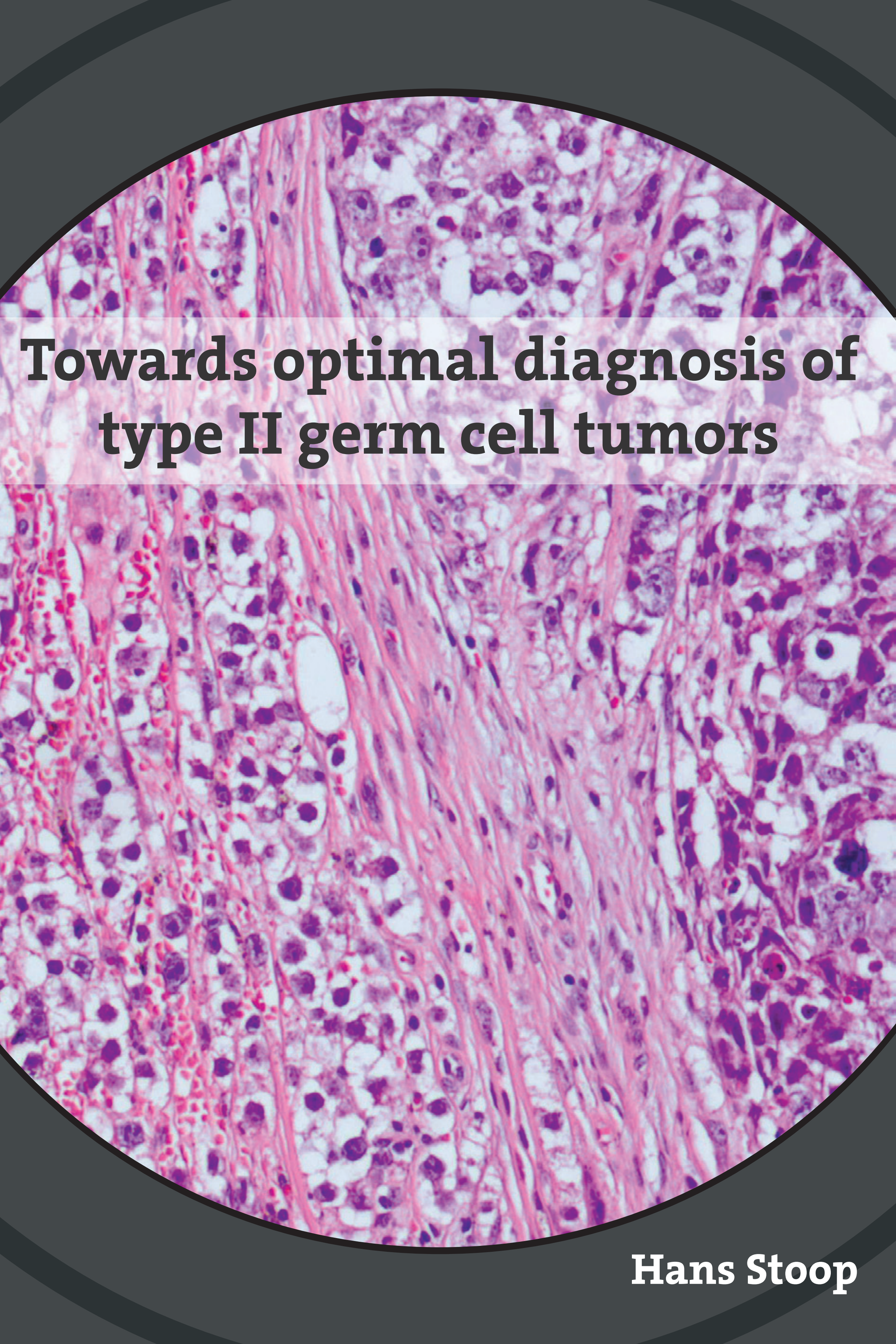

Towards optimal diagnosis of type II germ cell tumors

Hans Stoop

Towards optimal diagnosis of type II germ cell tumors

Optimale diagnostiek van type II kiemceltumoren

The work presented in this thesis was done at the Department of Pathology, Erasmus Medical Center, Josephine Nefkens Institute, Daniel den Hoed Cancer Center, Rotterdam, The Netherlands

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Towards Optimal Diagnosis of Type II Germ Cell Tumors

Optimale diagnostiek van type II kiemceltumoren

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Our wisdom comes from our experience,

and our experience comes from our foolishness

Sacha Guitry

Voor mijn ouders: Sjaak † en Lisa

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Abbreviations

AFP: a-fetoprotein
ChC: choriocarcinoma
CIS: carcinoma *in situ*
c-KIT: stem cell factor receptor (CD117)
dAP: direct alkaline phosphatase (staining)
DSD: disorders of sex development
EC: embryonal carcinoma
FFPE: formalin fixed paraffin embedded
GB: gonadoblastoma
GBY: critical region of the gonadoblastoma locus on Y chromosome
GCAP: germ cell alkaline phosphatase
GCL: germ cell lineage
H&E: haematoxylin and eosin (staining)
hCG: human chorionic gonadotrophin
IGCNU: intratubular germ cell neoplasia unclassified
IHC: immunohistochemistry
KITLG: c-KIT ligand (SCF)
LDH: lactate dehydrogenase
PGC(s): primordial germ cell(s)
PLAP: placenta like alkaline phosphatase
pSg: pre-spermatogonium
Sc: spermatocyte
SCF: stem cell factor (KITLG)
SCO: sertoli cell only
SE: seminoma
Sp: spermatogonium
Sz: spermatozoa
TDS: testicular dysgenesis syndrome
TE: teratoma
TGCT(s): testicular germ cell tumors
TIN: testicular intratubular neoplasia
TM: testis microcalcifications
TMA: tissue micro array
TNAP: tissue non-specific alkaline phosphatase
TSAP: tissue specific alkaline phosphatase
TSPY: testis specific protein Y encoded
YST: yolk sac tumor

General Introduction

1.1 Introduction

Primordial Germ Cells (PGCs) are the germ line stem cells in mammals that give rise to gametes at later life. These final mature germ cells, oocytes in females and spermatids in males, are designed to transfer genetic information to the next generation. Therefore they are specifically and optimally equipped, and undergo defined steps of maturation and specialization during their development. Much research on the fundamental aspects of germ cell development is done *in vivo* or *ex vivo* on animals, including Drosophila, zebrafish [1], and mouse [2-6]. These observations are extrapolated to the human situation, because of the limitations to study early human embryogenesis. However, this has restrictions, because of possible differences between mice and humans, including and specifically the germ cell lineage at early (embryonic) age [7]. Therefore direct investigation of human germ cell development is crucial to understand the various processes involved, and to prevent wrong interpretation due to the existence of interspecies variation(s). This is especially of relevance in the context of understanding the earliest events in the pathogenesis of human germ cell tumors (GCTs), for which significant differences exist between humans and animals [8]. In the next paragraphs a selection of relevant observations will be presented, informative for better understanding the pathogenesis of human GCTs. Because of this defined goal, the information given will be no review of the whole existing literature on this topic, for which other papers are available [8-10].

