2002).

At least in the rodent testes, gonocytal maturation is associated with adherence to Sertoli cells and relocation to the basement membrane via intermediate filaments and connexin 43 (Nagano *et al.*, 2000; Orth *et al.*, 2000; Perez-Armendariz *et al.*, 2001). In our series, we found no expression of connexin 43 and E-cadherin in fetal tubules. Neural cell adhesion molecule NCAM was expressed on the cell membrane of Sertoli cells in parallel to CK18 and both proteins were downregulated after the 19th gestational week, reflecting the progress of Sertoli cell

differentiation (Sharpe *et al.*, 2003). Similar to the results obtained from mouse testes (Nagano *et al.*, 2000), almost all of the pre-spermatogonia in our series were attached to the basement membrane of the tubules. In contrast, germ cells expressing OCT3/4, c-KIT or AP-2γ at the late stages of pregnancy were degenerated and located in the lumen of the tubules. This finding supports the idea that relocation of gonocytes is essential for their survival (Nagano *et al.*, 2000). However, the factors and pathways involved in human germ cell differentiation remain largely unknown and further exploration of this process is necessary.

In summary, we show that two immunohistochemically distinct populations of fetal germ cells exist during the second and third trimesters. Proteins which are associated with pluripotency, survival and proliferation including OCT3/4, c-KIT and AP-2γ as well as oncofetal marker M2A antigen are present in fetal germ cells with the morphology of gonocytes and intermediate cells. Their number gradually decreases after week 20, paralleled by an increasing number of MAGE-A4-positive pre-spermatogonia. Results presented in our study provide evidence of functional and temporal regulation of the differentiation process in fetal testes. They may also serve as a reference for further analyses investigating abnormal fetal germ cell maturation in chromosomal aberrations (Pauls et al., unpublished data). In addition, we found here that the monoclonal antibody D2-40 to glycosylated monomeric sialoglycoprotein M2A, which is expressed in fetal gonocytes and intermediate cells, is extremely robust to fixation artefacts and autolysis. The use of M2A might in future be also extended to detection of IGCNU and metastatic seminoma in diagnostically challenging cases (Pauls et al., unpublished data).

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Chapter 3

Transcription factor AP-2gamma, a novel marker of gonocytes and seminomatous germ cell tumors

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Abstract

Most germ cell tumors (GCTs) arise from intratubular germ cell neoplasias (IGCNUs, also referred to as carcinoma *in situ*), which are thought to originate from a transformed fetal germ cell, the gonocyte. However, the nature of the molecular pathways involved in IGCNU formation remains elusive. Therefore, identification of novel oncofetal markers is an important prerequisite to further our understanding of the etiology of this tumor entity. In the present study, we show that in humans AP-2gamma is expressed in gonocytes at weeks 12-37 of gestation, indicating a role of this transcription factor in fetal germ cell development. AP-2gamma and c-KIT, a known target of AP-2 transcription factors, were coexpressed in gonocytes, making a direct regulation possible. With increasing differentiation of fetal testis, gradual downregulation of AP-2gamma from the 12th to 37th week of gestation was observed. Furthermore, AP-2gamma was expressed abundantly in 25/25 IGCNUs, 52/53 testicular seminomas, 10/10 metastatic seminomas, 9/9 extragonadal seminomas and 5/5 dysgerminomas. In embryonal carcinomas and choriocarcinomas, focal staining only was observed. Spermatocytic seminomas, teratomas and yolk sac tumors as well as normal adult testis and various control tissues were negative for AP-2gamma. The expression pattern of AP-2gamma, like that of other oncofetal markers, supports the model of a gonocytal origin of IGCNUs and germ cell tumors. Finally, our results provide the basis for applying AP-2gamma.

Introduction

The most abundant malignancies among men aged 17-45 are GCTs (1). They comprise a heterogeneous group of neoplasms in terms of their histology, marker expression and age at manifestation. First described by Skakkebaek in 1972, the common precursor lesion of all GCTs, IGCNU (CIS, TIN, IGCN), arises from transformation of a gonocyte (2,,3). Markers commonly used for immunohistology, such as OCT3/4, PLAP and c-KIT, are expressed in gonocytes as well as IGCNUs and some stages of GCT, further supporting this model.

TFAP2C belongs to a family of 5 closely related genes that are involved in the morphogenesis of craniofacial, urogenital, neural crest and placental tissues (4). AP-2 transcription factors are believed to regulate the expression of several genes involved in cell growth and differentiation during development (5). AP-2 is essential for mammalian embryonic development because embryonic trophectodermal cells fail to proliferate in AP-2 knockout mice (6, 7). In transgenic studies, overexpression of AP-2 impaired differentiation of epithelial cells of the mammary gland and the seminal vesicle (8). Besides trophoblastic and neuroectodermal lineage, AP-2 can be detected in lung, testis and ovary (9,10). In human tumors, AP-2 is upregulated in certain stages of melanoma (11) and breast cancer.(12, 13).

In the present study, we investigated the expression of AP-2 during human fetal germ cell differentiation and development as well as in GCT. Analyzing human fetal tissues, we found AP-2 expressed in gonocytes at weeks 12-37 of pregnancy, indicating a role for this transcription factor during human germ cell development. Using a representative collective of GCTs, we found that AP-2 is highly expressed in classical seminomas and downregulated in nonseminomatous GCTs. Thus, AP-2 appears to be a distinctive immunohistochemical marker for GCT diagnostics.

Materials and Methods

Tissue samples

Thirty-one normal tissue samples of fetuses from gestational week 12 to 37 (2 × 12th, 3 × 13th, 15th, 16th, 17th, 18th, 3 × 19th, 20th, 4 × 21st, 2 × 22nd, 3 × 23rd, 3

× 25th, 27th, 30th, 31st, 33rd, 36th, 37th weeks of pregnancy) without maceration signs or autolysis were selected from tissue archives of the Section of Paidopathology, Department of Pathology, University of Bonn. Causes of death were spontaneous abortion (mainly amnion infection or placental problems), perinatal death or legitimate induced interruptions. All interruptions were performed in the Section of Neonatology, Department of Gynecology, University of Bonn. The developmental age of fetuses was assessed by the date of the last menstrual bleeding and correlated with the weight and size parameters of the fetus.

Tumor samples were collected from the files of the Department of Pathology and the Department of Neuropathology, University of Bonn Medical Center, and included 116 GCTs of different histology and localization and 25 IGCNUs (20 adjacent to a primary tumor and 5 sole neoplasms). There were 87 testicular, 13 metastatic and 11 primary extragonadal GCTs and 5 ovarian dysgerminomas (Table I). Tumors were diagnosed according to the WHO classification (14), and routine immunohistochemistry was performed using antibodies to PLAP, alpha-fetoprotein, human chorionic gonadotropin and receptor tyrosine kinase c-KIT.

Ten normal testes from patients with advanced prostate cancer and 5 testicular non-Hodgkin's lymphomas were added to the control group. To expand the series of control tissues and to study the expression of AP-2 in different tissues, 112 individual tissues were selected from the files of the Department of Pathology and 4 tissue arrays were prepared (Table II). Tissue microarrays were constructed from paraffin-embedded, formalin-fixed tissue using a Tissue Arrayer (Beecher Instruments, Silver Springs, MD). Single 1 mm cores were obtained from each paraffin block and placed in a predrilled slot of the recipient block.

Immunohistochemistry

Immunohistochemistry was performed on consecutive paraffin-embedded 4 m thick tissue sections using the ABC Kit (Vector, Burlingame, CA), as described (15). Briefly, dewaxed tissue sections were microwave-pretreated prior to incubation with

primary antibody. Sections were incubated overnight at 4°C with a polyclonal rabbit anti-AP-2 antibody (1:500, H77; Santa Cruz Biotechnology, Heidelberg, Germany).

The following well-defined antibodies were used to record possible differences of expression during fetal germ cell differentiation and in GCTs: polyclonal antibody to c-KIT (Dako, Hamburg, Germany; dilution 1:100) and MAb 8A9 to PLAP (Dako, dilution 1:50). The MAb to MAGE-A4 was kindly provided by Dr. G. C. Spagnoli (Department of Surgery and Research, University of Basel, Basel, Switzerland; dilution 1:50). For controls, we used buffer instead of the primary antibody.

Statistical analysis

Sections of fetal testicular tissues stained with AP-2, c-KIT or MAGE-A4 antibodies were prepared. Positive germ cells were counted in a minimum of 5 HPFs using ×400 magnification, with means and SDs calculated.

RNA preparation and analysis

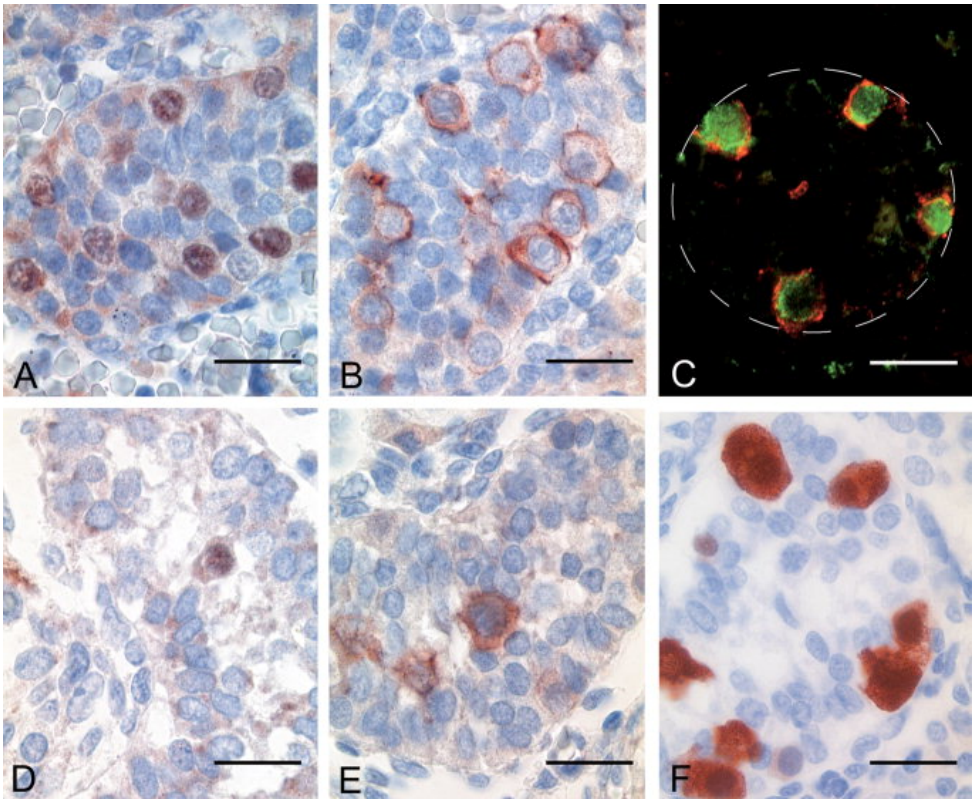
Total RNA was prepared from homogenized tissue samples essentially as described (8). Total RNA (10 g) was loaded onto a 1.2% agarose gel supplemented with 3.3% (w/v) formaldehyde and run in MOPS buffer (20 mM morpholinopropylsulfonate, 5 mM sodium acetate, 0.5 mM EDTA, pH 7.0). RNA was transferred to a nylon membrane (Hybond N+; Amersham, Aylesbury, UK) and sequentially hybridized to a 32P-labeled probe covering nucleotides 1-1116 of the human AP-2 cDNA (kindly provided by Dr. D. Taverna, Institute for Cancer Research and Treatment, Candiolo, Italy) and a 32P-labeled probe specific for the human Oct-4 gene that had been generated by RT-PCR amplification of RNA derived from human embryonic stem cells using the primers (forward) 5-gagaacaatgagaaccttcaggaga-3 and (reverse) 5-ttctggcgccggttacagaacca-3 (kindly provided by Drs. C. Benzing and O. Brüstle, Institute for Reconstructive Neurobiology, Bonn Medical School). Filters were washed twice in 2 × SSC, 0.1% (w/v) SDS; then in 1 × SSC, 0.1% (w/v) SDS; and finally twice at high stringency in 0.1 × SSC, 0.1% (w/v) SDS at 65°C for 10 min each.

Results

AP-2 expressed in gonocytes during fetal testis development

To establish the ontogenesis of AP-2, we studied its distribution in fetal testis and found AP-2-positive gonocytes at different stages of fetal development. High numbers of AP-2-expressing germ cells were seen at weeks 12-20 of fetal development, with an apparent peak around week 19 (Figs. 1a, 2a). Thereafter, expression was lower and decreased constantly after week 30 (Figs. 1d, 2a).

Figure 1



Immunohistochemical analysis of human fetal testis using antibodies directed against AP-2 (a,d), c-KIT (b,e) and MAGE-A4 (f) at the 13th (a-c) and 37th (d-f) weeks of gestation. Cells expressing AP-2 show brown nuclear staining; cells expressing c-KIT or MAGE-A4 show brown cytoplasmic staining. (c) Double-immunofluorescence analysis detected AP-2 (green) and c-KIT (red); cells expressing AP-2 display green nuclear staining, cells expressing c-KIT display red cytoplasmic staining; line in (c) indicates margin of the tubule. Scale bars (a-f) = 20 m.

To further specify the population of AP-2-positive germ cells, we performed double-immunofluorescence and immunohistochemistry with c-KIT and MAGE-A4 antibodies on the same tissue. Similar to AP-2, the highest number of c-KIT-positive gonocytes was found at 12-20 weeks of pregnancy (Figs. 1b, 2b). Afterward, expression continuously decreased to week 37 (Fig. 2b). In analogy to AP-2, few c-KIT-positive germ cells in the center of tubules were seen toward the end of the third trimester (Fig. 1d,e). Double-immunofluorescence revealed that most AP-2-immunopositive cells expressed c-KIT on their surface (Fig. 1c). In contrast to c-KIT and AP-2, the number of MAGE-A4-immunopositive cells was low until week 17. From there on, the number of MAGE-A4-positive gem cells increased rapidly; and in comparison to c-KIT and AP-2, perceptually more MAGE-A4-positive germ cells were present at week 37 (Figs. 1f, 2c).

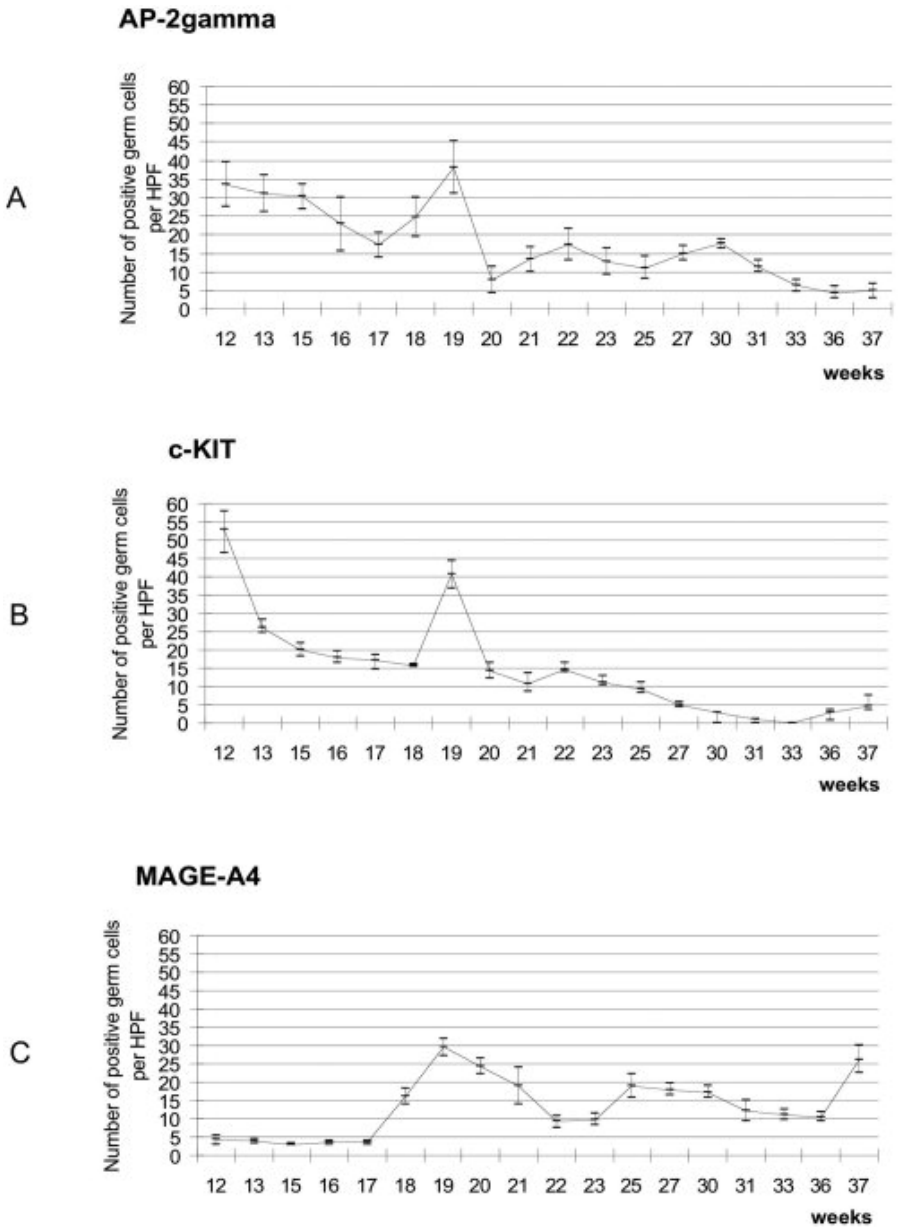
Table I. Expression of AP-2γ in 25 IGCNUs, 116 Invasive GCTs and 21 Controls

Tissue/histology	Histopathologic stage (UICC, TNM)/localization/remarks	Positive staining (approx. % positive cells)
IGCNU of testis		25/25 (100)
Seminomatous GCT		
Testicular classical seminoma	29 × stage pT1, 21 × stage pT2, 2 × stage pT3	52/52 (80-100)
Testicular anaplastic seminoma	pT2	0/1
Metastatic seminoma	Retroperitoneal lymph nodes	8/8 (60-100)
	Mediastinum	2/2 (60-100)
Extragonadal seminoma	Retroperitoneum	3/3 (70-100)
	Pinealis (germinoma)	6/6 (90-100)
Dysgerminoma	3 × stage pT1, 2 × stage pT2	5/5 (50-90)
Testicular spermatocytic seminoma	pT1	0/2
Nonseminomatous GCT		
Testicular embryonal carcinoma	10 × stage pT1, 7 × stage pT2	11/17 (5-10)
Metastatic embryonal carcinoma	Retroperitoneal lymph nodes	1/3 (5-10)
Testicular teratocarcinoma[1]	pT1	2/2 (<5)
Extragonadal teratocarcinoma[1]	ZNS	2/2 (<5)
Testicular choriocarcinoma	1 × stage pT1, 4 × stage pT2	5/5 (40-60)
Testicular yolk sack	pT1	0/3

tumor		
Testicular mature teratoma		0/5
Testicular controls		
Normal testis	Orchiectomy for prostate cancer	0/12
Testicular non-Hodgkin's lymphoma	B-cell lineage	0/5
Leydig cell tumor	Benign	0/4

[1] Expression in embryonal carcinoma element only.

Figure 2



Frequencies of AP-2- (a), c-KIT- (b) and MAGE-A4- (c) positive fetal germ cells during testicular development. Mean value of positive cells per HPF (y axis) at the corresponding gestational week (x axis) is given. Error bars show SD.

Distribution of AP-2 immunostaining in GCTs

AP-2 expressed in IGCNU and GCTs with seminomatous differentiation

Since gonocytes are believed to give rise to germ cell neoplasia, we next analyzed a broad collection of testicular, metastatic and extragonadal GCTs (Table I). Strong nuclear immunoreactivity for AP-2 was found in all neoplastic germ cells of 25 IGCNUs studied (Fig. 3a, Table I).

There was uniform nuclear staining in most tumor cells in 71 classical seminomatous GCTs, including 52 testicular, 10 metastatic and 9 primary extragonadal seminomas as well as 5 dysgerminomas (Fig. 3b,c, Table I). In contrast, 15 of these tumors were negative for PLAP (4 metastatic seminomas, 2 primary retroperitoneal seminomas, 2 germinomas and 7 testicular seminomas), and in 8 tumors c-KIT expression was weak or focal (7 classical testicular seminomas and 1 retroperitoneal seminomas metastasis). Seminomas that were PLAP-negative (Fig. 3d2) and c-KIT-positive (Fig. 3d3) were positive for AP-2 too (Fig. 3d1). Taken together, AP-2 expression could be detected in all GCTs with classical seminomatous differentiation. Our results indicate that AP-2 immunohistochemistry might be superior to PLAP and c-KIT in detecting various stages of seminomas.

In contrast to seminomas with classical histology, the one anaplastic testicular seminoma of our series was negative for AP-2 but focally immunopositive for c-KIT and PLAP. Also, both spermatocytic seminomas were negative for AP-2, c-KIT and PLAP but positive for MAGE-A4, a known marker for spermatocytic seminomas (16), further supporting a different phenotypic and developmental status of these seminomas.

Focal expression of AP-2 in nonseminomatous GCTs

In embryonal carcinomas and teratocarcinomas, AP-2 staining was focal in distribution and concentrated in the basal layer of carcinoma glands (Fig. 3e, arrow; Table I). In teratomatous elements of teratocarcinomas, scattered focal nuclear staining was seen in a few undifferentiated stromal cells, whereas differentiated teratoma elements lacked AP-2 expression (Fig. 3e, arrowhead).

PLAP was focally positive in 15 embryonal carcinomas, but staining was more adjacent to the lumenal surface of the embryonal carcinoma glands (not shown). In choriocarcinomas, trophoblastic giant cells were positive for AP-2 (Fig. 3f) and negative for PLAP (not shown). Yolk sac tumors were devoid of AP-2 and PLAP expression. These results suggest that AP-2 immunoreactivity is indicative of undifferentiated neoplastic germ cells within the nonseminomatous GCT.

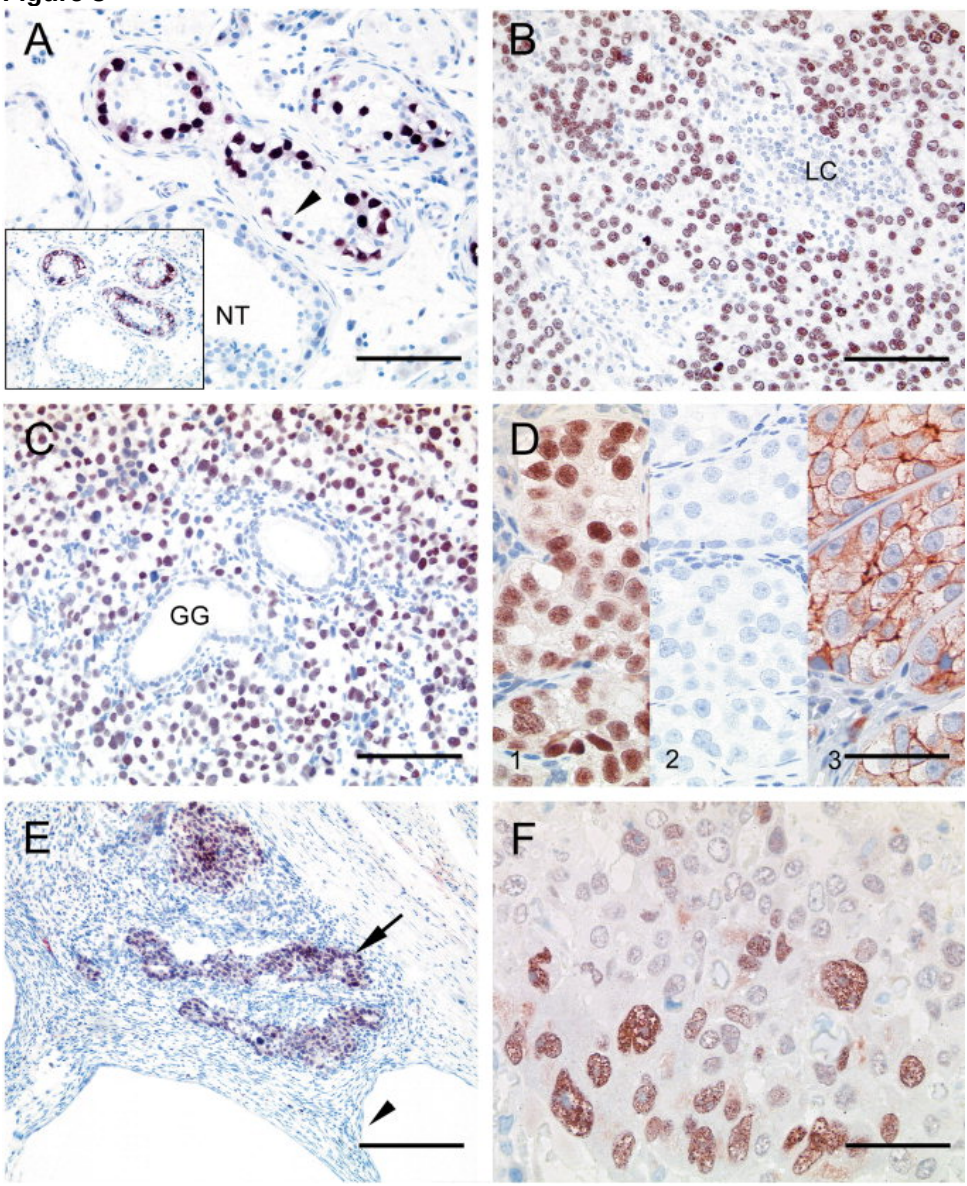
Distribution of AP-2 immunostaining in testicular controls and somatic tissues

Ten control sections of normal adult testis, obtained from patients with prostate cancer, and seminiferous tubules adjacent to GCTs with normal and impaired spermatogenesis were completely negative for AP-2. Here, unspecific, weak, granular cytoplasmic staining was observed in Leydig cells. To investigate whether AP-2 protein is expressed in other tumors besides GCT, we screened 108 individual samples of different normal and tumor tissues using tissue microarrays (Table II). Except for the breast, none of these control tissues showed positive staining for AP-2. As described (15), outer myoepithelial cells of normal ductuli lactiferi were immunopositive as were ductal invasive adenocarcinomas of the breast. Hence, AP-2 is not exclusively expressed in GCTs but can also be found in some tumors derived from somatic tissues.

RNA analysis of controls and GCTs

To verify the data obtained by immunohistochemistry, we prepared RNA from regular testis of 2 patients as well as IGCNU and its invasive seminoma from 2 patients and performed Northern blot analysis. As shown in Figure 4, strong expression of AP-2 was observed in invasive seminoma (Fig. 4, lanes 3b, 4b) and IGCNU (Fig. 4, lanes 3a, 4b) but was not detectable in regular testes (Fig. 4, lanes 1, 2). Next, the blot was probed with Oct-4, a known marker for IGCNU and GCT, and confirmed the results obtained with AP-2 (Fig. 4, OCT-4). This result demonstrates that the signal detected by immunohistochemical methods is indeed specific and results from elevated levels of AP-2 mRNA. The difference in signal intensity for AP-2 in IGCNU relative to seminomas is due to a larger portion of AP-2-negative, non-neoplastic tissue in these preparations.

Figure 3



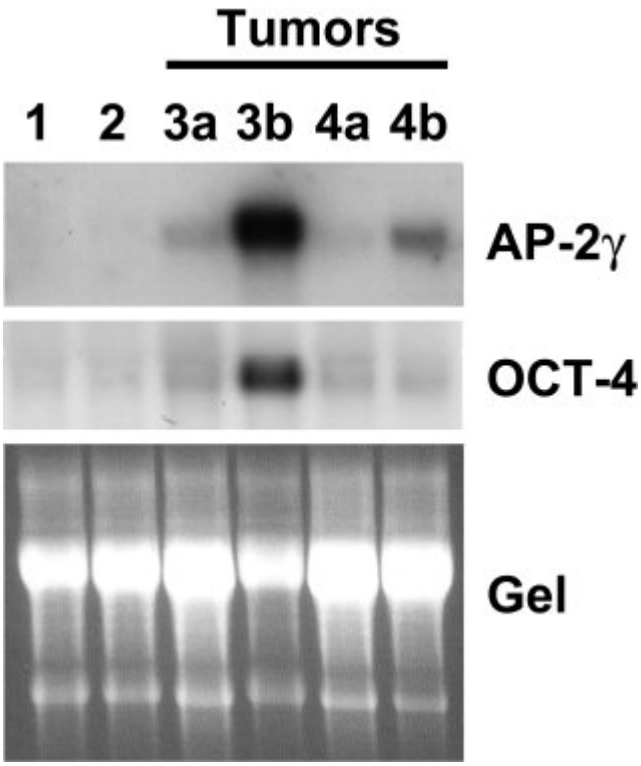
Immunohistochemical analysis using antibodies directed against AP-2 (a-c,d1,e,f), PLAP (d2) and c-KIT (inset in a,d3). Cells expressing AP-2 show brown nuclear staining; cells expressing c-KIT or PLAP show brown cytoplasmic staining. (a) IGCNU, positive for AP-2; notice Sertoli cells (arrowhead) and non-neoplastic seminiferous epithelium (NT) negative for AP-2. (inset) IGCNU positive for c-KIT in a serial section as control. (b) Testicular seminoma positive for AP-2; notice adjacent negative lymphocytes (LC). (c) Metastatic seminomas within the gastric wall, positive for AP-2; notice negative gastric glands (GG). (d) Testicular seminoma positive for AP-2 (1), negative for PLAP (2) and positive for c-KIT (3).(e) Teratocarcinoma. Focal expression of AP-2 in embryonal carcinoma element (arrow) but not in adjacent teratoma element (arrowhead). (f) Choriocarcinoma. Strong nuclear staining for AP-2 in giant cells. Scale bars: (a-c) = 100 m, (d,f) = 50 m, (e) = 200 m.

Table II. Description and Results of Tissue Array Immunohistochemistry for AP-2γ

Organ/tissue	Histology	*n* = 112	AP-2γ expression
Soft tissue	Dermatofibrosarcoma protuberans	3	0
	Fibrosarcoma	3	0
	Malignant fibrous histocytoma	8	0
	Myxofibrosarcoma	3	0
	Chondrosarcoma	2	0
	Chondroma	2	0
	Ewing sarcoma	2	0
	Osteosarcoma	2	0
	Chondrosarcoma	2	0
	Leiomyosarcoma	4	0
	Rhabdomyosarcoma	3	0
	Synovial sarcoma	2	0
	Malignant hemangiopericytoma	1	0
Skin	Dermatofibrosarcoma	1	0
	Melanoma	1	0
	Syringoma	1	0
	Pilomatrixoma	1	0
Uterus	Normal	2	0
	Adenofibroma	1	0
	Endometrial carcinoma	2	0
	Carcinosarcoma	1	0
Ovary	Normal	3	0
	Cystadenoma	1	0
	Adenocarcinoma	1	0
Breast	Normal	1	Basal cells of ductuli positive
	Adenocarcinoma	1	Positive
Prostate	Normal	3	0
	Adenocarcinoma	4	0
	Transitional cell carcinoma	1	0
Bladder	Normal	2	0
	Squamous carcinoma	1	0
	Transitional cell carcinoma	1	0
Kidney	Normal	2	0
	Clear cell carcinoma	2	0
Stomach	Adenocarcinoma	3	0
	Carcinoid	1	0
Oesophagus	Squamous carcinoma	1	0
	Adenocarcinoma	2	0
	Neuroendocrinal carcinoma	1	0

Small bowel/colon	Normal	2	0
	Adenocarcinoma	2	0
	GIST	10	0
Pancreas	Adenocarcinoma	2	0
Liver	Cholangiocarcinoma	1	0
	Hepatocellular carcinoma	1	0
Lung	Squamous carcinoma	1	0
	Small cell carcinoma	1	0
	Adenocarcinoma	1	0
Thyroid gland	Medullary carcinoma	1	0
	Papillary carcinoma	1	0
Parotid	Normal	1	0
	Basal cell adenoma	1	0
	Acinus cell carcinoma	2	0
	Adenoid-cystic carcinoma	1	0
Salivary gland	Adenoid-cystic carcinoma	1	0
	Acinus cell carcinoma	2	0
	Basal cell adenoma	2	0
	Salivary duct carcinoma	1	0

Figure 4



Northern analysis of AP-2 expression in normal testis (lanes 1,2), IGCNU (lanes 3a,4a) and seminoma (lanes 3b,4b). Total RNA (10 g) derived from tissues of 2 different patients were gel-electrophoretically separated, transferred to a nylon membrane and hybridized to an AP-2 (upper) or Oct-4 (middle) cDNA probe. The gel photo (lower) is shown as a loading control.

Discussion

GCTs originate from cells of the germ cell lineage. They comprise a heterogeneous group of neoplasms occurring mainly in the gonad but are also found within specific extragonadal sites. Here, we demonstrate that transcription factor AP-2 is expressed during male fetal development in gonocytes, IGCNUs and all GCTs with classical seminomatous differentiation.

We show that AP-2 can be detected in fetal germ cells at weeks 12-37 of pregnancy. Its expression generally declined with advancing age during the intrauterine period but persisted at lower levels throughout the third trimester. Previously, 3 distinct subpopulations of fetal germ cells were identified - gonocytes, intermediate germ cells and prespermatogonia - using immuno histochemical profiling with c-KIT and MAGE-A4. c-KIT is proposed to be a marker of early undifferentiated germ cells, the gonocytes, whereas MAGE-A4 marks the population of more differentiated fetal germ cells, the prespermatogonia (17). Here, expression of c-KIT and AP-2 overlaps during fetal germ cell development and, moreover, expression of both c-KIT and AP-2 can be detected in the same germ cells, whereas MAGE-A4 did not overlap with AP-2. This result together with morphologic criteria argues that AP-2 is expressed in the population of gonocytes. According to the morphologic criteria outlined by Wartenberg (18) and Robinson et al. (19) these cells would be regarded as M-prospermatogonia, which are an actively dividing germ cell population in fetal testis. Our findings are in line with a decreasing number of gonocytes, which subsequently differentiate to fetal prespermatogonia during late fetal development.

AP-2 and c-KIT are coexpressed in gonocytes, making direct transcriptional activation of c-KIT possible. Indeed, c-KIT has been described as a target gene of AP-2 transcription factors based on promoter studies (20, 21) as well as results obtained with melanoma cells (22). Furthermore, c-KIT represents a survival signal for germ cells in rats (23). Thus, in gonocytes, AP-2 might act as transcriptional activator of c-KIT, to keep the cells in a proliferative state.

It is believed that transformed gonocytes which persist into adulthood give rise to IGCNU, representing the common precursor lesion of all GCTs except spermatocytic seminomas (24) Indeed, we detected AP-2 expression not only in gonocytes but also in IGCNUs and in all classical seminomatous GCTs analyzed. Thus, expression of AP-2, like other oncofetal markers (c-KIT, PLAP, sACE, OCT 3/4) (25, 26, 27, 28) supports a gonocytal origin of IGCNU and GCT.

These markers are also important for the diagnosis of IGCNU and GCT. However, in contrast to PLAP expression, which is lost in a portion of seminomas during progession (29) (and present study) AP-2 expression was consistently high in seminomas independent of localization and pathologic stage. Also, using c-KIT immunohistochemistry on PLAP-negative tumors might not be sufficient to diagnose a metastatic seminoma as c-KIT is expressed in several somatic cell types and various somatic malignancies like GIST and lung, renal and ovarian carcinomas, raising the possibility of a misdiagnosis (30). Since AP-2 expression was consistently high in seminomas independent of their localization and pathologic stage, we suggest AP-2 as a potent marker for IGCNU and for the diagnosis of metastatic seminoma.

In contrast to its abundant expression in all classical seminomatous GCTs, spermatocytic seminomas were negative for AP-2, further supporting the model of a different origin of this seminoma type (14, 31). Also, compared to classical seminomas, AP-2 was downregulated in embryonal carcinomas and its expression was lost in yolk sac tumors and teratomas. This result is in agreement with the current model of progression of pluripotent embryonal carcinoma to more differentiated nonseminomatous GCTs (32).

However, trophoblastic giant cells in choriocarcinomas were positive for AP-2, though this tumor type also belongs to the group of differentiated nonseminomatous GCTs. In mice, AP-2 expression was found in cells of the trophoblast lineage (7) suggesting that reexpression of AP-2 in choriocarcinomas reflects regular developmental processes during placental differentiation.

The molecular role of transcription factor AP-2 is not fully understood. According to previous studies from us and others, AP-2 is essential in trophoblast cells during development (6, 7). AP-2-deficient embryos die due to a lack of proliferation of trophectoderm-derived extraembryonic tissues (7). Furthermore, studies on mice transgenic for AP-2 reveal a role in mammary gland tumorigenesis (8). There, forced overexpression leads to a block of differentiation (8) as well as enhanced tumor progression in a double transgenic model (33). Taken together, we propose that AP-2 serves as a molecule which keeps fetal germ cells at the undifferentiated, pluripotent stage by suppressing differentiation and supporting proliferation. Expression of AP-2 in gonocytes and all undifferentiated GCTs such as IGCNUs and seminomas further supports this hypothesis.

In conclusion, our study shows that transcription factor AP-2 is expressed in fetal germ cells during development and might be useful as a highly specific marker for diagnosis of IGCNU/seminoma/germinoma/dysgerminoma. This and other studies will help in broadening the knowledge of the molecular pathways affected in this type of tumor and in developing improved diagnostic and therapeutic reagents.

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Chapter 4

Expression of BLIMP1/PRMT5 and concurrent histone H2A/H4 arginine 3 dimethylation in fetal germ cells, CIS/IGCNU and germ cell tumors

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Abstract

Most testicular germ cell tumors arise from intratubular germ cell neoplasia unclassified (IGCNU, also referred to as carcinoma in situ), which is thought to originate from a transformed primordial germ cell (PGC)/gonocyte, the fetal germ cell. Analyses of the molecular profile of IGCNU and seminoma show similarities to the expression profile of fetal germ cells/gonocytes. In murine PGCs, expression and interaction of Blimp1 and Prmt5 results in arginine 3 dimethylation of histone H2A and H4. This imposes epigenetic modifications leading to transcriptional repression in mouse PGCs enabling them to escape the somatic differentiation program during migration, while expressing markers of pluripotency.

In the present study, we show that BLIMP1 and PRMT5 were expressed and arginine dimethylation of histones H2A and H4 was detected in human male gonocytes at weeks 12–19 of gestation, indicating a role of this mechanism in human fetal germ cell development as well. Moreover, BLIMP1/PRMT5 and histone H2A and H4 arginine 3 dimethylation was present in IGCNU and most seminomas, while downregulated in embryonal carcinoma (EC) and other nonseminomatous tumors.

These data reveal similarities in marker expression and histone modification between murine and human PGCs. Moreover, we speculate that the histone H2A and H4 arginine 3 dimethylation might be the mechanism by which IGCNU and seminoma maintain the undifferentiated state while loss of these histone modifications leads to somatic differentiation observed in nonseminomatous tumors.

Introduction

In males aged 15 – 34 years, type II testicular germ cell tumors (TGCT), i.e. seminomas and nonseminomas, are the most common malignancies with fatal outcome (1) accounting for up to 60% of all malignancies in young man. The incidence of this type of cancer has been steadily increasing throughout the last decades (2). The tumors arise from a neoplastic precursor, the carcinoma in situ (CIS)/intratubular germ cell neoplasia unclassified (IGCNU) and develop into seminoma and/or nonseminoma (including embryonal carcinoma, teratomas, yolk sac tumors and choriocarcinomas) (3). The IGCNU lesions are believed to arise by delayed or blocked maturation of primordial germ cells (PGC)/gonocytes during early fetal development (4). The recently identified markers for IGCNU and seminoma, namely the markers of pluripotency OCT3/4 and NANOG further support this model (5-10).

Expression of pluripotency genes is detected in embryonic stem cells (ES) and the inner cell mass of the early embryo. Additionally murine and human ES cells need to be cultured in the presence of factors inhibiting differentiation, although there are species specific differences (11,12). In PGCs, early gonocytes and IGCNU as well as seminoma lesions some of these markers of pluripotency are expressed, although differences have been reported (13,14). According to the current model, PGCs actively suppress somatic differentiation programs by epigenetic modifications, a mechanism which might also account for IGCNU and seminoma (15). Recent data in mice demonstrate that suppression of somatic differentiation programs in PGCs is mediated by a complex of two proteins, Blimp1 (B-Lymphocyte induced maturation protein-1; PRDM1) and Prmt5 (protein arginine methyltransferase-5). Upon arrival in the genital ridge the PGCs differentiate to become gonocytes and the Blimp1/Prmt5 complex is translocated in the cytoplasm and subsequently, Blimp1 is downregulated. Targeted deletion of Blimp1 leads to loss of PGCs short after specification due to differentiation. The Blimp1-deficient PGCs display an insufficient repression of markers indicative for somatic differentiation such as HoxB1 (16). Blimp1 is a transcriptional repressor harboring an N-terminal PR-SET domain, 5 zinc-finger domains and an acidic domain at the C-terminus. In murine PGCs the Blimp1/Prmt5 complex mediates symmetrical

methylation of histones H2A and H4 at arginine 3 (H2AR3me2s, H4R3me2s), resulting in widespread epigenetic modification leading to transcriptional repression (17).

In the present study, we investigated the expression of BLIMP1/PRMT5 during human fetal germ cell development and in testicular germ cell tumors. Analyzing human fetal tissues, we found BLIMP1/PRMT5 colocalized in gonocytes at weeks 12 – 19 of pregnancy, supporting a role in human germ cell development. Furthermore BLIMP1/PRMT5 is expressed in IGCNU and seminoma, but downregulated in nonseminomatous GCTs. Since the nuclear localization of BLIMP1 correlated with the presence of the histone modifications H2AR3me2s and H4R3me2, our data help in explaining the undifferentiated/fetal state of IGCNU and seminoma.

Material and Methods

Sample Handling and Characterization

Formalin fixed, paraffin embedded testicular tissues from 46 patients with GCTs (20 seminomas, 15 embryonic carcinomas, 5 Teratomas, 3 yolk sac tumors and 3 choriocarcinomas were collected for this study from archives of Departments of Pathology of University Medical Centers Bonn. Adjacent testicular parenchyma containing IGCNU were studied in 15 cases (32). All tumors were classified according to the WHO classification of tumors based on their histology by two independent pathologists. Fresh frozen samples of each of normal testicular tissues (n = 3), seminoma (n = 3), mixed germ cell tumors (n = 3), IGCNU (n = 5) and embryonal carcinomas (EC) (n = 3), as well as RNA extracts of TCam2 (33) and JKT-1 (34) cell lines, of which TCam2 resembles a seminoma-like cell-line (21-23), were additionally available for this study. Use of the tissue for scientific purposes was approved by the Institutional Regional Committee for Ethics.

RT-PCR and quantitative image analysis

Total RNA from at least three samples per tumor entity was extracted with TRIzol (Invitrogen, Karlsruhe, Germany) according to manufacturer's instruction. cDNA-syntesis was performed using SuperScript III reverse transcriptase (Invitrogen, Karlsruhe, Germany) and Oligo d(T)12–18(Invitrogen, Karlsruhe, Germany) and 100 ng of total RNA according to manufacturers instructions. PCRs were carried out in triplicates with following Primers: BLIMP1 F: 5'-GGGTGCAGCCTTTATGAGTC-3'; BLIMP1 R: 5'-CCTTGTTCA TGCCCTGAGAT-3'; PRMT5 F: 5'TTGCCGGC TACTTTGAGACT-3'; PRMT5 R: 5'-AAGGCAGGA AAGCAGATTGA-3'; GAPDH-F: 5'-TGGTATCGTGGAA GGACTCATG AC-3; GAPDH R: 5'-ATGCC AGTGAGCTTCCCGTTCAGC-3'. (β-Act: 25 cycles BLIMP1 and PRMT5: 30 cycles). After agarose gel electrophoresis of the PCR-products band intensity was measured after RT-PCR with the image analysis software ImageJ 1.37 v (National Institutes of Health, USA, http://rsb.info.nih.gov/ij/) in triplicates and normalized to the according GAPDH band.Co-Immunoprecipitation

Co-IP was performed with DYNABEADS® (Invitrogen, Carlsbad, USA) following manufacturers instructions. Immunopreciptation was performed with 1,5 µg anti-PRMT5 antibody (Chemicon, Temecula, USA) or anti-PRMT7 (Abcam, Cambrigde UK, 1:250). Western Blot with anti-BLIMP1 antibody followed (provided by H. M. Jäck).