1.2 Migration of primordial germ cells

In the third week of human embryonic development PGCs start to migrate by active amoeboid movement under response of mitogenic and survival factors from their origin in the posterior wall of the yolk sac along the hindgut, through the dorsal mesentery into the genital ridge [1,3,6,11-12]. This migration route can be visualized based on the fact that PGCs express a number of specific (embryonic) markers, involved in various biological mechanisms during this time window. Here, only those markers that have a (putative) value in diagnosis of human GCTs, i.e., alkaline phosphatase, OCT3/4, c-KIT, VASA, TSPY and SOX17 [13-17] will be referred to. Proper PGC development is dependent on the c-KIT pathway, in which both a survival (i.e., apoptosis suppressing) effect and well as a proliferative effect of the ligand (KITLG or Stem Cell Factor) can be identified [5]. In the rest of the introductory chapter the human embryonic gonadal male situation will be described, if not stated otherwise.

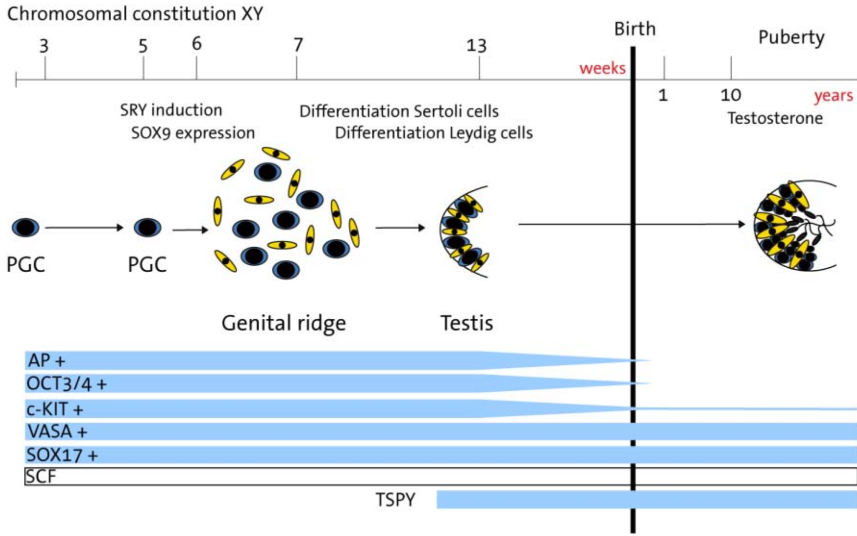
1.3 Gonocyte micro-environment

At the time the PGCs enters the genital ridge (later in the fifth week of development), they are called gonocytes. That moment, their migratory behavior ceases, but they continue to proliferate (the number at that time is approximately 1000 to 2000) [18]. At this specific moment of development, no differences between the sexes exist, related to both the germ cells itself as well as their micro-environment. However, early in the sixth week in the male constitution (XY), initiated by expression of the transcription factor SRY (testis determining factor), and subsequently SOX-9, Sertoli cells are formed, resulting in the formation of primitive sex cords, wherein the gonocytes interact with the supportive cells. This will be presented in more detail in the next paragraph.

1.4 Differentiation into testis

Under influence of SRY, of which the gene is mapped to the Y chromosome, the first differences between the male and female gonad are formed. In fact, the bipotential gonad is triggered to differentiate into the testicular direction by inducing a subset of somatic cells to differentiate into Sertoli cells, believed to act as the organizing center of the male gonad [19]. The presence of germ cells plays no part in this specific process in the male [20]. Parallel to the differentiation and proliferation of the Sertoli cells, the gonad increases in size due to increased proliferation and migration of other cells which will give rise to endothelial-, myoepithelial-, and Leydig cells [18,21-23]. During the 7th week of gestation the gonocytes become enclosed in the further developed sex cords, now referred to as primitive seminiferous cords, and enter mitotic arrest. At this time, the amount of germ cells is about 100.000 [24-26]. This situation will remain stable until a few days after birth. The gonocytes present have a high content of glycogen as in the stage of PGC, but still show expression of the other proteins, as mentioned before (alkaline phosphatase, OCT3/4, c-KIT, VASA, TSPY and SOX17) [27-30]. The gonocytes and Sertoli cells have initially a non-structured organization, but gradually the germ cells migrate toward the periphery of the further developing seminiferous tubules. In the 13th week the germ cells are predominantly localized at the basal side, and start to loose expression of the embryonic markers mentioned, with the exception of VASA and SOX17. They start to further enhance expression of TSPY (Testis Specific Protein on the Y chromosome) [31]. Contact between Sertoli cells and, the now referred to, pre-spermatogonia is required for proper regulation of proliferation and protection against apoptosis [32-34]. In fact, the gonocytes and pre-spermatogonia change their expression pattern gradually; proteins related to an embryonic stage are decreasing in level, and finally disappear. These different steps in the maturation of germ cells are schematically illustrated in Figure 1.

A



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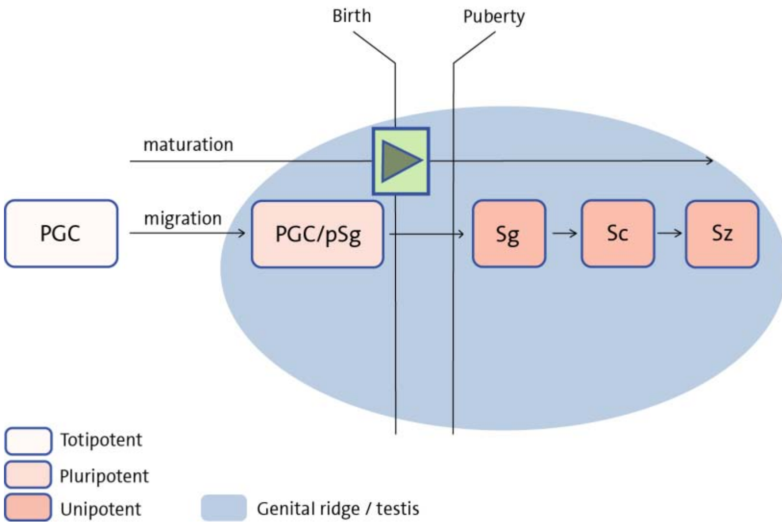

Figure 1. Maturation and migration of germ cells. (A) The different steps in the maturation of germ cells. Expression of the different diagnostic markers alkaline phosphatase (AP), OCT3/4, c-KIT, VASA, SOX17 and TSPY from the early embryonal period which starts when the PGCs originate from the epiblast. Later in the 5th week when the PGCs enter the genital ridge the induced Sertoli cells surround the PGCs which are now called gonocytes (GC) and the seminiferous tubule is formed. The expression pattern changes and the proteins related to the embryonic stage are declining in level and finally disappear. In puberty spermatogenesis starts; **(B)** Totipotent PGCs migrate to the genital ridge and start to mature. At the time of entering the genital ridge they are called gonocytes (being pluripotent) and subsequently when they are in contact with Sertoli cells and the basal lamina, are referred to pre-spermatogonia (pSg). In puberty the unipotent spermatogonia (Sg) form in succession spermatocytes (Sc) and spermatozoa (Sz).