Western Blot

For protein analysis Mini-PROTEAN Electrophoresis Cell and Mini Trans-Blot system was used (BioRad, Munich, Germany). Proteins were isolated using RIPA-buffer and prepared using standard protocol and finaly electrophoresed at 30 mA for 90 min. The gel was blotted onto a PVDF membrane in a BioRad blotting chamber overnight at 30 V at 4°C according to published protocols. After blocking in PBSTM (PBS, 0.1% v/v Tween 20, 5% low fat milk powder) primary antibodies (anti-BLIMP1 1:400 (kind gift from H. Jäck), anti-PRMT5 1:200, Chemicon International, USA) were incubated in PBSTM for 3 h at RT. The secondary antibodies (anti-rabbit-HRP, anti-mouse-HRP: DAKO, Hamburg, Germany) were diluted 1:2000. Finally the membrane was incubated in 2 ml PierceSuper Signal West Pico chemiluminescent substrate (Perbio, Bonn, Germany) and the signal was detected using Kodak X-Ray film (Kodak, Stuttgart, Germany).Array Analysis
DNA Array Dataset used to analyze BLIMP1/PRMT5 expression in Seminoma, embryonal carcinoma, TCam2 and JKT1 were generated as described [32].

Immunohistochemistry

For immunohistochemistry on paraffin-embedded tissue, dewaxed, 4-µm thick tissue sections were microwave-pretreated in citrate-buffer. Primary antibodies to PRMT5 (Upstate, Charlottesville, VA, 1:500), PRMT7 (Abcam, Cambrigde UK, 1:250) BLIMP1 (provided by H-M. Jäck, University of Erlangen, Germany 1:500) and H2AR3me2s/H4R3me2s (Abcam, Cambridge, UK, 1:2000) were used for detection. Immunohistochemistry was performed using the DAKO EnVision-AEC Kit and manufacturers protocol (DAKO, Hamburg, Germany) as previously described (7). Briefly, endogenous peroxidase was blocked for 5 min in 0.03% H2O2 (diluted in distilled water). Sections were washed in Tris-buffered saline

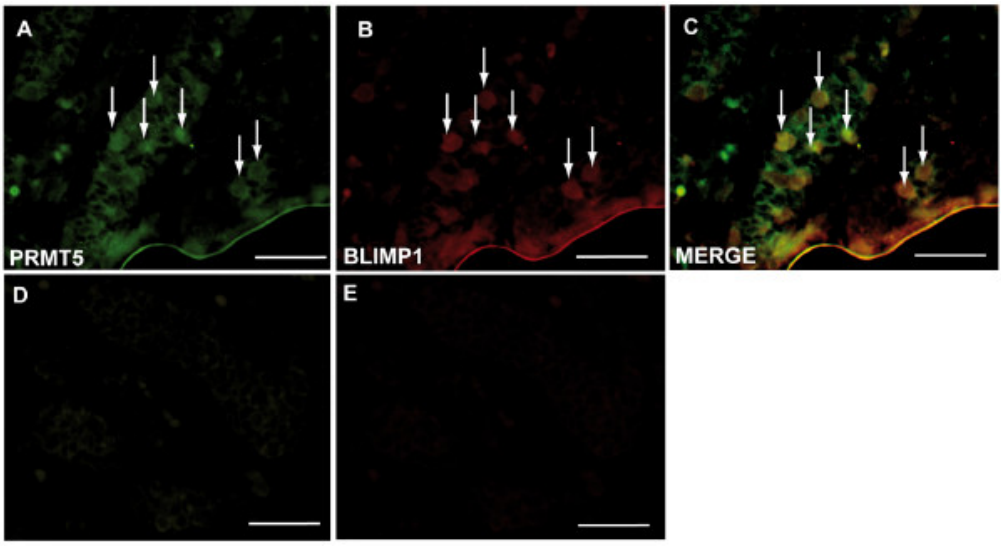
(TBS; 0.05 M Tris and 0.85% NaCl, pH 7.6) and incubated with primary antibodies overnight at 4°C. Thereafter, a HRP-labeled polymer conjugated with a secondary antibody was applied (DAKO EnVision-AEC KIT). Pictures were taken using a Leica microscope fitted with a JVC digital camera (Leica, Bensheim, Germany). Figures were assembled using Adobe CS3 software package. Merge of pictures was performed using ImageJ (NIH, US).

Results

Normal germ cell development

Data from murine embryos indicate, that the murine homologs of BLIMP1 and PRMT5, are expressed in PGCs from specification on up to their arrival in the genital ridge (16,17). Short thereafter, these cells differentiate to become gonocytes and the Blimp1/Prmt5 complex is translocated in the cytoplasm and subsequently, Blimp1 is downregulated. In order to test whether human BLIMP1 and PRMT5 are detected in human fetal PGCs/gonocytes, immunohistochemical analyses were performed on human fetal material. On the 12th week of pregnancy migrating gonocytes coexpressing PRMT5 and BLIMP1 were detected, (Fig.1, compare A to B, merged in C, arrows).

Figure 1: **Human fetal gonocytes at 12th week of pregnancy**

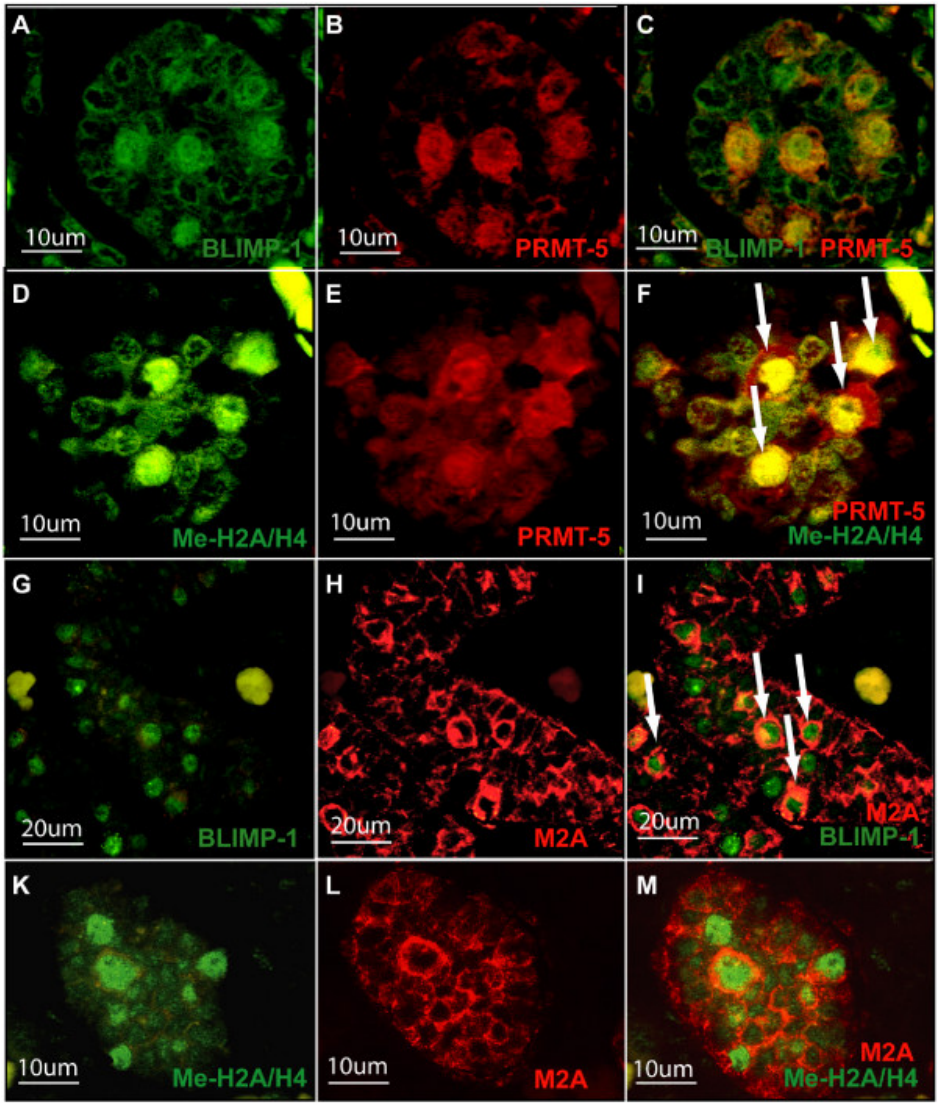


Sections of human fetal gonocytes at 12th week of pregnancy subjected to antibody staining towards BLIMP1 (A), PRMT5 (B) and overlay (C). D and E no primary antibody controls. Arrows indicate exemplary germ cells. Bar = 50 µm.

Next, testes from the 19th week of pregnancy were analyzed. By this time gonocytes gradually differentiate into prespermatogonia and migrate towards the periphery of the emerging seminiferous tubules to settle down in their niche (18). Both BLIMP1 (Fig. 2A) and PRMT5 (Fig. 2B) were detected at this stage in

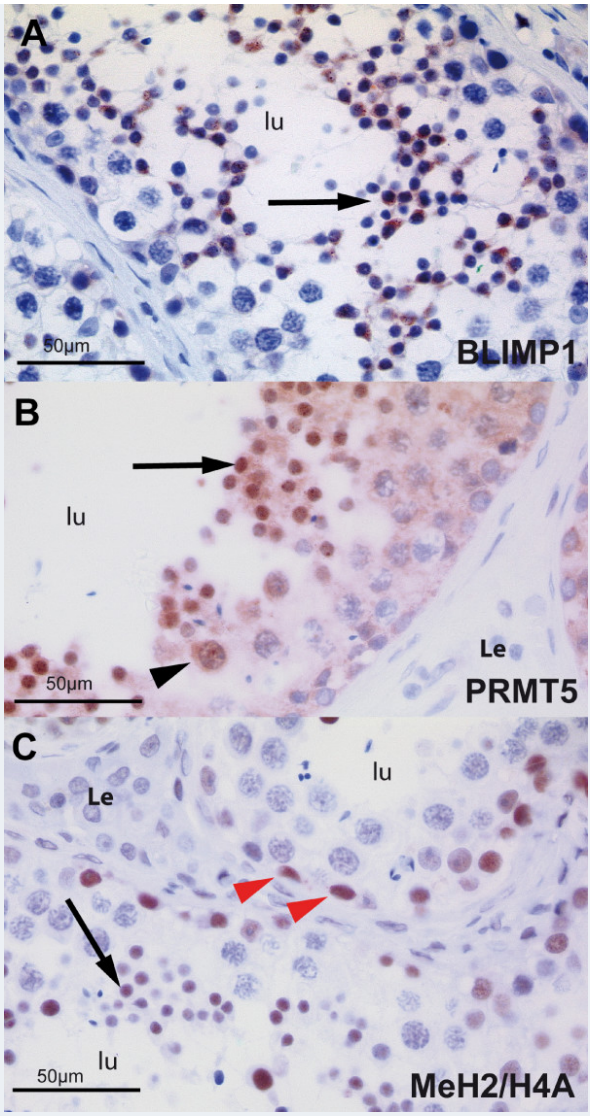
gonocytes. PRMT5, in contrast to BLIMP1, was detected both in the nucleus and in the cytoplasm. Since the murine Blimp1/Prmt5 complex has been described to mediate symmetrical dimethylation of arginine 3 on histone H2A and/or H4 tails (H2AR3me2s/H4R3me2s) (17) immunohistochemical analysis to detect this modification was performed (Fig. 2D). Co-staining of PRMT5 revealed that the cells displaying high nuclear levels of PRMT5 are in fact positive for the H2AR3me2s/H4R3me2s histone mark (Fig. 2E and 2F, merged). To further analyze the population of cells expressing BLIMP1 we performed double labeling experiments using BLIMP1 (Figure 2G) and the gonocytal markers M2A (19) (Figure 2H). BLIMP1/M2A double positive signals were detected in most gonocy tes (Figure (Figure2I arrows). Double labeling for H2AR3me2s/H4R3me2s (Fig 2K) combined with M2A (Fig. 2L) showed, that the M2A positive gonocytes displayed H2AR3me2s/H4R3me2s modifications (Fig 2M). Again, these findings were in accordance with the situation in mice, where the Blimp1 protein is downregulated and the H2AR3me2s/H4R3me2s methylation is gradually lost when germ cells proceed to prespermatogonia (17).

Figure 2 : Human fetal gonocytes at 19th week of pregnancy



Sections of human fetal gonocytes at 19th week of pregnancy subjected to antibody staining towards (A) BLIMP1, (B) PRMT5, (C) Merge of BLIMP1 and PRMT5, (D) PRMT5, (E) methylated H2A/H4, (F) merge of PRMT5 and methylated H2A/H4 (G) BLIMP1 (H) M2A antigen, (I) merge of BLIMP1 and M2A, (K) methylated H2A/H4, (L) M2A, (M) merge of methylated H2A/H4 and M2A.

Figure 3: Human adult testis



Sections of normal human adult testis stained for BLIMP1 (A), PRMT5 (B) methylated and dimethylated histones H2A/H4 (C). (A) A seminiferous tubule is shown with normal spermatogenesis. Spermatogonia, spermatocytes, and Sertoli cells are devoid of the staining, while nuclear and cytoplasmatic staining occurs in round spermatids (large arrow). (B) Staining with PRMT5 antibody shows low expression of PRMT5 in the nuclei of spermatocytes (arrowhead), and strong nuclear staining in round spermatids (large arrow). (C) Positive staining with Me H2A/H4 occurs in spermatogonia (red arrowheads) and round spermatids (arrow), but not in spermatocytes. lu lumen of the seminiferous tubule; Le Leydig Cells

Type II TGCTs

We next examined various TGCTs for the presence of BLIMP1/PRMT5 and H2AR3me2s/H4R3me2s. As shown in Figure 4, IGCNU show nuclear BLIMP1 staining (Fig. 4A), cytoplasmatic PRMT5 staining (Fig. 4B) and dimethylation of H2A/H4 (Fig. 4C). Seminomas show predominant nuclear BLIMP1 signal (Fig. 4D) sparse nuclear PRMT5 signal (Fig.4E) as well as a strong and homogenous signal for H2AR3me2s/H4R3me2s (Fig 4F). In embryonal carcinoma, expression of BLIMP1 (Fig. 4G) and PRMT5 (Fig. 4H) was weak and cytoplasmatic. As expected, histone H2AR3me2s/H4R3me2s methylation (Fig. 4I) was barely detectable and heterogeneous. Yolk sac tumors teratomas and choriocarcinomas stained focally and cytoplasmatic for BLIMP1 and PRMT5 (not shown). Focal cytoplasmatic expression of BLIMP1 and PRMT5 was also observed in differentiated parts of teratoma, while chorioncarcinomas were negative for both proteins. A summary of the results of the immunohistochemical studies is given in Table 1.

In order to quantify the expression of BLIMP1 and PRMT5 we performed RT-PCR analyses on normal testicular tissue as well as on various TGCTs. The RNA levels measured were first normalized to βActin and then calculated as relative expression with normal testicular tissue (N) set at 1. Expression of BLIMP1 was significantly higher in IGCNU ($p = 0.029$) containing testicular parenchyma and seminoma (Fig. 4K), but not in embryonal carcinoma (EC) ($p = 0.16$), which was comparable to normal testicular tissue. In contrast, PRMT5 was moderately higher in IGCNU ($p = 0.033$), while embryonal carcinoma ($p = 0,091$) and seminoma ($p = 0,091$) express a similar level of PRMT5 compared to normal testicular tissue (Fig. 4L). These data could be confirmed, using a whole genome expression DNA-Array as reported before (20). Here, the same pattern was observed (see Fig. M and and 4N).

Table 1: Expression of BLIMP1, PRMT5 and dimethylated histone H4/H2A in normal and neoplastic testicular tissues

	BLIMP1	PRMT5	H4R3me2s/H2Ame2s
Normal fetal testis			
Gonocytes	+++ (n)	+++ (n,c)	+++ (n)
Pre-spermatogonia	-	-	
Normal adult testis (N = 18)			
Spermatogonia	-	-	++ (n)
Pachytene spermatocytes	-	+ (n)	-
Round spermatids	+ (n,c)	++ (n)	++ (n)
Elongated spermatids	-	-	-
Testicular germ cell tumors			
IGCNU (N = 15)	+++ (n) 85–100%	+++ (c) 75–95%	+++ (n) 90–100%
Seminoma (N = 20)	++ (n) 10–75%	++ (n+c) 30–85%	++ (n) 20–80%
Embryonal carcinoma (N = 15)	+/-(c)* 15–80%	++ (c) 15–80%	(+)*
Teratoma (N = 5)	+ (n, c)	+ (n, c)	+ (n)
Chorioncarcinoma (N = 3)	+ (c)	-	-

N, number of cases; n, nuclear staining; c, cytoplasmatic staining

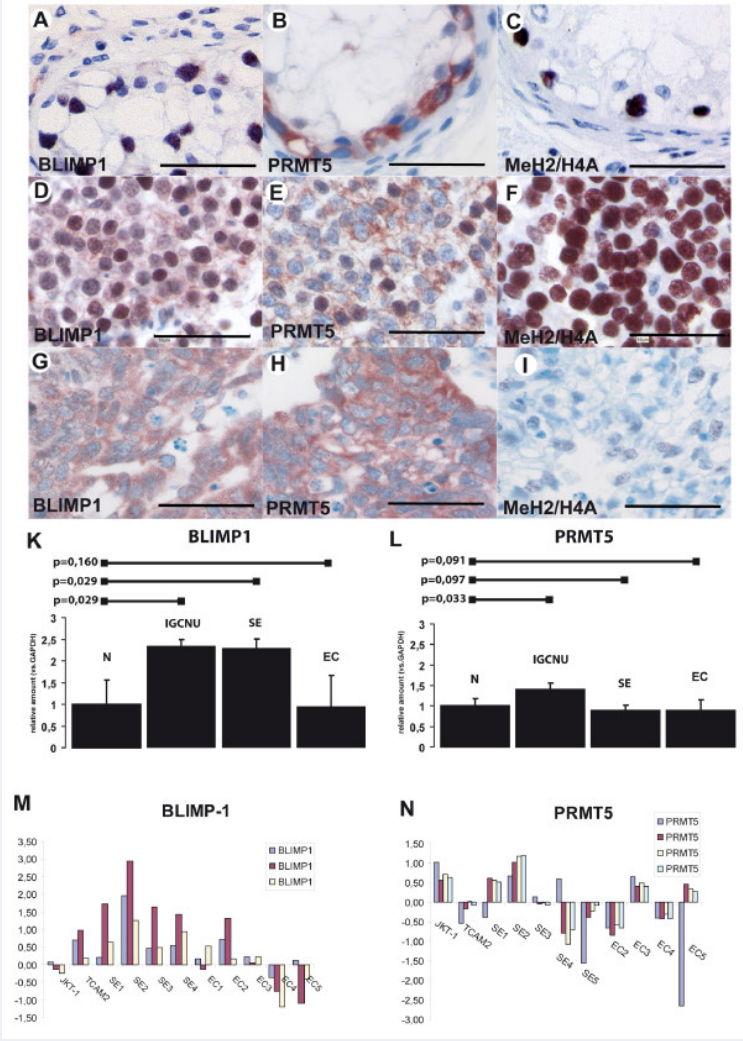
+ weak; ++ moderate; +++, strong expression; -, no expression detectable

*, only single tumor cells were detected as positive.

Finally, we asked whether BLIMP1/PRMT5 and modification of histone H2A and H4 could be detected in TCam-2, a cell line derived from a seminoma patient (21,22). Here, we were able to detect BLIMP1 in the nucleus, PRMT5 in the nucleus and the cytoplasm (Fig. 5A–C). RT-PCR analyses showed that BLIMP1 and PRMT5 are expressed in TCam-2 cells (Fig. 5E) and absent JKT1 cells, in agreement with Affymetrix data (Fig. 4M and 4N). Of note, the findings on the JKT-1 cell line are in concordance with the conclusion that it is not a seminoma cell line (22,23). Western blot analysis confirmed these results, showing that BLIMP1 and PRMT5, as well as the modified Histones H2A and H4 (Fig. 5F) can be detected.

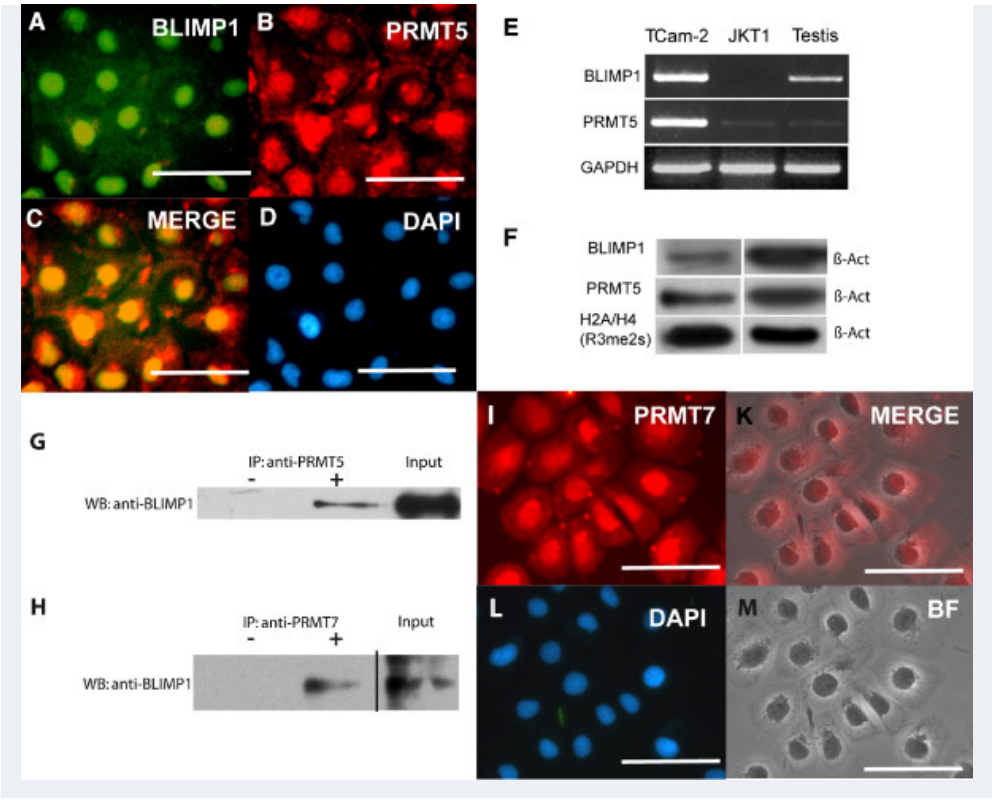
Next, we performed a CoIP on extracts from TCam-2 cells and were able to detect a signal for Blimp1 in material immunoprecipitated with PRMT5 antibody (Fig. 5G). This result demonstrates for the first time that PRMT-5 and BLIMP-1 interact biochemically.