1.5 Classification of germ cell tumors

The traditional classification of human GCTs is primarily based on histology. A new classification system, recognized by the World Health Organization (WHO), is based on various clinical, genetic and histological parameters which makes distinction into five relevant entities possible [35-36]. Identification of these subtypes will result in an improved clinical diagnosis and prediction of treatment response, as well as more straightforward research. The group of testicular GCTs comprises three of the five GCT entities. The so-called type I GCTs of the testis are predominantly diagnosed in neonates and infants [35-36], and will therefore not be discussed here. The types II and III are the majority (90%) of a diverse group of lesions in the testis at adult age. Other types of testicular tumors include the gonadal stromal tumors, i.e., Leydig cell tumors, Sertoli cell tumors and others [37], also not to be discussed here. The type II and III testicular GCTs can be distinguished from each other based on cell of origin, pathogenesis and clinical behavior. Because of the topic of this thesis, the type III GCTs will be referred to only briefly in the next paragraph, after which the type II GCTs will be discussed throughout.

1.5.1 Type III germ cell tumors

Spermatocytic seminomas, here referred to as type III GCTs [36], are a rare variant of testicular GCTs, accounting less than 1% of all cases in adults, with an incidence of about 0,2 per 100.000, without increase in the population [38]. According to the WHO classification system, the spermatocytic seminomas are a variant of seminoma [35]. However, they are predominantly found at elderly age, show a distinct morphology, and hardly metastasize [39-41]. Based on multiple observations, it is currently accepted that type III GCTs originate from a later maturation stage of the germ cell lineage, likely spermatogonia or spermatocyte [42-44]. Interestingly, recent observations indicate that gain of chromosome 9 is a recurrent anomaly, for which DMRT1 is a possible candidate, informative as diagnostic marker [45]. In addition, other informative markers for diagnosis of type III GCTs are reported [39,42-43].

1.5.2 Type II germ cell tumors

1.5.2.1 Introduction

Testicular type II GCTs, here referred to as TGCTs, are histologically and clinically subdivided into the seminomatous and nonseminomatous tumors [35]. The nonseminomas can be subdivided into the stem cell component, referred to as embryonal carcinoma, the somatic lineages (teratoma) and the extra-embryonic lineages (yolk sac tumor and choriocarcinoma). During the last 40 years the incidence of TGCTs doubled in Caucasian populations [46], while they are

significantly less frequent in Asian and African populations, independent of migration. Clear differences are also identified within Europe, with the highest incidence in Switzerland and Denmark [47]. In the Netherlands the incidence of TGCTs is about 6.6 per 100.000 corresponding to approximately 750 newly diagnosed cases each year. The incidence is still growing (4.8 per 100.000 in the period 1994-1998) [38,46,48-50]. In spite of this rise there is no increase in death of patients because of this disease. The reason is the availability of effective treatment options, including surgery, irradiation and/or chemotherapy, resulting in a 10 year survival of about 95% [51]. In the Netherlands, before 1977 (the pre-cisplatin period), the 10 year survival of a patient with a seminoma was 83%, and with nonseminoma 56% [38,52]. However, there are drawbacks related to the recent success leading to survival of 95%, including long term side effects of systemic treatment, like heart- and vascular disease, infertility, metabolic syndrome, and secondary malignancies [53]. Therefore, understanding of the earliest steps in the pathogenesis of TGCTs is of importance, because it will allow development of methods for early diagnosis and treatment, limiting treatment-related side effects. In the next paragraph the current knowledge about the etiology of TGCTs will be summarized.

1.5.2.2 Etiology and identified risk factors

As indicated, a remarkable geographical variation exists in the (increase of) incidence of TGCTs [47-48,54]. It is suggested that TGCT-development is related to a Western lifestyle [55]. Environmental exposures during early development have been postulated to play a significant role [47,55-60]. This is demonstrated by analysis of migration studies and the dip in the increasing trend during the Second World War. In addition, a number of defined risk factors is identified. These include cryptorchidism, in fact the first condition recognized as being involved with the etiology of this cancer [61-66]. Only 2-10% of the patients with a TGCT have a history of undescended testis [67]. This indicates that other risk factors must exist. One of these is infertility [68]. Within the overall Andrology clinic population, the incidence of TGCT (or the precursor of it, see below) is 1% [69]. In addition, hypospadias and a low or high birth weight are identified as risk factors [59,70-71]. Moreover, a previous (unilateral) TGCT is a risk factor as well. The cumulative risk to develop a contralateral TGCT ranges from 0.6 % to 8%. [72-74], being higher in patients with a nonseminoma compared to with a seminoma [73,75]. Interestingly, a role of the immune system has been suggested [76-78].

Besides these effects, a significant genetic component is identified [79-80]. This is demonstrated by the reported increased risk for this type of cancer in fathers and brothers, although this might also be related to a common micro-environment. However, no high penetrance predisposing genes were discovered by various linkage studies in TGCT-families[81-83]. Recently significant findings were made

using genome-wide association (GWA) studies. Independent investigations showed the presence of TGCT-predisposing alleles in or near a limited number of genes, i.e., *KITLG (SCF)*, *BAK1*, *SPRY4*, *TERT*, *ATF7IP*, and *DMRT1* [81-87]. Interestingly, the proteins encoded by these genes are (partly) known to have a role in early germ cell development, including migration, survival, micro-environment establishment and maturation.