Figure 4 : Human germ cell tumors.



Sections of neoplastic germ cells of IGCNU (A-C), seminoma (D-F), embryonal carcinoma (G-I) stained for BLIMP1 (A, D, G), PRMT5 (B, E, and H) and methylated histones H2A/H4 (C, F, I). In Figure A-C tubules with IGCNU are shown with consistent nuclear expression of BLIMP1 and Me H2A/H4 in neoplastic germ cells (A, BLIMP1; C, Me H2A/H4). PRMT5 is expressed in the cytoplasm of neoplastic germ cells (B). Notice that no expression is present in Sertoli cells. In Figures D-F expression in seminomas is presented. Notice the variation of the expression of BLIMP1, being low or moderate in the majority of the cells (D). PRMT5 is expressed in the cytoplasm of most seminoma cells, but some neoplastic cells also show nuclear staining (E). Figure F shows a strong nuclear staining of MeH2A/H4 in most seminoma cells. Size bar is 50 µm. Quantification of the relative expression of BLIMP1 (K) and PRMT5 (L) normalized to β-Actin and compared to normal testicular tissue. Bars above the graph indicate p-values. (M, N) Expression values for BLIMP1 (M) and PRMT5 (N) from independent Affymetrix expression analyses (as referred in 23). Data are plotted as Log2 (y-axis) after normalization. Abbreviations: Normal testicular tissue (N), IGCNU, seminoma (SE), embryonal carcinoma (EC).

Figure 5 : Analysis of TCam-2 seminoma cell line. (A-B)



Immunohistochemistry using the antibodies indicated. (C) Merge of (A) and (B). (D) Counterstaining with DAPI to detect nuclei. (E) RT-PCR cell lines TCam2 and JKT1 as well as Testis detecting expression of the indicated genes. (F) Western Blot of protein lysate from TCam2 cells detecting the proteins indicated. (G) Co-IP experiment using antibody to PRMT5 for IP and antibody to BLIMP-1 to detect potential interaction. – no Antibodyl; + IP using PRMT5 Antibody; Input Control. (H) Co-IP experiment using antibody to PRMT7 for IP and antibody to BLIMP-1 to detect potential interaction. – no Antibodyl; + IP using PRMT5 Antibody; Input Control. (I-M) Immunohistochemistry using the PRMT7 antibody (I), (K) Merge of (I) and (M), (L) Counterstaining with DAPI to detect nuclei, (M) brightfield image. Scale Bar indicates 25 µm.

We had shown, that nuclear BLIMP1 and methylated H2A and H4 are expressed in IGCNU and seminoma, yet these cells express either little or cytoplasmic PRMT5 (Fig. 4A–F). We speculated that another methytransferase cooperating with BLIMP1 might be able to compensate PRMT5 function and help in establishing this methylation pattern. PRMT7 which is like PRMT5 a type II methyltransferase seemed a potential candidate since both PRMT5 and PRMT7 have been demonstrated to mediate symmetric arginine dimethylation of sm Proteins required for the spliceosome (24). The CoIP experiment (Fig. 4H), demonstrates that BLIMP1 and PRMT7 interact biochemically. In addition PRMT7 shows a strong nuclear signal in TCam-2 cells (Fig. 4I–M). These results indicate that in germ cell tumors, both PRMT5 and PRMT7 might cooperate with BLIMP1 to establish dimethylation of H2A and H4.

Discussion

In this study, we analyzed the expression of the putative inhibitor complex of germ cell differentiation BLIMP1 and PRMT5 on mRNA and protein level and the presence of the resulting repressive histone modifications H2A/H4R3me2s in human fetal and adult germ cells as well as TGCTs. We found BLIMP1 and PRMT5 localized in the nuclei of gonocytes, and the latter also in the cytoplasm, and could show the presence of the resulting dimethylation of H2A/H4 at arginine 3. In IGCNU a strong nuclear signal of BLIMP1 and of H2K3me2s/H4K3me2s was detected, whereas PRMT5 signal was cytoplasmatic in IGCNU and heterogeneous in seminomas.

The expression in fetal gonocytes in humans described here is in concordance to the observations made in mouse (17) indicating a conserved role of the nuclear localized BLIMP1/PRMT5 complex between mouse and man. Recently the transcriptional repressor BLIMP1 has been shown to be a crucial determinant of the germ cell lineage in mice (16). This Krüppel-type zinc-finger containing protein interacts with the arginine methyl-transferase PRMT5 resulting in a symmetrical methylation at arginine 3 of histone H4 and H2A (H4R3me2s/H2Ame2s). The methylation in turn represses transcription (17) and therefore might be important for suppressing the somatic cell fate and keeping germ cells in a pluripotent state. In fact, in mice Blimp1-deficent germ cells show inconsistent repression of HoxB1, a hallmark of germ cell specification and fail to express Stella a marker of undifferentiated germ cells (16). Also, recent studies showed, that abrogation of the Drosophila melanogaster homolog of PRMT5, Capsuleen/dart5, is essential for germ cell specification and maintenance (25,26). Interestingly, Blimp1 expression is lost in PGCs which are cultured in the presence of basic FGF and LIF (15) and gradually become embryonic germ cells (27-29). Hence the BLIMP1/PRMT5 interaction resulting in H2A/H4 modification might lead to repression of premature differentiation during human fetal germ cell development. As a consequence prolonged expression of BLIMP1/PRMT5 could result in persistence of undifferentiated gonocytes into adulthood.

It is believed that those persisting gonocytes give rise to IGCNU the common precursor lesion of all type II TGCTs (4). Indeed, we detected BLIMP1 protein and

the characteristic modification of histones H2A and H4 not only in gonocytes but also in IGCNU and in seminoma supporting a PGC/gonocyte origin of IGCNU and therefore GCT (5,8,30,31). PRMT5 however, is not detectable in nuclei of IGCNU, and displays only a sparse nuclear localization in seminoma cells. We found that another type II protein arginine methyltransferase, PRMT7 is expressed in TCAM2 seminoma cells and that PRMT7 interacts with BLIMP1 as well. So we speculate that in IGCNU and seminoma, BLIMP1 recruits PRMT7 to compensate for the lack of nuclear PRMT5 to mediate H2A and H4 dimethylation.

Upon progression of IGCNU to nonseminomas signal intensity of BLIMP1 decreased and subcellular localization changed. As a consequence, H2A/H4 modification decreased and became heterogeneous in nonseminomas. Hence, the loss of the repressive histone modifications allows further uncontrolled differentiation observed in nonseminomas.

Conclusion

Taken together we propose the following model for development of germ cell neoplasia. First, coexpression and nuclear localization of the BLIMP1/PRMT5 complex leads to histone H2A/H4 dimethylation which results in transcriptional silencing of genes responsible for somatic differentiation in PGCs. Upon differentiation to prespermatogonia, this complex is downregulated and the H2A/H4 marks are lost. Aberrant constitutive histone H2A/H4 arginine 3 dimethylation allows the cells to escape the regular differentiation program resulting in their persistence into adulthood. These cells eventually progress into IGCNU, displaying the H2A/H4R3me2s modification as well. Since the subcellular localization of PRMT5 excludes PRMT5-dependent histone H2A/H4 modification in IGCNU we propose that BLIMP1 might act in cooperation with PRMT7. This mechanism persists in seminoma where the H2A/H4R3me2s modifications can be observed which explains the undifferentiated nature of the tumor cells. Translocation of BLIMP1 into the cytoplasm leads to breakdown of histone H2A/H4 dimethylation and subsequently to the activation of the differentiation programs and therefore the conversion from IGCNU into a nonseminomatous germ cell tumors.

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Chapter 5

c-KIT is frequently mutated in bilateral germ cell tumours and down-regulated during progression from intratubular germ cell neoplasia to seminoma

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Abstract

Testicular germ cell tumours (TGCTs) are the most frequent cancer type in young men; 5% of these patients develop a second TGCT in the contralateral testis. The pathogenesis of TGCT is closely linked to primordial germ cells (PGCs) or gonocytes. The receptor tyrosine kinase (c-KIT) is necessary for migration and survival of PGCs and is expressed in intratubular neoplastic germ cells (IGCNUs) and seminomas. We studied the frequency of *c-KIT* exon 11 and 17 mutations in 155 unilateral (108 seminomas and 47 non-seminomas) and 22 bilateral (18 seminomas, two embryonal carcinomas, two IGCNU) cases. While no mutations were detected in exon 11, the mutation frequency in exon 17 was significantly higher in bilateral (14/22, 63.6%) compared to unilateral TGCT (10/155, 6.4%) ($p < 0.001$). Different activating mutations (Y823D, D816V, D816H and N822K) were detected in bilateral TGCT. Y823D mutation was identical in both testes in three cases and quantitative pyrosequencing showed that up to 76% of the cells analysed in tumour samples carried this mutation. One bilateral synchronous seminoma revealed a S821F mutation in one testis and a Y823D mutation contralaterally. To study the role of c-KIT in TGCT progression, we compared its expression in 41 seminomas and adjacent IGCNUs. Immunohistochemical analysis revealed that c-KIT expression was significantly reduced in seminomas compared to IGCNUs ($p < 0.006$) and that there were no significant changes in *c-KIT* mRNA copy numbers in progressed compared to low-stage seminomas. In summary, our study shows that patients with *c-KIT* mutations are more prone to develop a bilateral TGCT and suggests that in a portion of bilateral TGCTs, *c-KIT* mutations occur early during embryonal development, prior to the arrival of PGCs at the genital ridge. Furthermore, our findings show that c-KIT down-regulation occurs during the progression of IGCNU to seminoma.

Introduction

Testicular germ cell tumours (TGCTs) are the most common cancer type in young men (1). All TGCTs except spermatocytic seminomas originate from intra- tubular germ cell neoplasia (IGCNU). Once IGCNU is established, it always leads to invasive TGCT (2). Several risk factors for IGCNU have been described, such as atrophy of the testis, mumps orchitis, familial predisposition, cryptorchism, gonadal dysgenesis and a history of TGCT in the contralateral testis (3); 5% of patients with TGCT will develop bilateral disease and epidemiological studies of bilateral TGCT revealed that 25% of TGCT patients show a latency period of up to 20 years, again stressing the fact that these patients should undergo close follow-up over a longer period of time (4). Surgical biopsies, still remaining the only reliable predictor for TGCT, should only be offered to high-risk patients with unilateral disease due to a high complication rate and a lack of relevance for other cases concerning the outcome (5). Nevertheless, it is of high interest to further define causal factors triggering development of bilateral TGCT.

The receptor tyrosine kinase (c-KIT) was first detected in TGCT by Strohmeyer et al in 1991 (6) and in IGCNU by Rajpert-De Meyts and Skakkebaek in 1994 (7). In human fetal testis, c-KIT is found in gonocytes (8-11), proposed cells of origin of IGCNU, is down-regulated after the 25th gestational week (12) and is not detectable or expressed at a very low level in fetal and adult spermatogonia (13). Detailed immunohistochemical studies showed that c-KIT is highly up-regulated in IGCNU (7, 14) and is retained in seminomas (7) but not expressed in non-seminomas (7, 15). Because the role of c-KIT in IGCNU and seminomas is as yet not clear, we studied its expression during the progression of IGCNUs and seminomas by immunohistochemistry and quantitative RT-PCR. Furthermore, our study aimed to clarify whether bilateral as opposed to unilateral TGCTs are highly associated with activating c-KIT mutations, as previously postulated, and whether other mutations beside those in codon 816 are involved (16). In contrast to the study of Looijenga (16), which identified activating codon 816 c-KIT mutations in 93% of 61 bilateral TGCTs, lower mutation rates of 17-28.5% were found in two previous studies: here, however, only a limited number of bilateral TGCTs were examined (17, 18). Therefore, we analysed 22 samples of bilateral (18 seminomas,

two embryonal carcinomas, two IGCNUs) and 155 samples (108 seminomas and 47 non-seminomas) of unilateral TGCT to detect genetic alterations in exons 17 and 11. In fact, the mutation frequency was significantly higher in bilateral compared to unilateral TGCTs ($p < 0.001$) and all identified mutations were localized within exon 17. Interestingly, Y823D activating mutation was frequently detected in bilateral TGCT and was identical in both sites in three patients. Results from our experiments also show that c-KIT is down-regulated in seminomas compared to their IGCNUs and indicate that it is not further up-regulated in progressive seminomas.

Materials and methods

Tissue samples

Formalin-fixed, paraffin-embedded testicular tissues from 155 patients with unilateral TGCT (107 seminomas, 37 embryonal carcinomas, six mixed malignant non-seminoma, three yolk sac tumours, one choriocarcinoma and one spermatocytic seminoma) and from 12 patients with either bilateral TGCT (18 seminomas and two embryonal carcinomas) or IGCNU (n = 2) in the contralateral testis were collected from the archives of the Departments of Pathology of University Medical Centers Bonn, Essen and Giessen. All tumours were classified according to the WHO classification, based on their histology, by two independent pathologists. Fresh frozen samples of 32 seminomas were additionally available for this study. Use of the tissue for scientific purposes was approved by the institutional Regional Committee for Ethics.

DNA preparation, PCR and sequencing

DNA extraction, polymerase chain reaction (PCR) and sequencing were performed as previously described (19). Briefly, genomic DNA was extracted after pre-treatment with proteinase K. The PCR reaction was carried out using Platinum Taq polymerase (Invitrogen) in a volume of 50 µl, containing 10 mm Tris-HCl, pH 8.3, 40 mm KCl, 1.0-2.5 mm MgCl2, 200 mm each dNTP, 20 pM each primer, and 0.25 U platinum Taq polymerase (Invitrogen). The following conditions were used: for exon 11 PCR, 3 min at 94 °C; 37 cycles of 40 s at 94 °C, 40 s at 52 °C, 40 s at 72

°C, 5 min at 72 °C; and for exon 17 PCR, 3 min at 94 °C; 37 cycles of 40 s at 94 °C, 40 s at 48 °C, 40 s at 72 °C, 5 min at 72 °C. The following PCR primers were used for direct sequencing: exon 11F, 5-CTATTTTTCCCTTTCTCCCC-3, 11R 5-TACCCAAAAAGGTGACATGG-3; exon 17F, 5-GGTTTTCTTTTCTCCTCCAAC-3; exon 17R, 5-AAACTAAAAATCCTTTGTAGGAC-3. Cycle se- quencing was performed using ABI PRISM Dye Terminator Sequencing Kit (Applied Biosystems, Weiterstadt, Germany) on a TC 9600 thermocycler (Perkin-Elmer, Germany) with 20 ng PCR products. All mutations were confirmed by a second independent round of DNA extraction, PCR and cycle sequencing.

Restriction endonuclease-mediated selective PCR (REMS-PCR) was performed as described previously [16], by generation of an AatII recognition site encompassing codon 816, using a modified 3 primer. PCR amplifications were carried out in a volume of 50 µl, as mentioned above, under the following conditions: 3 min at 94 °C; and 37 cycles of 45 s at 94 °C, 30 s at 45 °C, 45 s at 72 °C, 10 s at 72 °C. 20 µl each amplification product was digested with 5 U AatII (Fermentas, St. Leon-Rot, Germany) at 37 °C overnight. No cleaved PCR product of 106 bp was detected in the presence of mutations in codon 816, while wild-type PCR products were digested into 85 and 21 bp fragments, respectively.

PCR amplification primers for detection of Y823D mutation by pyrosequencing were developed as follows: 823F, forward biotinylated primer, 5-CAGCC-AGAAATATCCTCCTTACTC-3; and 823R reverse primer, 5-ACTGTCAAGCAGAGAATGGGTACT-3. Each PCR mix contained the forward and reverse primers (20 pmol each) and the PCR was performed under the following conditions: 3 min at 94 °C; 37 cycles of 40 s at 94 °C, 40 s at 65 °C, 40 s at 72 °C, 5 min at 72 °C. The PCR products (20 µl each) were sequenced using the Pyrosequencing PSQ96 HS System (Biotage AB), following the manufacturer's instructions, using the primer 5-TACTCACGTTTCCTTTAAC-3.

Exon 17 PCR products of selected samples of bilateral TGCT were cloned into a pCR 2.1-vector by TA cloning Kit (Invitrogen) and competent Escherichia coli

TOP10 were transformed with ligation products. Positive clones were identified by blue/white screening and three individual positive clones were grown in Luria-Bertani medium overnight. Plasmid DNA was isolated by the alkaline lysis (Birnboim-Doly) method. Plasmids were screened for correct inserts by restriction digestion with KpnI and XhoI enzymes. Inserts were sequenced using M13 reverse and forward primer sets.

RNA preparation and real-time PCR

Total RNA was extracted with Trizol (Invitrogen, Karlsruhe, Germany) from seminomas of different stages; 16 seminomas of pT1, 12 of pT2 and six of pT3 stages were collected. All samples had RNA integrity number (RIN) values > 8 (Agilent Bioanalyser 2000, Agilent Technologies). Quantitative PCR (QPCR) was performed on cDNA synthesized from 100 ng RNA using nonamer primers, and Omniscript Synthesis Kit (Qiagen, Hilden, Germany) using 5 µl PCR supermix from Abgene (Abgene, Hamburg, Germany) on the ABI 7900 detection system. The intron-spanning primer pairs were designed by Applied Biosystems (Assay ID Hs00174029-m1 for c-KIT). Primers were added to the reaction mixture at a final concentration of 200 nM. Thermal cycle parameters were: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All reactions were performed in triplicate with ß-actin as an internal control.

Immunohistochemistry

For immunohistochemistry, dewaxed, 4 µm-thick tissue sections were made from paraffin-embedded tissue and microwave-pretreated in citrate-buffer for antigen retrieval. Immunohistochemistry was performed using the Dako (Hamburg, Germany) EnVision-AEC Kit according to the manufacturer's protocol, using a monoclonal antibody to KIT (Dako) as previously described [12]. Briefly, endogenous peroxidase was blocked for 5 min in 0.03% H2O2 (diluted in distilled water). Sections were washed in Tris-buffered saline (TBS; 0.05 M Tris and 0.85% NaCl, pH 7.6) and incubated with primary antibodies overnight at 4 °C and thereafter with HRP-labelled polymer conjugated with a secondary antibody.

Evaluation and statistics

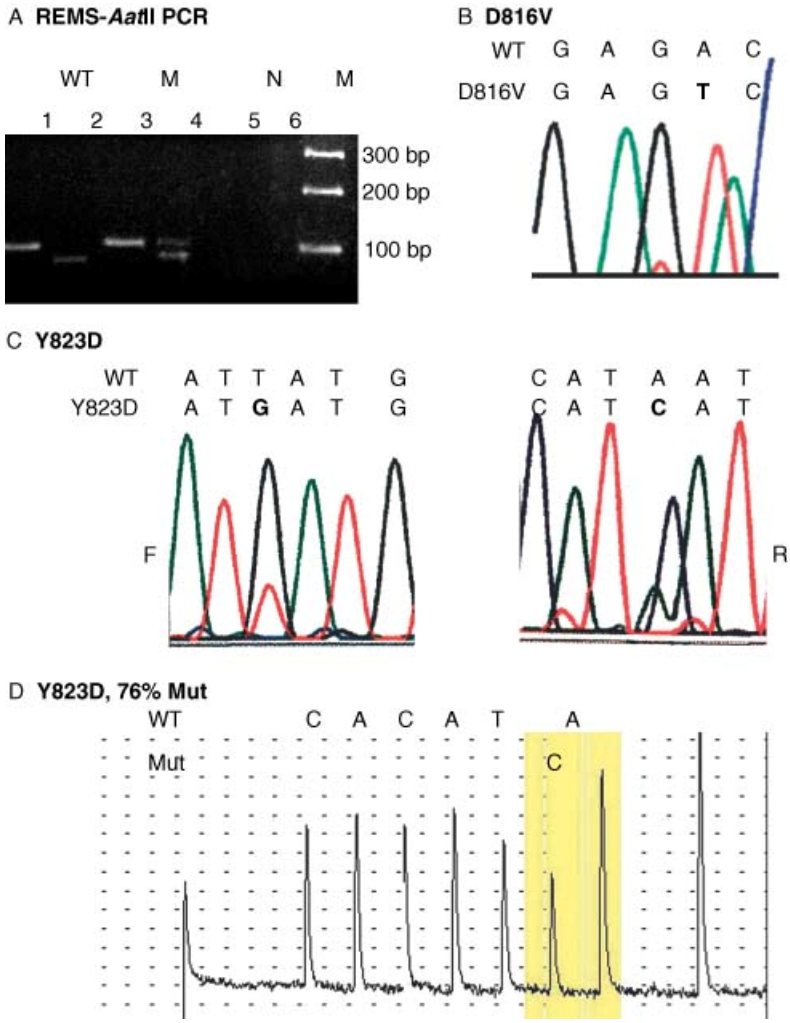
Immunohistochemical results from c-KIT staining were assessed independently by two pathologists in a semiquantitative manner. The quantity of immunoreactive tumour cells was estimated according to the following scheme: < 10%, 10-75%, > 75% of the tumour cells and None. The level of immunoreactivity was assessed by scoring of its predominant intensity: weak (+), moderate (++) and strong (++ +). The final immunoreactive score (IRS) was calculated as strong (3) when at least 75% of the tumour exhibited at least moderate immunoreactivity. In cases of weak immunoreactivity in < 10% of all tumour cells, the final IRS was considered as negative (0). The score of 2 was assigned if weak immunoreactivity was present in > 75% of tumour cells or if in 10-75% tumour cells strong or moderate staining was observed. All other cases received a score of 1. Statistical analyses were performed by SPSS software (SPSS Inc., Chicago, IL, USA) and included 2, Fischer's exact and Wilcoxon tests.