1.5.2.3 Compiling model of testicular germ cell tumor risk factors

Skakkebæk and coworkers proposed in 2001 existence of a Testicular Dysgenesis Syndrome (TDS), characterized by impaired spermatogenesis, cryptorchidism and hypospadias, associated with possible development of TGCTs [88]. It has been hypothesized that TDS is the result of anti-androgen and/or (xeno)estrogen action, possibly via the supportive cell function [89-91]. This may act via genetic- or epigenetic mechanisms. For example phthalates, which are widespread used, are classified as endocrine disruptors [92-95]. Exposure can lead to demethylation of promoter-associated CpG islands, a crucial regulator of gene expression, both in time and place [96]. A high estrogen level exposure during pregnancy has indeed been suggested [97], supported by a recent study [98]. Related to this model, another, so far not mentioned risk factor for type II GCTs is of relevance. These anomalies are described by the umbrella term Disorders of Sex Development (DSD), previously referred to as intersex [99]. It is characterized by discordance between genetic sex, gonadal sex and phenotypic sex, in which patients showing hypovirilization and/or gonadal dysgenesis are at increased risk [100]. This is directly related to the presence of the gonadoblastoma region of the Y chromosome (GBY), including the forementioned TSPY gene [101]. Another parameter for prediction of malignant transformation of the germ cell is the anatomical localization of the gonad, as expected based on identification of cryptorchidism as risk factor (see above). Overall, an abdominal gonad has a higher risk for malignant transformation than a scrotally localized gonad [for review 102-105]. To allow optimal diagnosis of type II GCTs, both in the testis, as well as gonads of patients with DSD, recognition of the precursor lesion of this type of cancer is required. Current knowledge related to this issue will be presented in the next paragraph.

1.5.2.4 Precursor of type II (testicular) germ cell tumors

Presence of atypical germ cells in the seminiferous tubules adjacent to a TGCT was detected already in 1896 [106]. They were seen as a product of the cancer or a representation of spread from the malignancy itself [107]. However, the association between these cells and progression to a TGCT was officially established by Skakkebæk in 1972 [108]. This observation was in line with the earlier suggestion that the various histological variants of TGCTs have a common precursor [109]. This precursor is referred to as carcinoma *in situ* (CIS) (the term to be used here), intratubular germ cell neoplasia unclassified (IGCNU) [110-111] and testicular intratubular neoplasia (TIN) [112]. Supportive data that a PGC/gonocyte is the origin of CIS is gathered by analysis of morphology, and protein and (m and mi)RNA expression levels, showing similarities to embryonic stem cells [113-116]. Based on these observations, as well as the specific risk factors identified (see above), the currently accepted pathogenetic model of type II (T)GCTs is that they originate early in embryogenesis, affecting a PGC/gonocyte, blocked in its process of maturation [117-118]. The presence of CIS predicts development of an invasive TGCT [119-120], which indicates that clinical interference is required. The CIS counterpart in a gonad of a DSD patient with a low level of testis development (referred to as "testicularization" [118]), is known as gonadoblastoma (GB) [121]. In fact, the germ cells in GB show the same characteristics of CIS cells, including their tendency to progression to an invasive type II GCT. A summary of the various relevant items presented, is given in Figure 2.

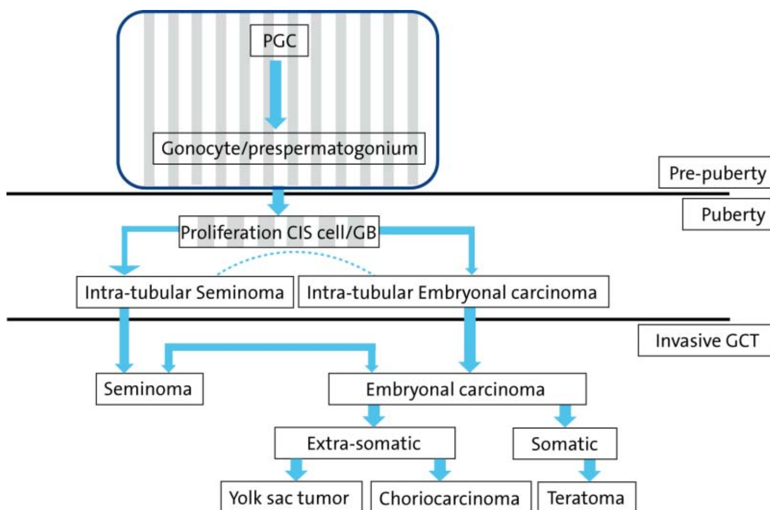

Figure 2. Pathogenesis of the type II (testicular) germ cell tumor. The arrested embryonal germ cell (pre-CIS cell) in the embryonal gonad, is supposed to start proliferating at puberty. Via the specific intratubular stage CIS/GB develops in an invasive germ cell tumor: seminoma and/or nonseminoma. The embryonal carcinoma, which is the stem cell component of the nonseminoma, can differentiate into a somatic (teratoma) and/or extra-somatic (yolk sac tumor and choriocarcinoma) entity.

1.6 Concluding remarks

An interplay between environmental - and genetic factors seems to be involved in the earliest pathogenetic events in development of type II (T)GCTs. This knowledge allows identification of risk populations to develop this type of cancer in the earliest stage of development possible, leading to early diagnosis and treatment, thereby preventing progression to a cancer. This set up requires availability of informative (highly sensitive and specific) markers and tools for detection of the earliest changes in the process of malignant transformation of a germ cell. This specifically applied on histological samples obtained by surgical biopsies of gonads at risk for this type of cancer. In addition, in certain situations, a non-invasive diagnostic test might be applicable.

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Chapter 2

Aims and outlines of the thesis

TGCT is by far the most common malignancy in Caucasian men aged 15-45 years, and the second cause of death in this age group. The combination of the rising incidence, identification of risk populations, knowledge of the precursor lesions, as well as the excellent prognosis of the patients, especially when diagnosed at an early stage of development, makes this an interesting type of cancer for early diagnosis and subsequent treatment. This will benefit the quality of life of the individual patient because it will prevent known long term sequalae of systematic treatment of the patients with invasive (metastatic) cancer using either chemotherapy or irradiation.

Within the Netherlands currently about 750 new TGCT patients are diagnosed per year, which has doubled since 1989. Histologically and clinically, TGCTs are subdivided into seminomatous and nonseminomatous tumors. The nonseminomas are further subclassified into embryonal carcinoma (being the stem cell population), teratoma (representing somatic lineages), yolk sac tumor and choriocarcinoma (representing extra-embryonic lineages). These can be pure or intermixed, even together with a seminoma component. Both seminomas and nonseminomas originate from a common precursor, known as CIS (IGCNU or TIN), which has characteristics of a primordial germ cell (PGC)/gonocyte. Indeed, a disturbed early testicular development (referred to as reduced testicularization) is a risk factor for development of TGCTs. Therefore understanding normal testicular development is of importance to shed light on the earliest events in the pathogenesis of TGCTs. For this reason, a series of normal male embryonic, fetal, and post-natal gonads are investigated, especially related to the expression of a number of proteins informative to distinguish the different maturation stages of germ cells. The results are described in **Chapter 3**, in which it is shown that CIS very likely finds its origin in germ cells which are arrested in development during the early embryogenesis, at the stage of PGC/gonocytes. The findings determine the window of opportunity for early diagnosis.

The possible histological composition of TGCTs indicates a pluripotent nature of the cell of origin. To further substantiate this hypothesis, it is investigated whether the tumors can also generate the germ cell lineage itself. Based on the application of a set of markers that have become available recently, supportive evidence is presented in **Chapter 4.** TGCTs are indeed omnipotent/totipotent, and are able to form all differentiation lineages as found during embryogenesis, including the germ line.