Results

Frequency of c-KIT exon 17 mutations in bilateral germ cell neoplasia

Up to three samples of each bilateral testicular germ cell neoplasia were screened for codon 816 mutations using the REMS-AatII PCR approach, as previously described [16]. In five samples (3a, 3b, 6a, 10a, 12b), REMS-AatII PCR yielded an undigested 106 bp product, indicating the presence of a codon 816 mutation (Figure 1A). In all other samples, complete digestion of the PCR products was achieved (85 and 21 bp products). Sequencing the PCR product revealed that three patients carried D816V (Figure 1B) and one patient a D816H mutation. In one IGCNU (case 3b), REMS-AatII PCR yielded an undigested 106 bp product, but no mutations were detected by direct DNA sequencing. Altogether, c-KIT exon 17 mutations were detected in 14/22 (63.6%) of bilateral samples by direct sequencing. A unique N822K mutation was detected in patient 8. In patients 2, 4 and 11, Y823D mutations were found in both testes; patient 7 with synchronous seminomas had an Y823D mutation in one side and S821F mutation in the other.

Figure 1: c-KIT exon 17 mutations



(A) Representative gel electrophoresis of REMS-PCR of wild-type and codon 816 mutant prior to (lanes 1 and 3) and after (lanes 2 and 4) AatII digestion. Notice complete digestion of the 106 bp PCR product in wild-type and incomplete digestion of the mutant sample. N, negative control where DNA was omitted from the PCR reaction (lane 5) and in the digestion (lane 6). M, DNA marker. (B, C) Automated sequencing data from amplicons with heterozygous D816V (B, forward), Y823D (C, forward and reverse) mutations. The wild-type sequence is shown above and the mutant sequence below. F, forward reaction; R, reverse reaction. (D) Representative results from pyrosequencing of the seminoma sample from patient 4a detecting the Y823D mutation in 76% of analysed cells

Y823D mutation was also detected in normal testicular parenchyma in patient 2, and in adjacent IGCNU in patient 7 (Figure 1C). To assure that no codon 816 mutations were overlooked by analysis of DNA extracts, all cases of germ cell neoplasia lacking codon 816 mutations (patients 1, 2, 4, 5, 6b, 7, 8, 9a) were re-examined by cloning of their exon 17 PCR amplification products. Again, no codon 816 mutations were detected, either by REMS-PCR of cloned PCR products or by direct sequencing.

We next verified Y823D mutation and quantified the number of mutant alleles carrying Y823D by pyrosequencing. To demonstrate assay reproducibility, we performed three runs per sample and compared the results in mutated and non-mutated samples (n = 50). While in non-mutated samples the amount of mutated DNA was < 5%, up to 76% of mutated alleles were detected in samples with Y823D mutation detected by direct DNA sequencing (Table 1, Figure 1D). All results, including REMS-AatIIPCR, direct DNA sequencing and pyrosequencing, are summarized in Table 1. The frequency of activating mutations in patients with bilateral disease was significantly higher compared to unilateral TGCT ($p < 0.001$, Fischer's exact test). Polymorphism I798I was detected in one case (8b).

Table 1. Summary of *c-KIT* exon 17 mutations in 12 patients with bilateral germ cell neoplasia

			Invasive TGCT		IGCNU		N	
Case	Age	Diagnosis	REMS-*AatII* PCR	DS	REMS-*AatII* PCR	DS	REMS-*AatII* PCR	DS
1a	35	S	-	None				
1b	34	S	-	None	-	None		
2a	35	S	-	Y823D (19%)			-	Y823D (18%)
2b	35	S	-	Y823D (19%)				
3a	41	S	+	D816V				
3b	41	IGCNU			+	None		
4a	38	S	-	Y823D (76%)				
4b	40	EC	-	Y823D (27%)				
5a	30	S	-	None	-	None		
5b	30	S	-	None	-	None		
6a	35	S	+	D816V				
6b	35	S	-	None				
7a	40	S	-	S821F	-	None		
7b	40	S	-	Y823D (23%)	-	Y823D (19%)		
8a	28	S	-	N822K				
8b	28	IGCNU			-	None (I798I)		
9a	34	EC	-	None			-	None
9b	35	S	+	D816V	-	None	-	None
10a	35	S	+	D816V	-	None	-	None
10b	35	IGCNU			na	na		
11a	36	S	-	Y823D (18%)	-	None		
11b	36	S	-	Y823D (19%)				
12a	33	EC	na	na				
12b	44	S	+	D816H				

DS, direct DNA sequencing; IGCNU, intratubular germ cell neoplasia; S, seminoma; EC, embryonal carcinoma; N, normal testicular parenchyma; na, not available; +, undigested PCR product in REMS-*AatII* PCR (codon 816 mutation); -, total digestion of PCR product in REMS-*AatII* PCR (wild-type codon 816).

Frequency of c-KIT exon 17 mutations in unilateral germ cell neoplasia

Whereas mutations in exon 11 of c-KIT were found in neither unilateral nor bilateral TGCT, exon 17 mutations were detected in 10/155 unilateral TGCTs (6.45%), including nine seminomas and one embryonal carcinoma (Table 2).

Table 2. Summary of *c-KIT* exon 17 activating mutations in unilateral germ cell neoplasia

Case	Disease-free (years)	TGCT					Contralateral testis	
		Age	Histology	Mutation	IGCNU	N	Histology	Mutation
1	3	58	S	D816V			SCO	
2	2	44	S	N822K			Normal	None
3	4	40	S	N822K	None	Y823D	Normal	
4	12	29	EC + S	D816V (S)*			Normal	
5	3	26	S	Y823D			Normal	
6	4	34	S	D816V	None		Normal	
7	8	48	S	D816V			SCO	
8	3	49	S	D816H			Normal	
9	3	26	S	C809S	None		Normal	
10	4	28	EC + S	Y823D (EC)*	Y823D	None	Normal	None

S, seminoma; EC, embryonal carcinoma; N, normal testicular parenchyma; SCO, Sertoli cell only.
* In case 4, mutation was detected only in the seminomatous component and in case 10 only in the embryonal carcinoma component of each TGCT.

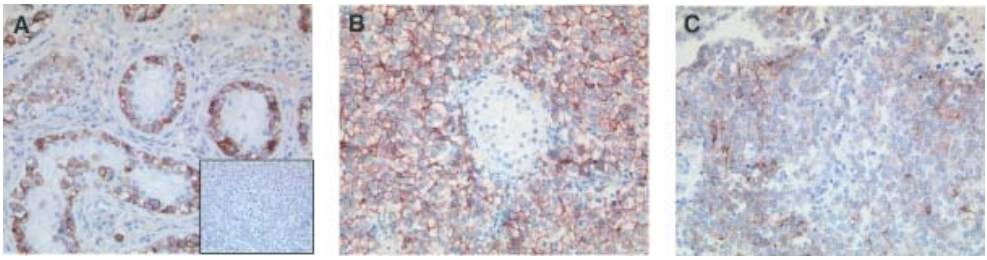
Histological examinations of contralateral biopsies from patients with unilateral TGCTs carrying c-KIT mutations showed normal testicular parenchyma with regular spermatogenesis in eight patients (2, 3, 4, 5, 6, 8, 9 and 10). In contrast, contralateral biopsies from patients 1 and 7 revealed a complete atrophy of the seminiferious tubules. All 10 patients were free of testicular masses in the contralateral testis up to 12 years after the diagnosis. Testicular contralateral biopsies of patients 2 and 10 were available for DNA extraction and direct sequencing. No exon 17 c-KIT mutations were detected in these samples. Interestingly, in patient 3, seminoma and normal parenchyma carried different

mutations: N822K mutation was detected in seminoma and Y823D in normal testis. In patient 10, Y823D mutation was detected in invasive TGCT consisting of an embryonal carcinoma and seminoma as well as in adjacent IGCNU but not in normal testicular parenchyma. Polymorphism I798I was detected in 52 unilateral TGCTs (33.5%).

Expression of c-KIT during progression of IGCNUs and seminomas

Expression of c-KIT in precursor lesions was compared to the expression in the adjacent seminomas by analysis of immunohistochemical results for KIT staining in 41 unilateral tumours. Altogether, IRS of 2 was detected in 12 and IRS of 3 in 29 IGCNU. In invasive seminomas, IRS of 1 was detected in five seminomas, IRS of 2 in eight, IRS of 3 in 21 and no expression of c-KIT was seen in 7 cases. By statistical analysis, KIT-expression was significantly reduced in seminomas compared to IGCNU ($p = 0.006$; Wilcoxon test), as shown by a representative example in Figure 2A. Detailed results of c-KIT expression in all individual cases are given in Table 3 and representative staining of c-KIT in IGCNUs and seminomas are shown in Figure 2.

Figure 2

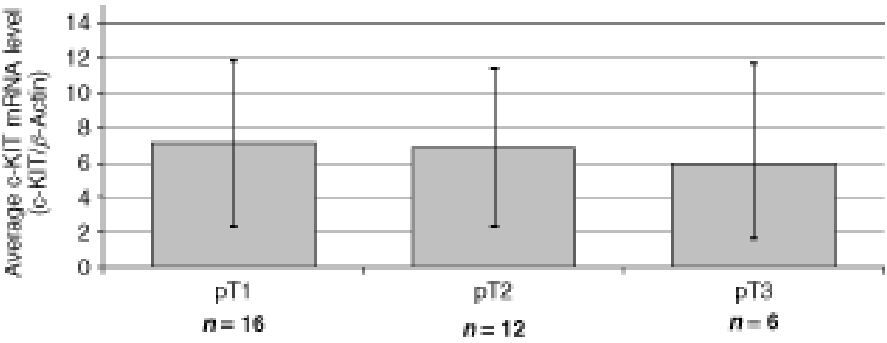


Examples of c-KIT staining. (A) c-KIT is strongly expressed in IGCNU (IRS of 3) but down-regulated and not detectable in adjacent invasive tumour (inset). (B, C) Examples of strongly (B) and moderately (C) c-KIT-positive seminomas (IRS of 3 and 2, respectively)

Furthermore, to check whether the progression of seminomas is associated with an increased expression of c-KIT, real-time PCR was performed from mRNA extracted from 34 fresh unilateral seminomas at different stages of the disease (pT1, n = 16; pT2, n = 12; pT3, n = 6). The average mRNA copy number compared to normal testicular parenchyma was 7.08 in pT1 seminomas, 6.8 in pT2 seminomas and 6.0 in pT3 seminomas (Figure 3). This finding shows that mRNA copy number does not increase during the progression of seminomas.

In addition, expression of c-KIT in mutated and wild-type seminomas was compared by immunohistochemical analysis. Among mutated seminomas (n = 22, 13 bilateral and nine unilateral), three had an IRS of 2, and 18 had an IRS of 3. Among wild-type seminomas (n = 70, unilateral), IRS of 0 was detected in nine cases, IRS of 1 in one case, IRS of 2 in 25 cases and IRS of 3 in 35 cases (Table 4). By statistical analysis, expression of c-KIT was significantly higher in mutated versus wild type seminomas (p = 0.007; Wilcoxon test).

Figure 3



Comparison of c-KIT mRNA levels between seminomas of stages pT1, pT2 and pT3 by real-time PCR normalized with ß-actin and compared to the expression in normal testis. Bars indicate ± S

Table 3. Results of c-KIT immunohistochemistry in IGCNU and adjacent seminomas, as calculated by immunoreactive score (IRS)

	IGCNU-IRS	
Seminoma-IRS	**2**	**3**
0	4	3
1	0	5
2	2	6
3	6	15

Table 4. Results of c-KIT immunohistochemistry in seminomas with c-KIT exon 17 mutation and in wild-type seminomas, as calculated by immunoreactive score (IRS)

IRS	Mutated	Wild-type
0	0	9
1	0	1
2	3	25
3	18	35

Discussion

Receptor tyrosine kinase c-KIT and its ligand Steel factor are not only important for proliferation, survival and differentiation of PGCs but also regulate their migration in vivo (20). Binding of Steel factor to c-KIT triggers survival of PGCs, while down-regulation of Steel factor activates apoptosis of PGCs in the midline (21). In adult mice mutant for c-KIT, decrease of spermatogonial proliferation and different spermatogenic alterations are detected (20), but in infertile human patients no alterations of the c-KIT gene have so far been detected (22). In contrast, c-KIT mutations occurred in TGCT and their frequency was investigated in previous studies by different techniques, including direct sequencing, SSCP and high-resolution melting amplicon analysis (16, 23, 24).

In previous studies not discriminating between unilateral and bilateral TGCT, most mutations have so far been detected in exon 17 in seminomatous germ cell tumours, with varying frequencies of up to 40.9% (23-26). A recent study focusing on codon 816 of c-KIT has shown that different mutations in this codon (D816V, D816H, D816Y) are highly associated with bilateral compared to unilateral TGCT (16), but following studies could not reproduce these results (14, 16, 17). Also in codon 816, gain-of-function mutations in various other loci of exon 17 were found previously in bilateral TGCT and included D820G and N822K mutations; however, only very limited numbers of bilateral TGCT were analysed (17, 18). Thus, to study the role of c-KIT in bilateral and unilateral germ cell neoplasia, we analysed 22 bilateral and 155 unilateral TGCTs. Here we demonstrate that the mutation frequency in exon 17 of c-KIT is in fact significantly higher in bilateral compared to unilateral TGCT (6.5% in unilateral versus 63.6% in bilateral TGCT; $p < 0.001$, Fischer's exact test). Interestingly, the frequency of codon 816 mutations in bilateral TGCT was lower in our study and in previous studies compared to the study of Looijenga et al(93%), although the additional sensitive technique of REMS-PCR was used in our study to detect any alterations within codon 816 (16). Surprisingly, Y823D mutation was most frequently found among bilateral TGCTs (31.8%) and occurred in three patients on both sites, as well as in normal testis and IGCNU. In contrast, D816V and N822K mutations were detected in only one of the

two tumours. All these findings indicate that in a subset of bilateral TGCTs, c-KIT mutations occur early in germ cells, most likely in migrating PGCs, and therefore are identical in both testes, while in others, gene alterations of c-KIT are generated in postmigratory or even in neoplastic germ cells. The clinical management procedure for patients with TGCT does not necessarily include histological examination of the contralateral testis. Because our study shows that c-KIT exon 17 mutations occur 10 times more frequently in bilateral compared to unilateral TGCT, the presence of c-KIT mutation would help to identify patients with higher risk for bilateralism, and a contralateral biopsy could reveal IGCNU or TGCT at early stages in these patients.

In accordance with most previous studies (18, 24, 25, 27), c-KIT mutations preferably occurred in seminomas, because 22/24 (91.6%) detected mutations were found in seminomas and only 2/24 (8.4%) in embryonal carcinomas. Except for the new mutations, S821F in one bilateral and a C809S in one unilateral seminoma, all detected mutations were auto-activating and led to a constitutive phosphorylation of c-KIT receptor in absence of SCF (24). Such gain-of-function mutations of c-KIT are mutually exclusive oncogenic events in mastocytosis and in gastrointestinal stromal tumours (GISTs) (28, 29, 30). This also seems to be the case for TGCT, as no other receptor tyrosine kinase except for c-KIT are altered in this malignancy (31). The consequence of c-KIT activation in germ cells awaits analysis, but detected mutations can potentially lead to malignant transformation, as it has been shown in haematopoietic cells (32).

Based on the finding of frequent amplification of the c-KIT gene (in up to 21% of seminomas), it was hypothesized that the KIT receptor plays an important role in the progression of IGCNU towards seminoma (18). Because gene amplification might lead to the overexpression of the corresponding protein, higher expression of c-KIT should be expected in invasive seminomas than in IGCNU. Surprisingly, analysis of the c-KIT expression in 41 seminomas and their precursor lesions revealed significant down-regulation of c-KIT expression in seminomas compared to IGCNU ($p = 0.006$). Furthermore, we did not observe an increase of KIT mRNA

levels in pT2/pT3 compared to pT1 seminoma by real-time RT-PCR. These findings are in line with previous studies that have detected a loss of c-KIT expression during tumour progression, suggesting a role in initiation but not progression of seminoma (3, 26, 33). In addition, the immunohistochemical analysis revealed that wild-type seminomas show a significantly lower c-KIT expression compared to seminomas with c-KIT mutation (Table 4), due to seminomas with low or missing c-KIT expression. This result also demonstrates that c-KIT expression is lost in a portion of seminomas. Our preliminary data indicate that the heterogeneous pattern of c-KIT expression in seminomas correlates with the availability of SCF and that SCF, in parallel with c-KIT, is down-regulated in seminomas compared to IGCNU. Thus, it is likely that activation of c-KIT by ligand binding plays a role in the initiation of IGCNU and that c-KIT activation is lost in progressed seminomas. Further studies are required to confirm this hypothesis.

In summary, our study shows that, in contrast to unilateral TGCT, bilateral TGCTs frequently carry activating c-KIT mutations at different positions of exon 17. Thus, detection of c-KIT exon 17 mutations could help to identify patients at high risk for development of TGCT in the contralateral testis. Furthermore, our results suggest that Y823D mutations occur in migrating PGCs and that persistent c-KIT activation may lead to malignant transformation of immature germ cells to IGCNU. Further investigations, including functional studies, are necessary to study the precise mechanism of action of c-KIT during the development and differentiation of germ cells.

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Chapter 6

TCam-2 but not JKT-1 cells resemble seminoma in cell culture

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Abstract

Of all malignancies diagnosed in men between 17 and 45 years of age, 60% are germ cell tumors (GCT). GCT arises from carcinoma in situ cells, which are thought to originate from a transformed fetal germ cell, the gonocyte. Seminoma together with embryonal carcinoma represents the most frequent subtypes of GCT. However, the nature of the molecular pathways involved in seminoma formation remains elusive. Therefore, analysis of appropriate cell culture systems is an important prerequisite for further understanding of the etiology of this tumor entity. Although several cell lines for embryonal carcinoma have been established and analyzed, so far only two cell lines from seminoma patients have been reported. In the present study, we have analyzed these seminoma cell lines (TCam-2 and JKT-1) and compared the gene-expression profiles with those of normal tissue and of seminoma and embryonal carcinoma by using DNA Array technology. We have found that TCam-2 clusters with the group of classical seminoma, whereas JKT-1 clusters with the group of embryonal carcinoma. Using reverse transcription/polymerase chain reaction, Western blot, and immunohistochemistry, we have confirmed the seminoma-like nature of TCam-2, whereas JKT-1 lacks expression for most of the genes detectable in GCTs, thus making doubtful the germ cell nature of this cell line. The data represent the first genome-wide expression analysis of the two cell lines and comparison/clustering with subgroups of germ cell tumors. Only TCam-2 seems to represent a suitable in vitro model for seminoma.

Introduction

The most abundant malignancies among male population between the ages of 17 and 45 years are germ cell tumors (GCTs; Adami et al. 1994). They comprise a heterogeneous group of neoplasms in terms of their histology, marker expression, and age of manifestation. First described by Skakkebaek in 1972, the common precursor lesion of all type II testicular GCTs (TGCT; Looijenga and Oosterhuis 2002), viz., the carcinoma in situ (CIS, TIN, IGCNU), arises from the transformation of a gonocyte (Skakkebaek 1972, 1978). In recent decades, the incidence of TGCT has increased annually by 3%–6% in the Caucasian population (Oosterhuis and Looijenga 2005). Of the Type II TGCTs, 50% manifest as pure seminomas, with uniform cells having morphology similar to that of cells in CIS. Seminoma formation is believed to be the default pathway of type II TGCT, and the development of a non-seminoma requires the re-activation of pluripotency (Oosterhuis and Looijenga 2005). Interestingly, some seminomas eventually develop into non-seminomas, such as embryonal carcinoma, teratoma, yolk sac tumor, or choriocarcinoma. Marker genes developed for the diagnosis of seminomas include the marker of pluripotency OCT3/4, placental alkaline phosphatase (PLAP), the receptor tyrosine kinase KIT, and transcription factor AP-2γ. Moreover, genome profiling studies by using various types of TGCTs have shed light on the molecular programs activated in these tumors (Skotheim et al. 2002, 2005; Okada et al. 2003; Sugimura et al. 2004; Almstrup et al. 2005a, b).

In order to improve our understanding of the biology of seminomas, the establishment and analysis of cell lines and animal models is mandatory. Although no animal models for seminoma are available to date, two groups have been successful in generating cell lines, namely JKT-1 and TCam-2 (Mizuno et al. 1993; Kinugawa et al. 1998), from seminoma patients. Whereas JKT-1 has been used frequently to study seminomas (Jo et al. 1999; Hatakeyama et al. 2004; Kobayashi et al. 2004; Roger et al. 2004, 2005; Shiraishi et al. 2005), TCam-2 has been utilized in a few cases only (Koshida et al. 2000; Kitazawa et al. 2006; Goddard et al. 2007). Since the original reports date from 1993 and 1998 respectively, the cell lines were analyzed with the marker sets known at that time. Further studies of

these lines would therefore benefit from a thorough side-by-side analysis of the two cell lines with DNA Array techniques and up-to-date markers used for classification of GCT.

Here we have subjected both cell lines to gene-expression profiling and compared the obtained data with that obtained from normal testicular tissue, seminoma, and embryonal carcinoma. Using 41 probes indicative for GCT, we demonstrate that TCam-2 clusters to pure seminoma, whereas JKT-1 clusters to embryonal carcinoma. Further analysis with reverse transcription/polymerase chain reaction (RT-PCR), Western blot, and immunohistochemistry shows that TCam-2 expresses markers specifically found in seminoma, whereas JKT-1 lacks the expression of genes commonly detected in GCT.

Materials and methods

Cell culture

TCam-2 (obtained from Dr. Janet Shipley, Institute of Cancer Research, Sutton, England) was grown in RPMI plus 10% fetal calf serum (FCS), 1% penicillin/streptomycin, 200 mM glutamine. JKT1 cells (obtained from Dr. Michiko Fukuda, The Burnham Institute, La Jolla, Calif.) were grown in minimal essential medium (MEM) plus 10% FCS, 1% penicillin/streptomycin, 200 mM glutamine. The EC cell line 2102EP (obtained from Dr. F. Honecker, Hamburg University Medical Center, Department of Oncology/Hematology, Hamburg, Germany) was grown in Dulbecco's modified Eagle's medium:F12 medium (1:1) plus 10% FCS, 1% penicillin/streptomycin, 200 mM glutamine. Knut1 ES cells derived in our laboratory were grown as published (Peitz et al. 2007). The cells were grown at 37°C and under 5% CO2.

Testicular tissues

All fresh testicular tissue samples used for microarray analysis were obtained immediately after orchidectomy. Use of the tissue for scientific purposes was approved by an institutional regional committee for ethics. From patients with overt

GCTs, three to five tumor samples and, if possible, macroscopically normal testicular tissue were excised, snap-frozen, and stored at −80° for RNA extraction. The orchidectomy samples were fixed in 4% phosphate-buffered formalin overnight at room temperature and embedded in paraffin. All frozen and paraffin-embedded tissues were stained by hematoxylin and eosin (H&E) and by immunohistochemistry with PLAP antibody. All tumors were classified according to the World Health Organization classification of tumors based on their histology and assessment of tumor or IGCNU amount.