The pluripotent (omnipotent/totipotent) nature of TGCTs initiated a series of studies in which markers known to be relevant to regulate maintenance of pluripotency in embryonic stem cells are investigated for their staining pattern in TGCTs. This especially for the earliest identified factor OCT3/4, also known as

POU5F1. To study the protein expression profile in normal gonadal development and tumors, immunohistochemistry is applied, both on individual sections, as well as tissue micro arrays. The results, as described in **Chapter 5 and 6**, demonstrate that OCT3/4 is an absolute marker for detection of the precursor lesions CIS and GB, seminomatous tumors and embryonal carcinoma. All tumor cells show a strong positive staining within the nucleus, while in the embryonal carcinoma cells also a cytoplasmic localization is identified. Upon differentiation, OCT3/4 is lost. Based on this highly reproducible finding, both using monoclonal and polyclonal antibodies, it is investigated whether OCT3/4 can also be used as marker for development of a non-invasive test, i.e., detection of CIS cells in semen. In **Chapter 7**, the results of the first study are presented showing that in principle CIS cells can be identified in semen using immunohistochemical detection of OCT3/4. The diagnostic application of OCT3/4 is not limited to TGCTs. In **Chapter 8** it is shown that OCT3/4 can also be used to diagnose extragonadal seminomatous tumors, including those located in the brain.

In **Chapter 9** the transcription factors SOX2 and SOX17 are shown to be very useful in the diagnostics of TGCTs. During embryogenesis they interact with OCT3/4, important for normal embryogenesis. Including these markers in the diagnostic workup of TGCTs, next to OCT3/4, will lead to an optimal diagnosis, and subsequent treatment of the patient. Patients with cryptorchidism and more severe DSD are high risk groups for the development of a TGCT. Diagnosis at young age in these cases is of eminent importance. A gonadal biopsy can give important information, however, especially in the forementioned cases, germ cell maturation delay can be present. This will result in an extended time window of expression of embryonic markers which are used for CIS detection in adults. In **Chapter 10** the value of the immunohistochemical detection of KITLG (SCF) for this group of young individuals is demonstrated. Early detection will benefit quality of life. In **Chapter 11** an enzymehistochemical method is described which makes it possible to examine frozen sections for the presence of CIS, seminoma or embryonal carcinoma. This method makes it possible to guide the decision, during the initial surgical intervention, to remove the gonad, or perform limited surgery.

During development of the gonads, especially related to the germ cell population, changes in DNA methylation by means of cytosine methylation, is a crucial regulatory mechanism for gene expression. Most investigations, also on TGCTs, are done using isolated DNA, which do not allow analysis of the cells under investigation in their natural environment. This is solved by means of immunohistochemistry, of which results are described in **Chapter 12**. PGCs/gonocytes are showing an overall demethylated pattern, while spermatogonia and later germ cell maturation stages become more methylated.

CIS, GB, and seminomas are also hypomethylated, while the differentiated nonseminomatous elements are hypermethylated and embryonal carcinoma show an intermediate pattern. Cisplatin refractory seminoma shows a hypermethylated pattern. Cell line investigations support the model that cFLAR (also known as cFLIP) might be a regulator of cisplatin sensitivity in TGCTs. In **Chapter 13**, the results obtained are discussed in a broader perspective regarding their possible impact in the diagnostic workup of TGCTs.

Chapter 3

Pathobiological implications of the expression of markers of testicular carcinoma *in situ* by fetal germ cells

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Abstract

Several proteins, such as the placental/germ cell alkaline phosphatases (PLAPs), the stem cell factor receptor c-KIT, and the transcriptional regulator and marker of pluripotency OCT3/4, have been found in both normal immature and malignant germ cells, known as carcinoma in situ/intratubular germ cell neoplasia unclassified (CIS/ITGCNU). In the present study, immunohistochemical methods were used to evaluate the expression of these markers in a series of male gonads from fetuses from the second and third trimesters, and neonates. In addition to these markers, the presence of VASA (a protein specific for the germ cell lineage), TSPY (the testis-specific protein, Y-encoded), and the proliferation index (Ki-67 antigen) was analyzed. All these proteins are reported to be present both during spermatogenesis and in CIS/ITGCNU. Positive staining for VASA with varying intensity was found in all germ cells, while TSPY was predominantly located in the pre-spermatogonial cells at all developmental ages. In contrast, the markers PLAP, c-KIT, OCT3/4, and Ki-67 were more frequent at earlier developmental stages and decreased gradually with time, although they could occasionally be detected in germ cells of neonates. These findings are in line with a declining number of gonocytes during fetal development, concomitant with an increase in the number of pre-spermatogonia. The latter have lost the immature germ cell phenotype. These findings are compatible with the hypothesis that CIS/ITGCNU arises from developmentally arrested germ cells, most likely primordial germ cells/gonocytes, at an early time point during intrauterine development.

Introduction

In humans and rodents, amongst other species, the embryonic precursors of the gametes are known as primordial germ cells (PGCs) (see ref 1 for a review). These cells are set aside to an extra-embryonic localization early during embryonic development and around the fifth and sixth weeks of human development, they migrate to the area where the genital ridge will be formed [2]. Subsequently, gonadal and sexual differentiation occur in the sixth and seventh weeks [3]. With regard to testicular development, the germ cells at this particular stage are referred to as gonocytes, which are predominantly found in the central areas of the newly formed tubules. Starting around the 14th week of development, these gonocytes gradually migrate towards the tubular periphery. Once the cells are in close contact with the basal lamina of the tubule, they are referred to as pre-spermatogonia. Maturation is a gradual process; at the 20th gestational week, the fetal testis predominantly contains pre-spermatogonial germ cells [4]. During the first few years after birth and until puberty, morphological and functional changes occur, including a change from large, immature germ cells (pre-spermatogonia) to adult type A spermatogonia [5–7]. With the onset of puberty, spermatogonia undergo further spermatogenic maturation and after meiotic divisions, finally produce spermatozoa.

On the basis of multiple findings, it has been hypothesized that CIS/ITGCNU, the common precursor lesion of adult testicular germ cell tumours (TGCTs), originates early during fetal development (see ref 8 for a review). This is illustrated by the presence of a number of markers common to CIS/ITGCNU and immature germ cells, including germ cell/placental alkaline phosphatases (PLAPs), the proto-oncogene receptor c-KIT, and the transcriptional regulator and marker of pluripotency OCT3/4. CIS/ITGCNU cells phenotypically and ultrastructurally resemble PGCs/gonocytes [9–11]. Moreover, epidemiological data support the hypothesis that the initiating event of TGCT development occurs during the fetal period [12]. In spite of these data, an alternative origin of TGCTs has been suggested, in which the pachytene spermatocyte, a cell not found during the prepubertal period, has been suggested to be the target of transformation [13].

In order to investigate the origin of CIS/ITGCNU further, we undertook an extensive study of the presence of a number of markers during normal fetal testicular development. This will help us to define further the emerging phenotype of CIS/ITGCNU and shed light on the possible limitations of these markers for early diagnosis of CIS/ITGCNU in high-risk neonates and infants.

Material and methods

Tissue samples

Use of tissues for scientific reasons was approved by an institutional review board (MEC 02.981). The samples were used according to the 'Code for Proper Secondary Use of Human Tissue in The Netherlands', as developed by the Dutch Federation of Medical Scientific Societies (FMWV) (version 2002).

Human fetal gonads from 27 males from the second and third trimesters after spontaneous or induced abortions (gestational age 15–40 weeks), or from premature and term neonates that had died shortly after birth, and one infant who died from cot death at the age of 6 weeks were obtained from post-mortem sections in our department. Testes were dissected and fixed in 10% formalin and processed into paraffin wax. To ensure satisfactory quality, poor preservation of tissue samples assessed by haematoxylin and eosin staining led to exclusion from this analysis. Cases showing conditions that possibly affect gonadal development, such as trisomy 13, 18 and 21, hydrocephalus, maldeveloped kidneys, or gross intrauterine growth retardation, were excluded from the study. Gestational ages were calculated in relation to the mother's last menstrual cycle and were in accordance with the foot length and the crown–heel length measurements at autopsy, showing a maximum variation of 2 weeks.

Table 1. Antibodies (source) and detection method used for immunohistochemistry

Antibody	Company	Code	Pre-treatment	Dilution	Secondary antibody (1 : 200)(biotinylated) Code	Visualization
PLAP	Cell Marque	CMC203	HIAR*	1:200	Dako E0413	ABCplx-ap†
c-KIT	Dako	A4502	HIAR	1:500	Dako E0431	ABCplx-ap
OCT3/4	Santa Cruz	sc-8629	HIAR	1:1000	Vector BA9500	ABCplx-hrp‡
VASA	Provided by D Castrillon [43]		HIAR	1:2000	Dako E0431	ABCplx-ap
TSPY	Provided by Y Lau [39]		None	1:3000	Dako E0431	ABCplx-ap
Ki-67	Dako	A047	HIAR	1:50	Dako E0431	ABCplx-hrp

* Heat-induced antigen retrieval [42].
† ABC complex, alkaline phosphatase, Dako Code: K0391.
‡ ABC complex, horseradish peroxidase, Dako Code: K0377.

Histochemical and immunohistochemical staining

For immunohistochemistry, sections were incubated with the primary antibody overnight at 4^0C (PLAP, c-KIT, TSPY, VASA) or for 2 h at room temperature (OCT3/4, Ki-67). The antibodies used are indicated in Table 1. Sections were counterstained with haematoxylin.

Double staining was performed by using a combination of the same detection method but with different substrates: Fast blue/naphthol ASMX phosphate (F3378 and N500; Sigma, Steinheim, Germany) for blue staining and 3-amino-9-ethyl-carbazole (A.5754 and D4254; Sigma, Steinheim, Germany)/H_2O_2 for red staining, without counterstain. Endogenous peroxidase activity and/or endogenous biotin was blocked using 3% H_2O_2 (for 5 min) and/or a blocking kit for endogenous biotin (Vector Laboratories, Burlingame, CA, USA) to prevent background staining [14].

For quantification, cell numbers showing a positive signal were counted in five to ten cross-sections of seminiferous tubules by two different observers (FH and HS) who were blinded to the gestational age at which the material was sampled.

Results

The presence of a number of well-known and novel markers for CIS/ITGCNU during normal fetal testicular development was studied. The available data on these markers are summarized in Table 2. The results are reported separately for the known CIS/ITGCNU markers (PLAP, c-KIT, and OCT3/4) and the other markers analysed (VASA, TSPY, and Ki-67). Subsequently, the results of double staining are described. Representative illustrations are shown in Figure 1. Figure 2 summarizes the results of each marker for each case individually. For the majority of cases, intra-individual comparison of the expression of CIS/ITGCNU markers (PLAP, c-KIT, and OCT3/4) gave consistent results, reflecting the state of maturation of one particular case. The data for the whole population are depicted graphically in Figure 3 (grey bars) and compared with findings published so far (black bars).

Table 2. Overview of markers for carcinoma in situ/intratubular germ cell neoplasia unclassified (CIS/ITGCNU), proliferation, and germ cell-specific factors

Marker/ Antigen	CIS/ITGCNU (intensity)	References
Glycogen	+++	Nielsen and Lein, 1974 [20]
PLAP	+++	Beckstead, 1983 [44]; Jacobsen and Norgaard-Pedersen, 1984 [45]
c-KIT	+++	Rajperts-De Meyts,d Skakkebæk, 1994 [46];Strohmeyer et al, 1995 [47]
OCT3/4	+++	Looijenga et al, 2003 [27]
VASA	+	Zeeman et al, 2002 [48]
TSPY	++	Schnieders et al, 1996 [38]; Lau et al, 2000 [39]
Ki-67	+++	Datta et al, 2000 [30]

Immunohistochemical detection of PLAP, c-KIT, and OCT3/4

Positive staining for PLAP, c-KIT, and OCT3/4 was seen specifically in the germ cells, while no staining was present in Sertoli, Leydig, or interstitial cells (see Figure 1).

The highest number of germ cells staining for PLAP was seen in the earliest stages of fetal development examined and decreased continuously through-out the following weeks with advancing gestational age. Within the tubules of earlier stages, PLAP was predominantly detected in gonocytes, and to a lesser extent in pre-spermatogonia (see Figure 1A). After birth, PLAP could still be found occasionally (see Figure 2A), with maximally one positive cell per visual field. These cells were almost exclusively located in the centre of the tubules. Mainly during the second trimester, a large number of germ cells were positive for c-KIT, which, like PLAP, declined gradually throughout gestation. Again, positive germ cells were detectable at term, albeit at low numbers. Within the tubules, c-KIT was seen in both gonocytes and pre-spermatogonia (see Figures 1B and 2B).

OCT3/4 resulted in nuclear staining of germ cells at all gestational ages. With advancing gestational age, a constant decrease in cells staining for OCT3/4 was found (see Figure 2C). High numbers with an average of four to six cells per tubule, mainly gonocytes, were seen throughout the first half of the second trimester (Figure 1C). Throughout the second half of the second trimester, the average number of positive cells decreased to less than three cells per tubule. At term, only a few positive germ cells were detectable, mainly located in the centre of the tubules.