RNA preparation

To ensure sample purity, serial sections were taken, and the amount of target tissue estimated in the first and last section. Total RNA was extracted from normal testis (n=3), seminomas (n=5), and embryonal carcinomas (n=3). RNA was extracted either with TRIzol (Invitrogen, Karlsruhe, Germany) or with RNAeasy (Qiagen, Hilden, Germany). RNA quality was assessed with a Agilent Bioanalyzer 2000 (Agilent Technologies, Palo Alto, Calif.), and only samples with an RIN value of >8 were used for further analysis.

Whole-genome gene-expression array analysis

Probe preparation, hybridization, image generation, and analysis were carried out according to the manufacturer's guidelines for the AB1700 Microarray system. Briefly, 2 µg total RNA were translated in vitro and labeled with digoxigenin-11-uridine-5′-triphosphate (Roche Diagnostics, catalog no. 03 359 247 910) and purified with the rt-IVT-Kit (Applied Biosystems). Digoxigenin-labeled cDNA probes were hybridized at 70°C for 16 h to the Whole Human Genome Survey Microarray V2.0 (Applied Biosystems) with 32,878 transcripts containing 60-mer DNA probes representing 29,098 genes. Visualization was achieved by incubating the microarray with an anti-digoxigenin alkaline phosphatase conjugate (Roche Diagnostics, catalog no. 11 093 274 910). The Applied Biosystems 1700 Chemiluminescent Microarray Analyzer was employed to create and analyze images and to perform basic quality control and feature extraction.

Bioinformatic data processing and analysis

Using Bioconductor (http://www.bioconductor.org/docs/faq/) R software and the AB1700 Data Analysis script (Yongming Andrew Sun, Applied Biosystems), all probe sets with FLAG >5000 were removed, and samples with more than 50% missing values were excluded from the analysis. Missing values were replaced with average signals from replicate arrays within the same subgroup. The data was normalized by quantile normalization and transformed to log2 scale.

Western blot

For protein analysis, we used the Mini-PROTEAN Electrophoresis Cell and Mini Trans-Blot system (BioRad, Munich, Germany). Protein was prepared by standard protocols and electrophoresed at 30 mA for 90 min. Gels were blotted onto a polyvinylidene fluoride membrane in a BioRad blotting chamber overnight at 30 V at 4°C according to published protocols. After the membrane had been blocked in PBSTM (phosphate-buffered saline, 0.1% v/v Tween 20, 5% low fat milk powder), it was incubated in primary antibodies (antibodies raised against: alpha fetoprotein [AFP], A008, 1:1,000; PLAP, 8A9, 1:50; Kit, A4502, 1:400; D2-40, M3619, 1:150; all from DAKO, Hamburg, Germany; AP-2γ, 6E4/4, 1:200; from Upstate, USA, New York; Nanog, N-17, sc-30331, 1:200; OCT3/4, c-10, sc-5279, 1:200, all from Santa Cruz, Heidelberg, Germany) in PBSTM for 3 h at room temperature, followed by secondary antibodies (anti-rabbit-horseradish peroxidase [HRP], anti-goat-HRP, anti-mouse-HRP; all from DAKO, Hamburg, Germany) diluted 1:500 (except for Nanog, 1:2,000). Finally, the membrane was incubated in 2 ml PierceSuper Signal West Pico chemiluminescent substrate (Perbio, Bonn, Germany, product no. 34080), and the signal was detected by using Kodak X-Ray film (Kodak, Germany).

RT-PCR protocol

RNA was isolated from cells and tissues by using TRIzol as described (Jager et al. 2003). For RT-PCR, 1 µg DNAseI (NEB, Frankfurt, Germany)-digested RNA template was used. First-strand cDNA synthesis and PCR were performed according to manufacturer's manual (Invitrogen, Karlsruhe, Germany). PCR was carried out at 95°C for 4 min, followed by 30 cycles of 94°C for 30 s, 61°C for 30 s,

and 72°C for 30 s, and finally at 72°C for 10 min. The forward (F)and reverse (R) primers used were: AFP-F: 5′-AGCTTGGTGGTGGATGAAAC-3′, AFP-R: 5′-CCCTCTTCAGCAAAGCAGAC-3′; AP-2γ-F: 5′-CCCACTGAGGTCTTCTGCTC-3′, AP-2γ-R: 5′-AGAGTCAC ATGAGCGGCTTT-3′; D-glyceraldehyde-3-phosphate dehydrogenase [GAPDH]-F: 5′-TGGTATCGTGGAAGGACTCATG AC-3′, GAPDH-R: 5′-ATGCCAGTGAGCTTCCCGTTCAGC-3′; MAGE-F: 5′-GAGCAG ACAGGCCAACCG-3′, MAGE-R: 5′-CGGACTGCGTCTCAGGAA-3′; PLAP-F: 5′-GGTGAACCGCAACTGGTACT-3′, PLAP-R: 5′-CCCACCTTGGCTGTAGT CAT-3′; OCT4-F: 5′-CGAAAGAGAAAGCGAACCAG-3′, OCT4-R: 5′-GCCGGT TACAGAACCACACT-3′; DAZL-F: 5′-ATGTTAGGATGG ATGAAACTGAGATTA-3′, DAZL-R: 5′-CCATGGAAATTTATCTGTGATTCTACT-3′; VASA-F: 5′-AGAAA GTAGTGATACTCAAGGACCAA-3′, VASA-R: 5′-TGA CAGAGATTAGCTTCTTC AAAAGT-3′; BOULE-F: 5′-TATAAGGATAAGAAGCTGAACATTGGT-3′, BOULE-R: 5′-CGAAGTTACCTCTGGAGTATGAAAATA-3′; bone morphogenetic protein-2 [BMP-2]-F: 5′-TCTGACTGACCGC GTTACTC-3′, BMP-2-R: 5′-TCTCTGTTTCAGGCCGAACA-3′.

Immunohistochemistry

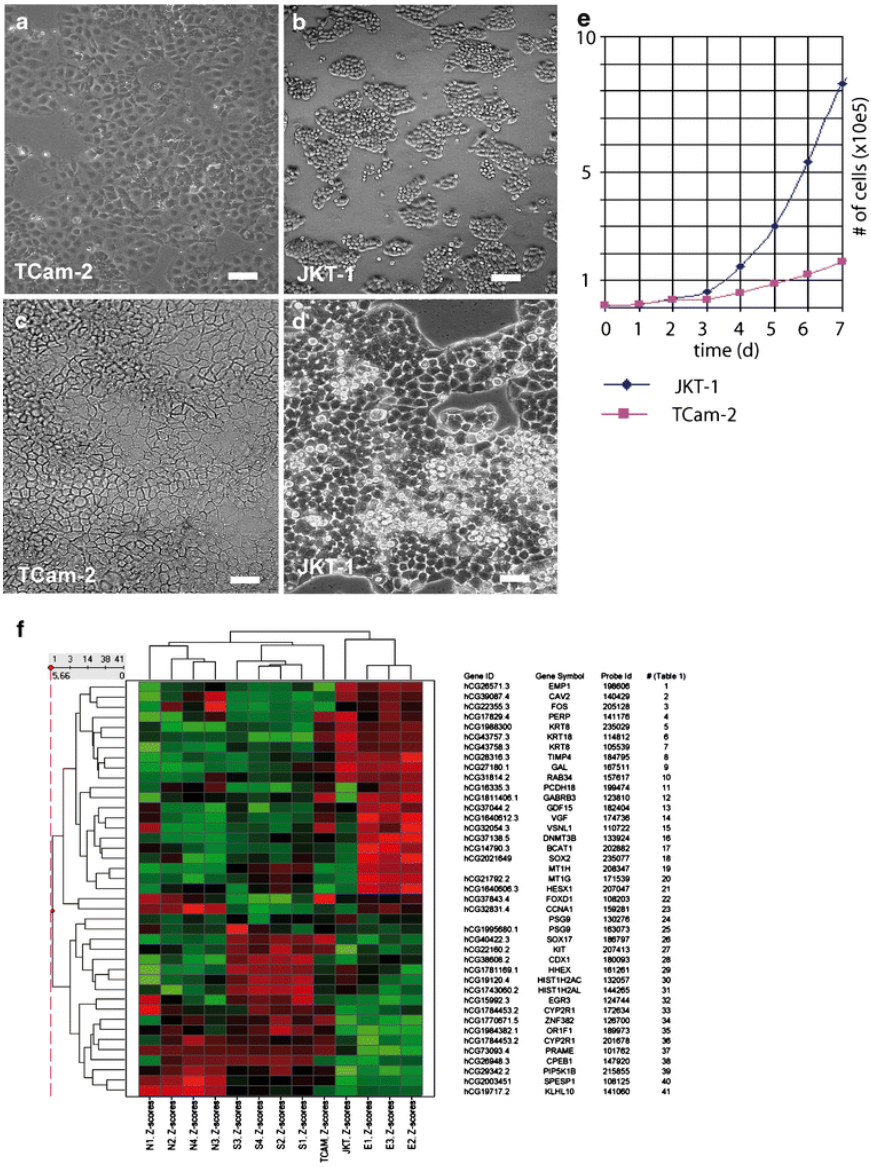
The following primary antibodies were used for immunofluorescence: anti-AFP, diluted 1:200, A0008; anti-PLAP, diluted 1:50, 8A9; anti-ck18, diluted 1:50; anti-KIT, diluted 1:200, A4502; anti-D2-40, diluted 1:150, M3619 (all from DAKO); anti-AP-2γ, diluted 1:200, H77; anti-NANOG, diluted 1:200, N-17; anti-Oct3/4, diluted 1:100, C-10 (all from Santa Cruz). Detection of primary antibodies was performed by using Alexa-488 goat anti-mouse, diluted 1:500; Alexa-488 donkey anti-goat, diluted 1:500; and Alexa-594 goat anti-rabbit, diluted 1:500 (all Invitrogen, Karlsruhe, Germany).

Results

Morphology and growth parameters

When comparing the morphology and growth characteristics of TCam-2 and JKT-1 with each other, several differences and a few similarities could be observed. After attachment to the culture flask surface, TCam-2 cells appeared polygonal and flat in shape, whereas JKT-1 cells are round (Fig. 1a,b). After a few days in culture, JKT-1 cells became polygonal in shape and looked similar to TCam-2, but still a few round cells remained (Fig. 1d). TCam-2 cells seemed to need either cell-cell-contact or a certain cell density for optimal growth, because when the culture was initiated at low cell density, the cells required two to three times longer to reach exponential growth phase compared with a culture starting with high cell density. This property could not be detected when culturing JKT-1. After 5 days in culture, TCam-2 formed areas of high cell density in which cells were compressed and tightly clustered (Fig. 1c). JKT-1 cells grew in a looser formation (Fig. 1d). TCam-2 cells displayed higher adherence to the tissue culture surface compared with JKT-1 cells, suggesting a increased amount of desmosomes in TCam-2 cells. TCam-2 cells had larger nuclei and enlarged cytoplasm when compared with JKT-1 cells (see Supplemental Data S1, online). Both cell lines grew as a monolayer and displayed contact inhibition. To determine the doubling time, 1-2×104 cells were seeded onto 35-mm cell culture dishes, and the cell number was determined every day (Fig. 1e). The calculated doubling times were 27 h for JKT-1 cells and 58 h for TCam-2 (see Supplemental Data S2). Hence, TCam-2 and JKT-1 displayed highly diverging basic growth characteristics in vitro.

Figure 1

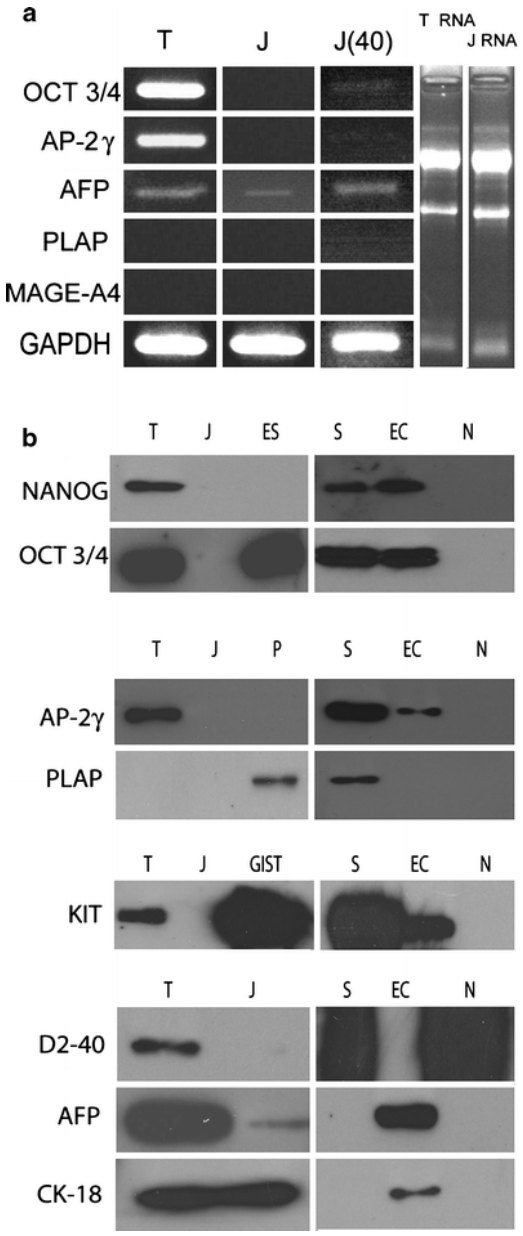


a–d Comparison of TCam-2 (a, c) and JKT-1 (b, d) cells after 3 days (a, b) and 5 days (c, d) of culture. Bar 50 µm. e Growth curve of the cell lines. f Unsupervised hierarchical clustering of normal cells (N; n=4), seminomas (S; n=4), embryonal carcinomas (E; n=3), and JKT-1 and TCam-2 cell lines with 41 probes selected to differentiate between seminomas and embryonal carcinomas was performed to show similarity of Z-scores between the different tumour entities and cell lines entities. An unweighted averaged (UPGMA) method and Euclidian distance were used to generate the heat map in Spotfire; GeneID, Genesymbol, Probe ID, and number (#) are given right (see also Table 1) DNA array analysis

In order to determine global gene-expression patterns, RNA was extracted from TCam-2 and JKT-1 cells, and DNA array analysis was performed comparing the two cell lines with normal germ cell tissue and material derived from GCT. Initially, unsupervised hierarchical clustering of seminomas, embryonal carcinomas, and normal tissues (UPGMA unweighted average, Euclidean distance) was performed by using Spotfire Decision Site for Functional Genomics (Spotfire, Europe, Göteborg, Sweden). This showed a convincing clustering of the samples into groups of the different entities. The two cell lines JKT-1 and TCam-2 clustered with the tumor samples, but no separation between seminoma and embryonal carcinoma was possible (not shown). The failure of the unsupervised cluster method with 32,878 transcripts to determine whether the cell lines were more closely related to seminoma or embryonal carcinoma might have been attributable to genes contributed by the tumor samples derived from the stroma and by an inflammatory infiltrate that were not present in cell culture samples. To overcome this, we used Panther (Celera Discovery systems) and the Gene ontology (GO) consortium database to identify and remove genes related to the immune response and unclassified genes. A significance analysis of microarrays (SAM 2.21; Tusher et al. 2001) was used to extract genes differentially expressed between seminoma and embryonal carcinomas.

Finally, a list of 41 probes (corresponding to 39 gene products, see Table 1) that were published and validated by ourselves and others (see Table 1 online) was used to perform a hierarchical cluster analysis and showed that TCam-2 clustered to the group of seminoma, whereas JKT-1 displayed an expression pattern related to embryonal carcinoma (Fig. 1f). Relative expression values of the 41 probes are shown in Supplemental Data S3 (online).

Figure 2



a RT-PCR analysis of cDNA of TCam-2 (T) and JKT-1 (J) for expression of the genes indicated (J(40) extended PCR protocol with 40 cycles). The gel to check for RNA quality is depicted right (T RNA, J RNA). b Western blot analysis of 20 µg TCam-2 and JKT-1 proteins probed with the antibodies indicated left (T TCam-2, J JKT-1, S seminoma tissue, EC embryonal carcinoma, ES murine embryonic stem cells, N normal testes tissue, P placenta, GIST gastro-intestinal stroma tumour). β-actin expression was analysed as a loading control for each experiment (data not shown)

RT-PCR, Western blot, and immunohistochemistry

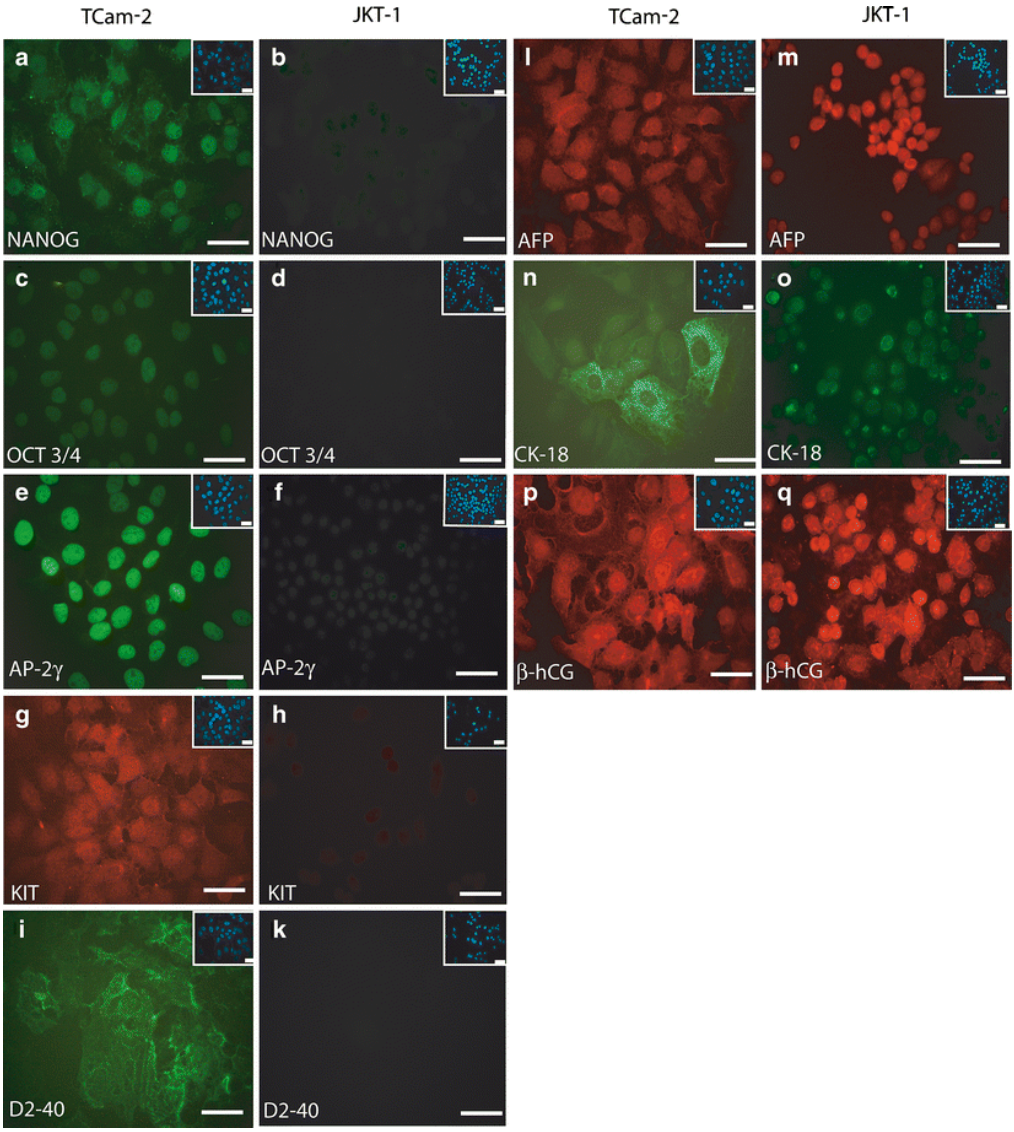
In order to confirm the data generated by the DNA array analysis, we extracted RNA from TCam-2 and JKT-1 cells and tested for the expression of the marker of pluripotency OCT3/4, the marker of seminomas AP-2γ, AFP, PLAP, and MAGE-A4, the marker of spermatocytic seminoma (Fig. 2a). Bands for OCT3/4, AP-2γ, and AFP could be detected in TCam-2 cells (Fig. 2a), whereas JKT-1 was positive for AFP and displayed only a weak signal for OCT3/4 and AP-2γ after application of an extended RT protocol (Fig. 2a, J(40)).

However, the detection of RNA does not necessarily indicate that the respective protein is being made. For example, miRNA might inhibit translation, or a short half-life of the message might result in reduced protein levels.

Hence, we performed Western blot analyses in order to determine the protein levels of several marker genes upregulated in GCT. The markers of pluripotency NANOG and OCT 3/4 and the transcription factor AP-2γ, the receptor tyrosine kinase KIT, AFP, D2-40, and cytokeratin 18 (CK18) were expressed (Fig. 2b) In TCam-2 cells. On the other hand, only AFP and CK18 protein could be detected in JKT-1 cells (Fig. 2b).

The results obtained by Western analysis were further corroborated by immunohistochemistry. Again, TCam-2 cells were positive for NANOG, OCT3/4, AP-2γ, D2-40, AFP, and CK 18 (Fig. 3), whereas only AFP and CK18 could be detected in JKT-1 cells (Fig. 3). Interestingly, both cell lines were positive for βHCG, a marker usually found in choriocarcinoma (Fig. 3p,q).