An increased number of germ cells positive for PLAP, c-KIT, and OCT3/4 were seen in testes from three second-trimester fetuses with chromosomal abnormalities, ie trisomy 21 and 18: these were not included in the series presented (data not shown). These findings are in line with previous reports [15,16] and support the model that chromosomal abnormalities can interfere with normal germ cell maturation.

Immunohistochemical detection of VASA, TSPY, and Ki-67

VASA-positive germ cells were found at all gestational ages and after birth. Although the staining intensity was variable, pre-spermatogonia showed consistently stronger staining than gonocytes (see Figure 1D). The number of positive cells per tubule decreased only slightly with gestational age, resulting in a different overall staining pattern compared with the factors described above: VASA was still found in germ cells at term and in neonates (see Figure 2D). Nuclear and cytoplasmic TSPY was predominantly observed in putative pre-spermatogonia, based on their peripheral localization within the tubules, at all gestational ages. No decrease in staining was found at term or in the first few weeks after birth (see Figure 2E). On average, three to five cells per tubule showed positive staining, most often seen in pairs or groups of germ cells (Figure 1E). In contrast to the other markers described so far, TSPY was not restricted to germ cells, but was also detected in Leydig cells at all ages examined.

Ki-67 showed nuclear staining in a high number of cells, both within and outside the tubules, throughout the whole period of testicular development investigated. In developing tubules, until 24 weeks of gestational age, Ki-67 was predominantly seen in gonocytes (Figure 1F); after 24 weeks, both basally and centrally located germ cells showed staining in roughly equal numbers. The number of positive cells decreased steadily with advancing age, and perinatally, only a few intratubular cells in the tubules remained positive (see Figure 2F). These were mostly centrally located in the tubules, whereas the majority of pre-spermatogonia seemed to have entered a quiescent phase at that time.

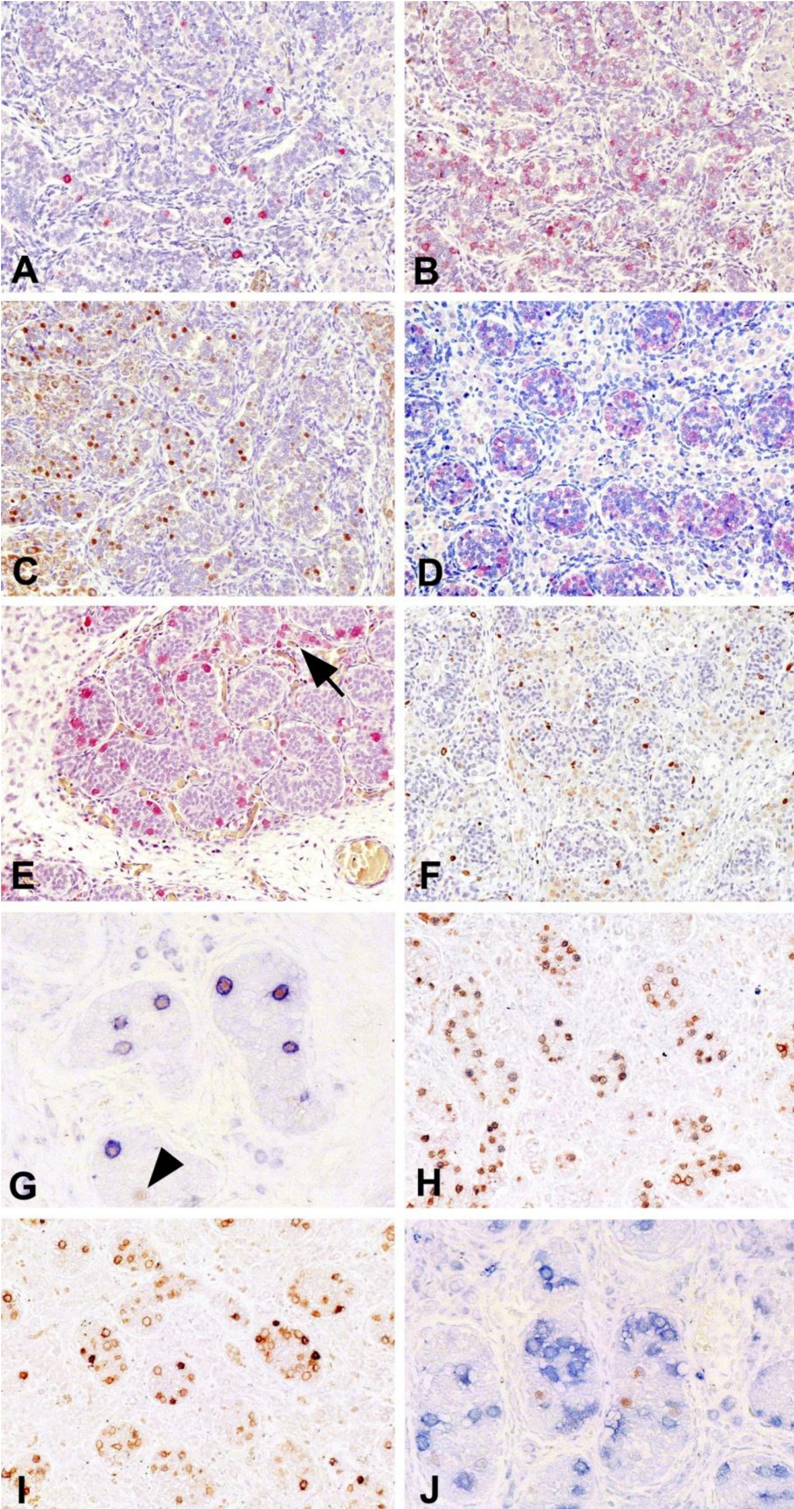
Results of double staining

Double staining was performed to assess correlations or differences in the presence of various markers in germ cells at different gestational ages. A total of seven cases at 15, 18, 21, 24, 27, 30, and 35 weeks' gestational age were stained for four different combinations: OCT3/4 and PLAP; OCT3/4 and c-KIT; PLAP and c-KIT; and OCT3/4 and VASA (Figures 1G–1J, respectively). The results of all double-staining experiments were in accordance with the single staining results. Staining for OCT3/4 and PLAP revealed that at all stages, a higher number of germ cells were positive for OCT3/4 than for PLAP. PLAP was never detected in germ cells negative for OCT3/4 and the presence of OCT3/4 was more frequently

observed at later stages of gestational development, when PLAP was almost undetectable. In contrast, OCT3/4 and c-KIT were found in comparable numbers of germ cells at earlier stages (second trimester), but c-KIT remained positive in more germ cells than OCT3/4 at later stages (third trimester). In agreement with these findings, double staining for PLAP and c-KIT showed that at all gestational ages more germ cells were positive for c-KIT than for PLAP. PLAP was never detected in germ cells negative for c-KIT, but due to an earlier loss of PLAP, the ratio of cells showing only c-KIT expression versus the number of cells positive for both factors increased from approximately 3 : 1 in the second trimester to approximately 9 : 1 during the third trimester.