Figure 3

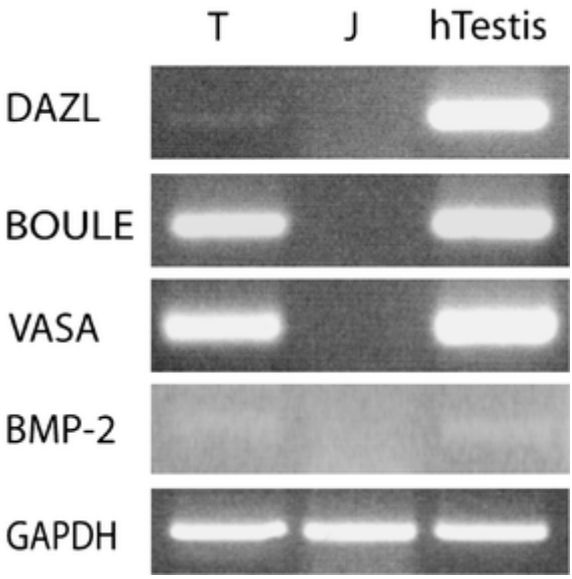


Immunohistochemistry of TCam-2 (**a**, **c**, **g**, **i**, **n**, **p**) and JKT-1 (**b**, **d**, **f**, **h**, **k**, **m**, **o**, **q**) cells for NANOG (**a**, **b**), OCT3/4 (**c**, **d**), AP-2γ (**e**, **f**), KIT (**g**, **h**), D2-40 (**i**, **k**), AFP (**l**, **m**), CK-18 (**n**, **o**), βHCG (**p**, **q**). *Upper right insets*: DAPI (4,6-diamidino-5-phenylindole) staining of the nuclei (*blue*). *Bars* 10 μm Taken together, the data indicated that TCam-2 cells represented a seminoma-like phenotype, whereas the classification of JKT-1 required further analysis.

Expression of AFP and CK-18 in JKT-1 suggested a yolk-sac-like character of the cells. Korkola et al. (2006) reported that yolk-sac tumors displayed high levels of BMP-2 compared with other GCTs, thus serving as a classifier for this tumor entity. However, BMP-2 could not be detected in JKT-1 cells by using RT-PCR (Fig. 4, compare with TCam-2).

Since JKT-1 was negative for most of the GCT markers tested, we analyzed the levels of general germ cell markers VASA, DAZL, and BOULE via RT-PCR (Ezeh et al. 2005). Again, JKT-1 was negative, whereas TCam-2 was positive for these markers (Fig. 4).

Figure 4



RT-PCR analysis of cDNA of TCam-2 (*T*) and JKT-1 (*J*) cells and human testis (*hTestis*) for expression of genes indicated *left*

Discussion

In vivo studies of material derived from tumors can provide insights regarding the complex genetic and cellular interactions of tumors. To address mechanistic issues of tumor behavior further, a simplified in vitro system is mandatory.

For seminoma (a subgroup of GCTs), many research groups including our own have failed to establish an appropriate cell line. Hence, to date, only two lines TCam-2 and JKT-1 have been derived from seminoma (Mizuno et al. 1993; Kinugawa et al. 1998). Where several laboratories have utilized mainly the JKT-1 cell line (Jo et al. 1999; Hatakeyama et al. 2004; Kobayashi et al. 2004; Roger et al. 2004, 2005; Shiraishi et al. 2005) as a surrogate for seminoma, TCam-2 (Koshida et al. 2000; Kitazawa et al. 2006) had been used rarely. Since the cell lines were originally published, various novel marker genes have been described making the identification and classification of TGCTs more precise and powerful. In this study, we have performed a side-by-side comparison of the two cell lines and analyzed basic parameters, such as morphology and growth characteristics. Fundamental differences in cell adhesion and doubling time have become apparent, with TCam-2 growing more slowly (doubling time: 58 h) compared with JKT-1 (doubling time: 27 h), data that are in accordance with previous publications (Mizuno et al. 1993; Kinugawa et al. 1998).

In this study, we have used a whole-genome approach to compare the gene-expression profiles of TCam-2 and JKT-1 with those of samples from normal and testicular tumor tissue. The analysis has revealed that TCam-2 clusters with the group of seminomas, whereas JKT-1 clusters with embryonal carcinomas. Of note, the dendrogram algorithm clusters the seminomas and the embryonal carcinomas together in two distinct groups, and the cell lines lie in their immediate neighborhood. This indicates that the cell lines are closely related to the respective tumor entity but not completely identical. This difference might also be attributed to the likelihood that the samples generated from tumors represent a mixture of tumor and normal tissue displaying a heterogeneity that cannot be modeled in cell culture systems. On the other hand, any cell that is taken out of its physiological context

and placed into an in vitro culture system might adapt to its new environment and consequently change its expression profile, conserving only a part of its original expression pattern.

Further analysis with RT-PCR, Western blot, and immunohistochemistry has confirmed these findings. The original publication of the TCam-2 cell line did report its immunoreactivity with respect to 5G9 (an anti-testicular-cancer monoclonal antibody) or 4B3 (PTHrP); here, we have extended the study to demonstrate the expression of OCT3/4, NANOG, AP-2γ, KIT, CK18, VASA, DAZL, and D2-40 by using RT-PCR, Western blot, and/or immunohistochemistry, thereby adding further evidence to the seminomatous nature of TCam-2. However, in our hands, TCam-2 expresses AFP but not PLAP, whereas the original publication found the cells to be AFP-negative and PLAP-positive (Mizuno et al. 1993). This might be an indicative of a certain drift of the cell culture over the years in vitro from 1993 to present. Since some cells within a seminoma are known to be positive for AFP, a known marker for yolk-sac tumors, and since not every seminoma is positive for PLAP (Franke et al. 2000), these result do not impact on the overall suitability of TCam-2 as a cell culture model for seminoma. In addition, TCam-2 displays the characteristic gain of chromosomes 9p and 12p, as shown by comparative genomic hybridization (Goddard et al. 2007). Goddard et al. (2007) have also shown the expression of OCT3/4 and KIT in TCam-2 cells, further confirming our data. However, of note, TCam-2 cells express BOULE, which is usually detected in adult meiotic germ cells (Ezeh et al. 2005), and βHCG, a marker for choriocarcinoma, indicating a distinct difference in the gene-expression pattern of this cell line compared with that of seminoma cells in situ.

JKT-1, on the other hand, does not cluster to seminomas but rather seems to group with embryonal carcinomas. Like TCam-2, JKT-1 was originally reported to be AFP-negative and PLAP-positive but has been found here to be AFP-positive and PLAP-negative. Furthermore, JKT-1 lacks expression of KIT and displays low levels of AP-2γ. Since these markers discriminate between seminoma (AP-2γ+ and

KIT+) and non-seminoma (AP-2γ low, KIT-; Hoei-Hansen et al. 2004; Almstrup et al. 2005b; Pauls et al. 2005), JKT-1 cannot represent a seminoma-like cell type.

Moreover, our Western blot and immunohistochemical data have revealed a lack of expression of NANOG, OCT3/4, and D2-40 in JKT-1 cells. Lack of pluripotency markers and the absence of PLAP and KIT expression has been reported for a rare GCT predominantly found in older patients, viz., the spermatocytic seminoma (Rajpert-De Meyts et al. 2003). However, JKT-1 lacks expression of MAGE-A4, a marker characteristic for this type of seminoma (Rajpert-De Meyts et al. 2003). We have been able to obtain a weak band for OCT3/4 indicative of low expression by using an extended RT protocol (40 cycles instead of 35 cycles), and one can argue that the expression of the OCT3/4 gene might have fallen below the detection threshold of Western blot and immunohistochemistry. Since low levels of OCT3/4 and NANOG have been reported in yolk sac tumors and choriocarcinoma (Korkola et al. 2005), we have investigated whether JKT-1 displays increased levels of BMP-2, a genetic classifier for yolk-sac tumors (Korkola et al. 2005). However, JKT-1 shows no signal for BMP-2 either, also excluding this possibility. We further demonstrate that JKT-1 lacks expression of the germ cell markers DAZL (Lifschitz-Mercer et al. 2002) BOULE, and VASA (Ezeh et al. 2005), raising the question as to whether JKT-1 can be regarded as a GCT line at all. Hence, the data presented here suggest that JKT-1, although initially clustering to embryonal carcinomas, does not express a convincing set of markers indicative for GCTs and germ cells. Recent data published by Jong et al. (2007) further strengthen this notion. Experiments with JKT-1 as a model system for seminomas must therefore be undertaken with an awareness of these apparent discrepancies.

In summary, these analyses clearly show that the TCam-2 should be preferred over JKT-1 whenever a cell line with a seminoma-like nature is required.

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Chapter 7

General discussion, concluding remarks and future prospects

General discussion

To highlight the pathogenesis of CIS and TGCTs, it is crucial to specify the normal differentiation process of human embryonic and fetal germ cells as well as to identify factors involved in disturbed embryonic/fetal germ cell differentiation. Significant differences were previously observed between murine and human germ cell development, and only few studies had been performed in human tissues. However, functional studies in mouse indicate a leading role of specific genes in specification, migration and differentiation of PGCs. Transcription factor Blimp1 and arginine-specific histone methyltransferase Prmt5 are supposed to be crucial in specification and maintenance of pluripotency in mouse PGCs [1, 2]. Loss of the transcription factor AP-gamma has been shown to induce a complete loss of PGCs after their specification [3]. The SCF-c-KIT pathway regulates survival, proliferation and migration of mouse and human PGCs [4]. Thus, it is of great importance to characterize these genes in human tissues, because all these genes might have a major role in the maintenance of gonocytes to adulthood and therefore in formation and maintenance of CIS.

The findings presented in **chapter 2** indicate that fetal germ cell differentiation in human males is a tightly regulated process, in which the gonocytes are prone to differentiate to pre-spermatogonia in a specific period of time. We detected proteins which are associated with pluripotency, survival and proliferation, including OCT3/4, c-KIT and AP-2gamma, as well as the oncofetal marker M2A antigen in gonocytes predominantly between the 12th and 26th gestational weeks, but not in the postnatal testis (Figure 1-3, Figure 5). These genes were consistently co-expressed in gonocytes, but not in pre-spermatogonia (Figure 1-3). The number of gonocytes decreases after week 20, paralleled by an increasing number of pre-spermatogonia. The latest population matched the morphological criteria proposed by Fukuda [5] and expressed the melanoma associated antigen MAGE-A4, which is a specific marker for normal premeiotic germ cells [6]. These results are in accordance with previous studies, which examined expression of respective markers during fetal development in normal male gonads [6-11]. We found that only two different populations of fetal germ cells exist during the second and third trimester by morphology and

immunohistochemistry: gonocytes and pre-spermatogonia. However, other studies proposed the existence of several additional subgroups of fetal germ cells by morphology and immunohistochemistry [12, 13], for which we did not find support in this study. Differences between our study and the previous reports might be the result of different tissue fixation (Bouin fixative versus formalin), different antigen retrieval techniques and application of a highly sensitive immunohistochemical detection method in the present study.

Furthermore, we found that the number of gonocytes and pre-spermatogonia varied significantly depending on the developmental stage. In particular, their expression was strongly increased in the 18th/19th week (Figure 5). We believe that this peak reflects a burst of proliferation activity of gonocytes as we found an increase in both Ki-67 expression and the overall germ cell number, while there was a transient decrease in the ratio of Sertoli cells to germ cells at this point of development. In general, the number of gonocytes decreased continuously during the second and third trimester, while the number of MAGE-A4 positive germ cells increased to the same degree. The differentiation process obviously progressed after the 25th week, when over 50% of germ cells expressed the spermatogonial marker MAGE-A4.

The study presented in chapter 2 suggests that persistence of immature gonocytes beyond the postnatal period indicates a significant disturbance of the differentiation process and that the second/third gestational trimester is critical for germ cell differentiation. This observation supports experimental data and epidemiological studies also indicating that the affected population of cells leading eventually to the invasive cancer is only present during a limited time window [14, 15]. Furthermore, the results presented can serve as a reference for normal fetal germ cell development compared to abnormal germ cell differentiation seen in cryptorchidism and DSD. Our study strengthen the link between CIS/TGCTs and gonocytes, as it was found in a recent study by others by gene expression profiles of microdissected cell populations and CIS [16].

Recent work of group of Prof. Schorle (Institute of Pathology, Bonn, Germany) showed that the transcription factor AP-2gamma is specifically expressed in murine PGCs and gonocytes from E7.25 to E12.5. Using a

conditional deletion approach for AP-2gamma they found that PGCs are lost shortly after specification, which resulted in sterile animals, independently of sex [3]. These findings suggested a significant role of AP-2gamma in germ cell biology. Because nothing was known so far about the expression of this gene in human (T)GCTs, we studied its expression of which the results are presented in **chapter 3**, and in addition to fetal tissues (Figure 1 and 2) performed an extensive study in GCTs, predominantly TGCTs (Table 1, Figure 3). A study from another group investigating AP-gamma expression in (T)GCTs which was published just before our study, came to similar results [17]. AP-2gamma expression was consistently high in CIS and in seminomas independent of their localization and pathologic stage (Figure 3), suggesting AP-2gamma as a potent marker for CIS and for (metastatic) seminoma. AP-2gamma was downregulated in embryonal carcinomas and its expression was lost in yolk sac tumors and teratomas. This result is in agreement with the current model of nonseminoma development, in which genes associated with ES cells are downregulated during progression of embryonal carcinoma to yolk sac tumor, choriocarcinoma and teratoma [18]. However, trophoblastic giant cells in choriocarcinomas were positive for AP-2gamma. Indeed, in mice, AP-2gamma expression was found in cells of the trophoblast lineage [19] and therefore this expression may reflect regular developmental processes during placental differentiation.

The expression pattern described for AP-2gamma in mice and human embryonic germ cells is similar, suggesting that this protein has conserved functions in both species. The molecular role of the transcription factor AP-2 is not fully understood, and its function in mouse germ cells and TGCTs has been studied in the recent paper and in ongoing experiments by the group of Prof. Schorle. The paper of Weber *et al*. showed that PGCs generated *in vitro* from ES cells lacking AP-2gamma upregulate somatic genes (Hoxa1, Hoxb1) and lack of expression of germ cell genes (Nanos3 and Dazl) demonstrating that the somatic gene program is induced in AP-2gamma deficient PGCs. Consistently, AP-2gamma downregulation in TCam-2 resulted in specific upregulation of HOXA1, HOXB1, MYOD1, and HAND1, indicative of mesodermal differentiation [3]. These results implicate that AP-2gamma is required for PGC and seminoma maintenance by

suppression of (mesodermal) differentiation, and suggest that loss of AP-2gamma expression may enable the activation of somatic developmental programs, leading to differentiation towards nonseminoma. Moreover, results presented in the manuscript of Weber *et al.* suggest that AP-2gamma is a downstream target of BLIMP1 (B-lymphocyte induced maturation protein-1; PRDM1), a putative regulation of specification in PGCs. This Krüppel-type zinc-finger containing protein interacts with the arginine methyl-transferase PRMT5 resulting in a symmetrical methylation at arginine 3 of histone H4 and H2A (H4R3me2s/H2Ame2s). The methylation in turn represses transcription and therefore suppresses the somatic cell fate and keeps PGCs in a pluripotent state.

In **chapter 4** we report on the detection of nuclear expression of the transcription factor BLIMP1 and protein arginine methyltransferase-5 PRMT5 in gonocytes and showed the presence of the resulting dimethylation of H2A/H4 at arginine 3 (Figure 1 and 2) (in absence of BLIMP1, PRMT5 in normal adult testis, Figure 3). These findings are in concordance to the observations made in mouse. In CIS, a strong nuclear signal of BLIMP1 and of H2K3me2s/H4K3me2s was detected, whereas PRMT5 signal was low in CIS and heterogeneous in seminomas (Figure 4 and 5, Table 1). Instead, we found expression of another type II protein arginine methyltransferase, PRMT7, which also interacts with BLIMP1, as demonstrated in TCam2 (Figure 5). This indicates that in CIS and seminoma, BLIMP1 recruits PRMT7 to compensate for the lack of nuclear PRMT5 to mediate H2A and H4 dimethylation. Upon progression of CIS to nonseminomas, signal intensity of BLIMP1 decreased and subcellular localization changed. As a consequence, H2A/H4 modification decreased and became heterogeneous in nonseminomas (Figure 4). Hence, we suggest that the histone H2A and H4 arginine 3 dimethylation might suppress differentiation of CIS and seminoma, while loss of these histone modifications might lead to reprogramming and differentiation to embryonal carcinomas and the various subtypes of differentiated nonseminomas. Our results strongly suggest that functions of BLIMP1 and AP-2gamma are conserved in PGCs and seminomas, and that AP-2gamma is a downstream target of BLIMP1 [3]. Interestingly, recent work of West and colleagues using a unique *in vitro* ES cell differentiation strategy showed that

BLIMP1 is induced by LIN28 by inhibition of let-7 maturation [20]. Functions of LIN28 are currently studied in TCam2 in the group of Prof. Looijenga.

While functions of BLIMP1, PRMT5 and AP-2gamma are their specification of PGCs from the epiblast and prevention from somatic differentiation, c-KIT regulates their migration and survival. Expression of receptor tyrosine kinase c-KIT in CIS and seminomas was described already more than 10 years ago [21, 22], but its role in malignant germ cells is still not clear. Recently it has been proposed that c-KIT gain of function mutations occur in TGCTs and are predominant in bilateral tumors [23] , although initially it was not confirmed by others [24, 25]. In **chapter 5**, we studied functions of c-KIT in TGCTs by sequencing of exon 17 and 11 of c-KIT in 155 unilateral and 22 bilateral tumors and analyzing c-KIT expression during progression of TGCTs. Our study showed that, in contrast to unilateral TGCT, bilateral TGCTs frequently carry activating c-KIT mutations at different positions of exon 17 (64% in bilateral versus 7% in unilateral TGCTs, $p < 0.001$, Fischer's exact test) (Figure 1, Table 1 and 2; Figure 1 below). In accordance with most previous studies [25-27], 22/24 (91.6%) of detected mutations were found in seminomas and only 2/24 (8.4%) in embryonal carcinomas. Surprisingly, the Y823D mutation was most frequently found among bilateral TGCTs (31.8%) and occurred in three patients on both sites, as well as in normal testis and CIS.

All these findings indicate that in a subset of bilateral TGCTs, c-KIT mutations occur early in germ cells, most likely in migrating PGCs, and therefore are identical in both testes, while in others, gene alterations of c-KIT are generated in post-migratory gonocytes or CIS. The consequence of c-KIT activation in PGCs/gonocytes awaits analysis, but detected mutations can potentially lead to malignant transformation, as it has been shown in haematopoietic cells [28]. Till now, the clinical management procedure for patients with TGCTs does not necessarily include histological examination of the contralateral testis. Because of the results in our study, identification of exon 17 mutation would help to identify patients with higher risk for bilateralism, and a contralateral biopsy could reveal CIS or TGCT at early stages in these patients.

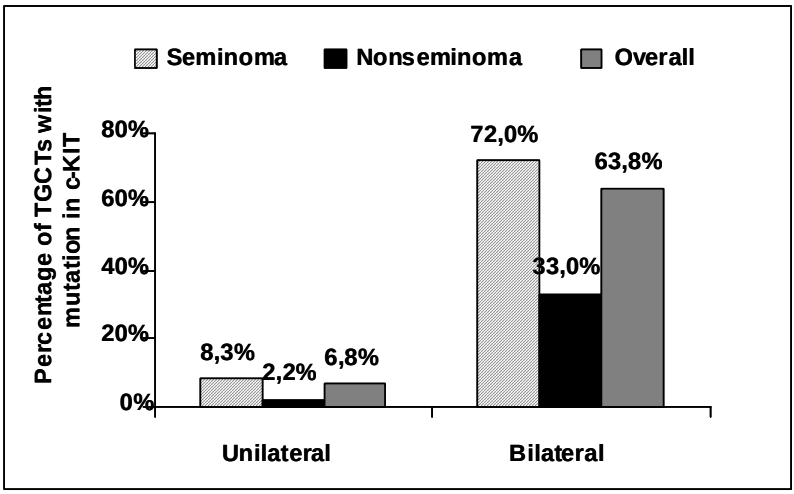

Figure 1 represents meta-analysis of data as demonstrated in chapter 5.

Based on the finding of frequent amplification of the c-KIT gene (in up to 21% of seminomas), it was hypothesized that the c-KIT receptor plays an important role in the progression of CIS towards seminoma [25]. Because gene amplification might lead to the overexpression of the corresponding protein, higher expression of c-KIT should be expected in invasive seminomas than in CIS. Surprisingly, analysis of the c-KIT expression in 41 seminomas and their precursor lesions revealed significant down-regulation of c-KIT expression in seminomas compared to CIS ($p = 0.006$) Furthermore, we did not observe an increase of c-KIT mRNA levels in pT2/pT3 compared to pT1 seminoma by real-time RT-PCR (Figure 2 and 3, Table 3). In summary, these findings are in line with previous studies that have detected a loss of c-KIT expression during tumour progression, suggesting a role in CIS initiation (constitutative activation of c-KIT could provide PGCs and gonocytes with survival advantage and thus initiate CIS) rather than in TGCTs progression [29, 30]. Further investigations, including functional studies, are necessary to study the precise mechanism of action of c-KIT during the development and differentiation of germ cells.

The results discussed till here were based mainly on functional experiments in mouse models and on expression analysis of human tissues (BLIMP1, PRMT5, AP-2gamma and others). To address mechanistic issues of tumor behavior further, a simplified *in vitro* system is mandatory. Seminomas are very difficult to culture

(observations in groups of Prof. Schorle and Prof. Looijenga), and only two cell lines derived from seminomas, TCam-2 and JKT-1, were described so far [31, 32]. Thus, functional studies in seminomas could only be performed after extensive characterization of these two known seminoma cell lines, which is presented in **chapter 6**. Previously, several laboratories have utilized mainly the JKT-1 cell line [33-38] as a surrogate for seminoma, while TCam-2 [39, 40] was used rarely. Since the cell lines were originally published, various novel marker genes have been described making the identification and classification of TGCTs more precise and powerful. We used this knowledge and performed a side-by-side comparison of the two cell lines and analyzed basic parameters, such as morphology and growth characteristics. Fundamental differences in cell adhesion and doubling time have become apparent, with TCam-2 growing more slowly (doubling time: 58 h) compared with JKT-1 (doubling time: 27 h) (Figure 1), data that are in accordance with previous publications [31, 32]. Using a whole-genome approach we compared the gene-expression profiles of TCam-2 and JKT-1 with those of samples from normal testis and TGCTs. The analysis revealed that TCam-2 clusters with the group of seminomas, whereas JKT-1 clusters with embryonal carcinomas (Figure 1).

Further analysis by RT-PCR, Western blot, and immunohistochemistry demonstrated the expression of seminoma genes OCT3/4, NANOG, AP-2gamma, c-KIT, VASA, DAZL, and M2A, in TCam2 (Figures 2, 3, 4), thereby adding further evidence to the seminomatous nature of TCam-2. In addition, Goddard *et al.* (2007) have also shown the expression of OCT3/4 and c-KIT in TCam-2 cells, further confirming our data [41]. Data presented in our study and by others [42] suggest that JKT-1 does not exhibit characteristics of seminoma and does not express a convincing set of markers indicative for TGCTs and germ cells.

Concluding remarks and future prospects

Analyzing the results of the studies in chapter 2, 3 and 4, and ongoing studies, we come to the conclusion that specific programs are active in CIS/TGCTs, and functions of specific genes we studied are conserved in PGCs and TGCTs. Based on our results we propose the following model for normal germ development and CIS formation: during normal embryogenesis, co-expression and nuclear localization of the BLIMP1/PRMT5 complex leads to histone H2A/H4 dimethylation which results in transcriptional silencing of genes responsible for somatic differentiation in PGCs. AP-2gamma is a target gene of BLIMP1, and its expression adds to suppression of differentiation in PGCs/gonocytes. Upon normal differentiation to pre-spermatogonia in fetal testis, the BLIMP1/PRMT5 complex is downregulated and the H2A/H4 marks are lost. In contrast, aberrant expression of BLIMP1 leads 1) via activation of PRMT5 to persistent histone H2A/H4 arginine 3 dimethylation and 2) AP-2gamma expression. This process leads to survival of gonocytes and to progression into CIS. Downregulation of BLIMP1 and AP-2gamma in seminoma and CIS might be a prerequisite of differentiation of embryonal carcinoma to teratoma, choriocarcinoma and yolk sac tumor. Figure 2 gives a schematic summary of the proposed actions of BLIMP1, PRMT5 and AP-2gamma, integrated in a current model of TGCT development.

Also, our studies showed that transcription factor AP-2gamma can be used as a highly specific marker for CIS/seminoma/germinoma/dysgerminoma. Next, detection of exon 17 c-KIT mutation could be used in future to reveal patients at risk for developing bilateral TGCTs. Here, one of the next points of interest is the role of c-KIT in PGCs and relevance of abnormal germ cell migration for the development of extragonadal GCTs.

The group of Prof. Schorle in collaboration with Prof. Looijenga is in the process of generating an *in vivo* model with constitutional activation of the c-KIT pathway by constructing a transgene mouse carrying D816V mutation in exon 17 of the c-Kit gene. We propose that in contrast to the wild type, in this model PGCs migrating in the midline will survive due to suppression of apoptosis by activating c-Kit and give rise to extragonadal GCTs in mouse.

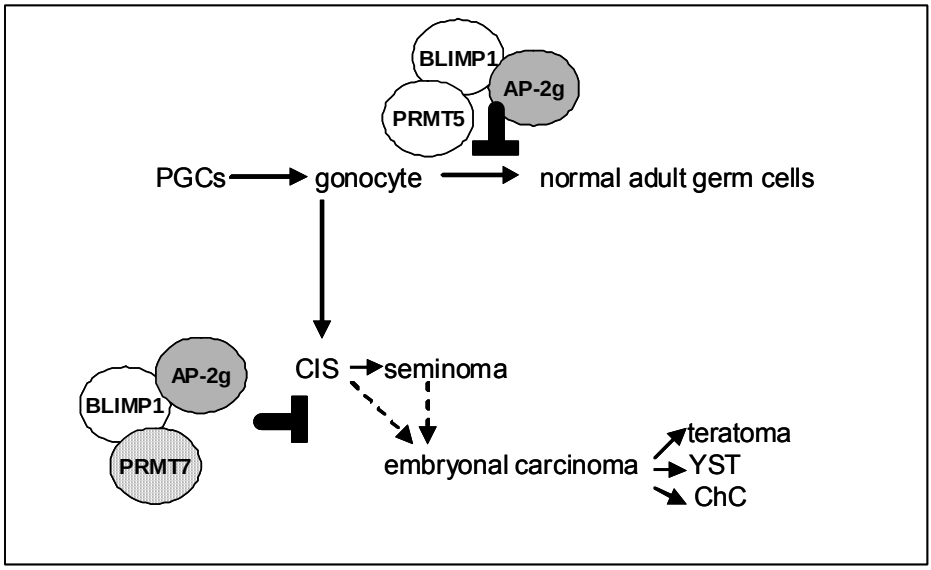

Figure 2. Schematic summary of functions of BLIMP1, PRMT5 and AP-2gamma. BLIMP1/PRMT5 complex (via histone H2A/H4 dimethylation) and activation of AP-2gamma lead to suppression of differentiation in PGCs/gonocytes. Upon normal differentiation to pre-spermatogonia in fetal testis, AP-2gamma and BLIMP1/PRMT5 complex are downregulated. Aberrant expression of BLIMP1/PRMT5 and AP-2-gamma leads to persistence of gonocytes and to CIS formation. In CIS and seminoma, BLIMP1 interacts with PRMT7 instead of PRMT5. Similar to PGCs/gonocytes, BLIMP1/PRMT7 complex as well as AP-gamma expression repress their reprograming to nonseminoma.YST: yolk sac tumor, ChC: choriocarcinoma.

For this we acquired financial support of “Deutsche Krebshilfe”. Characterization of a seminoma cell model was a prerequisite for future functional studies including studies on differentiation of seminoma to a nonseminoma. Last, our findings further strengthen the hypothesis of the embryonic/fetal origin of CIS.

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Chapter 8

Summary/samenvatting, acknowledgement and appendix

Summary

Chapter 1

Five groups of human GCTs have been classified, including male and female, benign and malignant, as well as gonadal and extragonadal tumors. Within this classification, malignant seminomatous and nonseminomatous GCTs in males and females are designated as type II GCTs (table 1, general introduction). The incidence of type II testicular GCTs, i.e., TGCTs, is continuously rising, probably due to effects of environmental compounds. All the different histological subtypes of TGCTs are derived from a precursor lesion CIS, which represents malignant intratubular germ cells with uniform morphology. The pathogenesis of TGCTs is not completely understood, but is closely linked to embryonic germ cells. CIS is proposed to develop from transformed immature germ cells, the gonocytes. As reported in general introduction, many characteristics of type II (T)GCT can be linked to ES cells (sensitivity to chemotherapy, gain of the short arm of chromosome 12 upon *in vitro* culturing, expression of stemness genes, including OCT3/4, NANOG and SOX2, overall low mutation rate). Recent data show that specific genes, including BLIMP1, PRMT5, AP-2gamma and c-KIT are crucial for normal PGC development. Studies reported below were done because knowledge about normal germ cell development is essential to understand the pathogenesis of GCTs and the expression of the genes mentioned above might be crucial in CIS/TGCTs formation.

Chapter 2

In this chapter we present our results obtained from investigation of 61 normal fetal gonadal tissues from the second and third trimester, by histology and immunohistochemistry with antibodies detecting the oncofetal proteins c-KIT, AP-2gamma, M2A and the spermatogonial marker MAGE-A4, We in fact found a consistent co-expression of the transcription factors OCT3/4 and AP-2gamma, c-KIT and M2A in fetal male germ cells, predominantly between the 12^{th} and 26^{th} gestational weeks, and we addressed this expression exclusively to the gonocytes. After the 25th week of gestation, a second population of germ cells, matching the morphological criteria of pre-spermatogonia expressed MAGE-A4, which is a

marker of adult spermatogonia. These more mature germ cells lost their pluripotent potential as demonstrated by the downregulation of the pluripotency related proteins and acquired a spermatogonial phenotype.

Chapter 3

AP-2gamma belongs to the family of AP-2 transcription factors which are involved in the embryonic development of different organs and regulate cell type proliferation and differentiation. The AP-2gamma knock-out mouse is infertile, and shows a complete loss of spermatogenesis in the adult testis. We showed that AP-2gamma is expressed in human gonocytes at weeks 12-37 of gestation, indicating a role of this protein in human fetal germ cell development. With increasing differentiation of fetal testis, gradual down-regulation of AP-2gamma from the 12th to 37th week of gestation was observed. Furthermore, AP-2gamma was expressed abundantly in CIS as well as in testicular, metastatic en extragonadal seminomas and dysgerminomas of the ovary. In contrast, in embryonal carcinomas and choriocarcinomas, only a focal staining was observed. Spermatocytic seminomas, teratomas and yolk sac tumors as well as normal adult testis and various control tissues were negative for AP-2gamma. We did not detect any expression of AP-2gamma protein in malignancies other than GCTs. Because of a high sensitivity and specificity of AP-2gamma, this marker could be used a diagnostic marker of CIS and seminomas in surgical pathology.

Chapter 4

The germ cell lineage is discriminated from the somatic cells during early development by repression of the somatic cell fate in which transcription factor BLIMP1 plays a major role. BLIMP1 interacts with protein arginine methyltransferase PRMT5, and this complex mediates the symmetrical methylation of histones H2A and H4 at arginine 3 (H2AR3me2s, H4R3me2s). This process results in the widespread epigenetic modification and transcriptional repression. We showed that BLIMP1 and PRMT5 were expressed and arginine dimethylation of histones H2A and H4 was detected in human male gonocytes at weeks 12-19 of gestation. Moreover, BLIMP1/PRMT5 and histone H2A and H4 arginine 3

dimethylation was present in CIS and most seminomas, while downregulated in embryonal carcinoma and other nonseminomatous tumors.

Chapter 5

We found that bilateral TGCTs had 10 times higher rate of activating mutations within the c-KIT than the unilateral TGCTs. Only 6.4 % of unilateral TGCTs, but 63.6 % of bilateral TGCTs had an activating c-KIT mutation. All mutations were detected in exon 17 of c-KIT and were identified as activating point mutation leading to constitutional phosphorylation of tyrosine-kinase. Importantly, in the subset of bilateral TGCTs, we detected the same activating mutations (D816V und Y823D) in both independently developed synchronic or metachronic tumors. This indicates that mutations in c-KIT gene can occur in the early stages of germ cell development, before or during the migration of the PGCs into the gonads. Immature germ cells which had acquired with constitutively activated SCF-c-KIT pathway could gain survival advantage, which could lead to persistance of gonocytes beyond the normal maturation process. Our findings might have a practical importance, because detection of c-KIT exon 17 mutations could help to identify patients at high risk for development of TGCT in the contralateral testis. In addition, we described the analysis of the c-KIT expression in primary seminomas and their precursor lesions and compared this expression in metastatic seminomas. We found a significant down-regulation of c-KIT in seminomas compared to CIS by immunohistochemistry. Furthermore, we did not observe an increase of c-KIT mRNA levels in pT2/pT3 compared to pT1 seminoma by real-time RT-PCR. These findings indicate that c-KIT might not play a significant role in the progression of TGCTs.

Chapter 6

Till recently, only cell lines representative for nonseminomas, i.p. embryonal carcinomas were available (NT2, Tera-1, 833KE, NCCIT, and 2102Ep). Because seminomas are very difficult to culture and dedifferentiation towards

nonseminoma might occur during culturing, we compared both known seminoma cell lines TCam-2 and JKT-1 with seminoma and embryonal carcinoma by whole genome-RNA expression profiles and analysis of specific proteins. We found that the cell line TCam-2 clusters with the group of seminoma, whereas JKT-1 clusters with the group of embryonal carcinoma. Using reverse transcription/polymerase chain reaction, Western blot, and immunohistochemistry, we confirmed the seminoma-like nature of TCam-2 cells, whereas JKT-1 cells lacked the expression of the genes detectable in seminoma specifically and TGCTs in general, thus making doubtful the germ cell origin of this cell line. Thus, only TCam-2 seems to represent a suitable *in vitro* model for seminoma.

Chapter 7

In the general discussion, we discuss our findings in relation to the current literature. We end this chapter with general remarks and integration of our findings in the current pathogenetic model.

Samenvatting

Hoofdstuk 1

Bij de mens worden vijf verschillende typen kiemceltumoren (KCT) onderscheiden, waaronder die van de man en de vrouw, die goedaardig en kwaadaardig zijn, alsook die gonadaal als extragonadaal gelokaliseerd zijn. In deze classificatie worden de kwaadaardige seminomateuze en niet-seminomateuze kiemceltumoren in mannen en vrouwen type II kiemceltumoren genoemd. De incidentie van type II testiculaire kiemceltumoren, i.e., TKCT, vertoont een continue toename, waarschijnlijk als gevolg van omgevingsfactoren. Alle verschillende histologische varianten van TKCTs ontstaan uit de voorloper CIS, dat kwaadaardige intratubulair kiemcellen met een uniforme morfologie aanduidt. De pathogenese van TKCTs is niet volledig begrepen, maar vertoont overeénkomsten met embryonale kiemcellen. CIS wordt verondersteld te ontstaan uit getransformeerde onrijpe kiemcellen, de primordiale kiemcellen/gonocyten. Zoals in de algemene introductie besproken, kunnen veel karakteristieken van type II (T)KCT gekoppeld worden aan ES cellen (gevoeligheid voor chemotherapie, extra copiën van de korte arm van chromosoom 12 na *in vitro* kweek, expressie van stamcel genen, zoals OCT3/4, NANOG en SOX2, en een lage mutatie frequentie). Recente gegevens tonen aan dat specifieke genen, waaronder BLIMP1, PRMT5, AP-2gamma en c-KIT cruciaal zijn voor normale primordiale kiemcel (PKC) ontwikkeling. De te presenteren studies zijn uitgevoerd omdat kennis omtrent normale kiemcel ontwikkeling essentieel is voor het begrijpen van de pathogenese van KCT, en de expressie van de hierboven genoemde genen zouden cruciaal kunnen zijn in CIS/TKCT ontwikkeling.

Hoofdstuk 2

In dit hoofdstuk presenteren wij de resultaten verkregen door het bestuderen van 61 normale foetale gonadale weefsels van de tweede en derde trimester, met behulp van immunohistochemie met antilichamen gericht tegen de oncofoetale eiwitten c-KIT, AP-2gamma, M2A en de spermatogoniale merker MAGE-A4. Wij vonden een consistente co-expressie van de transcriptie factoren

OCT3/4 en AP-2gamma, c-KIT en M2A in foetale mannelijke kiemcellen, voornamelijk tussen de 12de en 26ste ontwikkelingsweek, en wij toonden aan dat dit geheel terug te voeren is op gonocyten. Na de 25ste week van ontwikkeling, een tweede populatie van kiemcellen, welke morfologische gelijkenissen vertoont met pre-spermatogonia welke MAGE-A4 tot expressie brengen, een merker voor spermatogonia. Deze meer uitgerijpte kiemcellen hadden hun pluripotentie vermogen verloren zoals aangetoond door de verminderde expressie van de pluripotentie gerelateerde eiwitten en het verkregen spermatogoniale karakter.

Hoofdstuk 3

AP-2gamma behoort tot de familie van AP-2 transcriptie factoren welke betrokken zijn in embryonale ontwikkeling van verschillende organen door middel van het reguleren van cel proliferatie en differentiatie. AP-2gamma knock-out muizen zijn steriel, en vertonen compleet verlies van spermatogenese in de volwassen testis. Wij toonden aan dat AP-2gamma tot expressie komt in humane gonocytes in de periode van de 12de tot de 37ste week van ontwikkeling, dat een rol van dit eiwit in de ontwikkeling van foetale kiemcellen suggereert. Tijdens het proces van kiemcel uitrijping in de foetale testis was er een graduele afname in AP-2gamma in de 12de tot de 27ste week waar te nemen. AP-2gamma was tevens aanwezig in CIS als ook testiculaire, metastatische, en extragonadal seminomen en dysgerminomen van het ovarium. Daaréntegen was enkel een focale aanwezig te zien in embryonaal carcinoom and choriocarcinomas. Spermatocytaire seminomen, teratomen en dooierzaktumoren alsook normale volwassen testis en verschillende controle weefsel waren negatief voor AP-2gamma. Wij vonden geen expressie van AP-2gamma eiwit in maligniteiten anders dan KCT. Op grond van de hoge sensitiviteit en specificiteit van AP-2gamma, kan deze merker gebruikt worden in de diagnostiek van CIS en seminomen in de chirurgische pathologie.

Hoofdstuk 4

De kiemcellijn wordt tijdens vroege ontwikkeling afgesplitst van de somatische cellen door onderdrukking van somatische differentiatie waarin onder

andere de transcriptie factor BLIMP1 een belangrijke rol speelt. BLIMP1 vormt een interactie met het arginine methyltransferase eiwit PRMT5, en dit complex medieert de symmetrische methylatie van de histon eiwitten H2A and H4 op positie arginine 3 (H2AR3me2s, H4R3me2s). Dit proces resulteert in een uitgebreide epigenetische modificatie en transcriptionele onderdrukking. Wij toonden aan dat BLIMP1 en PRMT5 aanwezig zijn in mannelijke gonocyten in de ontwikkelingsperiode van 12de tot 19de week, geassocieerd met arginine dimethylatie van de histonen H2A en H4. Tevens werd BLIMP1/PRMT5 en histon H2A en H4 argininine 3 dimethylatie gevonden in CIS en de meeste seminomen, terwijl deze minder aanwezig tot afwezig waren in embryonaal carcinoma and andere gedifferentieerde nonseminomateuze componenten.

Hoofdstuk 5

Wij toonden aan dat tweezijdige TKCT een 10 maal zo hoge frequentie van activerende c-KIT mutaies te zien geven dan éénzijdige tumoren. Maar 6.4% van de éénzijdige TKCT, maar 63.6% van de tweezijdige TKCT bevatten een activerende c-KIT mutatie. Alle mutaties werden aangetroffen in exon 17 van c-KIT en werden geïdentificeerd als activerende puntmutaties, leidend to constitutionele fosforylatie van tyrosine-kinase. Van belang was de bevinding dat in een subgroep van de tweezijdige TKCT dezelfde activerende mutaties (D816V en Y823D) aangetoond werden, in zowel de synchrone als metachrone tumoren. Dit impliceert dat mutaties in c-KIT ontstaan in vroege stadia van kiemcel ontwikkeling, voor of tijdens het proces van migratie van de PKC in de gonaden. De onrijpe kiemcellen met een constitutionele geactivieerde SCF-c-KIT signaalroute kunnen overlevingsvoordeel vertonen, dat kan leiden tot behoud van gonocyten zelfs nadat onder fysiologische condities deze cellen uitgerijpt zouden zijn. Deze observatie kan van belang zijn in de praktijk, omdat het aantonen van c-KIT exon 17 mutaties informatief kan zijn voor het identificeren van patiënten met een hoog risico op het ontwikkelen van een TKCT in de contralaterale testis. Tevens bestudeerden wij de expressie van c-KIT in primaire seminomen en de bijgehorende voorlopers, en vergeleken dit met gemetastaseerde seminomen. Wij toonden een significante afname in c-KIT aan in seminomen ten opzichte van CIS door middel van

immunohistochemie. Er werd geen toename van c-KIT mRNA aangetoond in pT2/pT3 ten opzichte ban pT1 seminomen door middel van quantitatieve RT-PCR. Deze bevindingen suggereren dat c-KIT geen rol hoeft te spelen in de progressie van TKCT.

Hoofdstuk 6

Tot voor kort waren enkel cellijnen beschikbaar afkomstig van nonseminomen, voornamelijk embryonaal carcinoom (NT2, Tera-1, 833KE, NCCIT, and 2102Ep). Omdat seminomen erg moeilijk zijn om te kweken en mogelijk dedifferentiatie naar nonseminoom kan vertonen tijdens deze bewerking, vergeleken wij beide seminoom cellijnen TCam-2 en JKT-1 met seminoom en embryonaalcarcinoom door middel van totaal genoom-RNA expressie profielen en analyse van specifieke eiwitten. Wij vonden dat de cellijn TCam-2 clusterde met de groep van seminomen, terwijl JKT-1 clusterde met de groep van embryonaalcarcinomen. Met gebruikt van reverse transcriptie/polymerase ketting reactie, Western blotting, en immunohistochemie, bevestigden wij de seminoom-achtige karakteristieken van TCam-2 cellen, terwijl JKT-1 cellen geen expressie van de meeste genen te zien gaven aantoonbaar specifiek in seminoom, en TKCT in het algemeen, op grond waarvan de kiemcel oorsprong van deze cellijn ter discussie staat. Met andere woorden, enkel TCam-2 lijkt representatief als *in vitro* model voor seminoom.

Hoofdstuk 7

In de algemene discussie worden onze bevindingen besproken in relatie tot de huidige literatuur. We besluiten dit hoofdstuk met algemene overwegingen en suggesties voor toekomstig onderzoek.

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Curriculum Vitae

Katharina Biermann/Pauls was born 15.10.1973 in Dsheskasgan, Kasachstan (which belongs to the former Sowjet Union). Though of Russian state affilation, she is german natinality, which allowed her together with her parents to move to Germany in 1990. Living in Germany (Altenkirchen, Westerwald) she graduated at the “Westerwald” gymnasium in 1994 and entered medical school in Giessen, Germany. During the studies, she gained interested in pathology and molecular medicine by performing scientific work about testicular cancer at the Department of Pathology at Giessen/Marburg University Medical Center. After her graduation in 2001, she finished her doctoral thesis (in Germany -Dr. med, with “summa cum laude”, awarded as the best thesis 2001 of Giessen Medical Faculty) and performed one year internship at the Department of Internal Medicine, LMU University Munich. In 2002, she started as a pathology resident at the Department of Pathology, University Bonn. During the pathology training of 5 years period, she continued working on molecular pathology of germ cell tumors and in 2009 she successfully finished the “Habilitation” at the University of Bonn. Her projects were supported by several grants from “Deutsche Krebshilfe”and “Deutsche Forschungsgemeinschaft” which were primary written by herself. She finished her training in 2008, and the same year started her work as a pathologist at Josephine Nefkens Institute Erasmus MC Rotterdam.

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Abbreviations

AMH	anti-Müllerian hormone
AFP	alphafetoptotein
BLIMP1 (PRDM1)	B-lymfocyte induced maturation protein-1
CIS	carcinoma in situ
DSD	disorders of sex development
ES	embryonic stem cells
GCTs	germ cell tumors
HCG	human chorionic gonadotropin
HDAC	histone de-acetylase
HPF	high power field
IGCNU	intratubular germ cell neoplasia unclassified
LDH	lactate dehydrogenase
MIS	Müllerian inhibiting substance
PGCs	primordial germ cells
PLAP	placental alkaline phosphatise
PRMT5	arginine-specific histone methyltransferase 5
PRLND	retroperitoneal lymph node dissection
RT-PCR	reverse transcriptase polymerase chain reaction
RT	radiotherapy
TGCTs	testicular germ cell tumors
TDS	testicular dysgenesis syndrome
TIN	testicular intratubular neoplasia