An inverse correlation was found between the presence of OCT3/4 and the intensity of VASA. Gonocytes were positive for OCT3/4, but only weakly positive for VASA during the second trimester, whereas pre-spermatogonia staining strongly for VASA mostly lacked OCT3/4 (Figure 1J).

Figure 1. Results of the immunohistochemical analysis of different markers in fetal germ cells. **(A)** Fetal testis (15 weeks of development); PLAP (red signal) is seen in gonocytes and pre-spermatogonia. **(B)** Same case; c-KIT staining (red signal) is seen in a large number of gonocytes and pre-spermatogonia. **(C)** Same case; OCT3/4 (brown nuclear signal). **(D)** Same case; VASA (red signal). Note the variation in staining, with weaker signal intensity in gonocytes (more centrally in tubules) than in pre-spermatogonia (on the basal membrane of tubules). **(E)** Testis of a neonate (6 weeks); TSPY (red nuclear and cytoplasmic signal), mainly in pre-spermatogonia. Note also the nuclear and cytoplasmic staining of Leydig cells (arrow). **(F)** Fetal testis, 24 weeks: Ki-67 (brown nuclear signal) is seen both in tubules and in interstitial cells. (**G)** Fetal testis, 21 weeks: double staining for OCT3/4 (red nuclear signal) and PLAP (blue cytoplasmic signal). OCT3/4 and PLAP are co-expressed in the majority of immature germ cells, and occasional cells are positive for OCT3/4 but negative for PLAP (arrow-head). Note higher magnification. **(H)** Fetal testis, 15 weeks: double staining for OCT3/4 (blue nuclear signal) and c-KIT (red membranous signal). Note co-expression of OCT3/4 and c-KIT in the majority of germ cells. **(I)** Fetal testis, 15 weeks: double staining for PLAP (blue cytoplasmic signal) and c-KIT (red membranous signal). Note that co-expression of both markers results in a dark, almost black signal. The majority of germ cells express only c-KIT, whereas only a minority are positive for both markers. **(J)** Fetal testis, 21 weeks: double staining for OCT3/4 (red nuclear signal) and VASA (blue cytoplasmic signal). Germ cells showing a strong intensity for VASA are negative for OCT3/4 and are more often found on the basal lamina of the tubule. Note higher magnification.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Discussion

The aim of this study was to examine the differential presence of a number of factors known to be present in both human fetal germ cells and CIS/ITGCNU, the pre-invasive stage of TGCTs. In addition, we studied a number of factors associated with the proliferation and differentiation of germ cells, allowing us to correlate our findings with the fate of these cells during the second and third trimesters and the first weeks after birth.

Overall, the presence of all CIS/ITGCNU markers decreases during the second and third trimesters (see Figure 2, left panels). Nevertheless, with increasing age, expression was down-regulated differentially. This resulted in a specific expression pattern for each of these factors during fetal male germ cell development. We interpret these data in the context of a gradual process of maturation, extending into the first year of life, which is in accordance with earlier reports [17–20]. The results of the different double-staining experiments indicate that PLAP is the first factor that is down-regulated during the development of normal germ cells, followed by OCT3/4 and finally c-KIT. The fact that CIS/ITGCNU shows high expression of PLAP points towards an early origin of the precursor cell during intrauterine development. In the following paragraphs, the most interesting findings of the individual markers included in this study will be discussed in detail, predominantly in the context of understanding the pathobiological consequences of our findings.

c-KIT is a type III receptor tyrosine kinase, of which stem cell factor (SCF) is the ligand (see ref 21 for a review). The c-KIT/SCF system has been found to be involved in survival and proliferation of migrating germ cells in mice [22]. Our recent finding of the presence of activating mutations affecting one specific site of the receptor in the majority of bilateral TGCTs is in agreement with this model and supports activation of c-KIT as an early initiation event in the pathogenesis of TGCT [23]. Here we show a high presence of c-KIT at the early stages of germ cell development. Its expression generally declines with advancing age during the intrauterine period, but persists throughout the second and third trimesters and to a lesser extent after birth. This is in contrast to earlier reports, in which the authors concluded from a rapid decrease of c-KIT at 10–13 weeks that malignant transformation takes place early during fetal development, possibly even before week 10 [10]. However, more in line with the observation of this study, the detection of c-KIT in germ cells at later stages of development has been described in a recent report [24]. The discrepancies between the different studies might be due to differences in the sensitivity of the antibodies and the detection methods used.

OCT3/4 is a transcriptional regulator, exclusively found in pluripotent human and mouse embryonic stem cells and early germ cells (see refs 25–28 for a review). Recently, it has been described to be present in specific subtypes of TGCTs, including CIS/ITGCNU [27,29], in the context of which it is an informative diagnostic marker [27]. Here we show, in contrast to our earlier more limited study, that OCT3/4 can still be present during the first weeks after birth, albeit at very low frequency. In neonates, OCT3/4 is almost exclusively expressed in gonocyte-like cells located centrally in the tubules, and not in pre-spermatogonia.

Ki-67 is a nuclear antigen, which is studied in CIS/ITGCNU and TGCTs [30,31]. This antigen is informative in the assessment of proliferation, including germ cells in normal human testis, both during the fetal period and in newborns. Conflicting data on Ki-67 in germ cells at term have been reported [7,32]. To define further the population of proliferating germ cells during the intrauterine period, we assessed the expression of Ki-67 at different gestational ages. Throughout the second trimester, there was a decrease in the number of positive cells, which remained constant at a somewhat lower level at later ages. Interestingly, proliferation was seen in two different populations of germ cells at different time points. Ki-67 was predominantly seen in gonocytes until the end of the second trimester (ie around weeks 24–26). Thereafter, both basally and centrally located cells expressed Ki-67 in roughly equal numbers, suggesting that, in addition to gonocytes, the pool of more differentiated pre-spermatogonia also expands during the late second and third trimesters, extending into the first weeks after birth.

Human TSPY, the testis-specific protein, Y-encoded, gene, is organized as a repetitive gene family mapped to the critical region of the gonadoblastoma (GBY) [33–35] locus on the short arm of the Y chromosome [36,37]. It is mainly found during early spermatogenesis and has been suggested to play a normal role in spermatogonial proliferation and an oncogenic role in early germ cell tumourigenesis [38,39]. Expression of *TSPY* sequences has been found by RNA analysis in prenatal and adult testes [37,40,41]. To our knowledge, we describe for the first time the presence of TSPY protein in human germ cells during male gonadal development. Staining was often seen in groups of pre-spermatogonia throughout the second and third trimesters and in neonates. Although little is known about the function of this protein, a role in the regulation of the proliferation of germ cells is tempting, as has been suggested before [39].

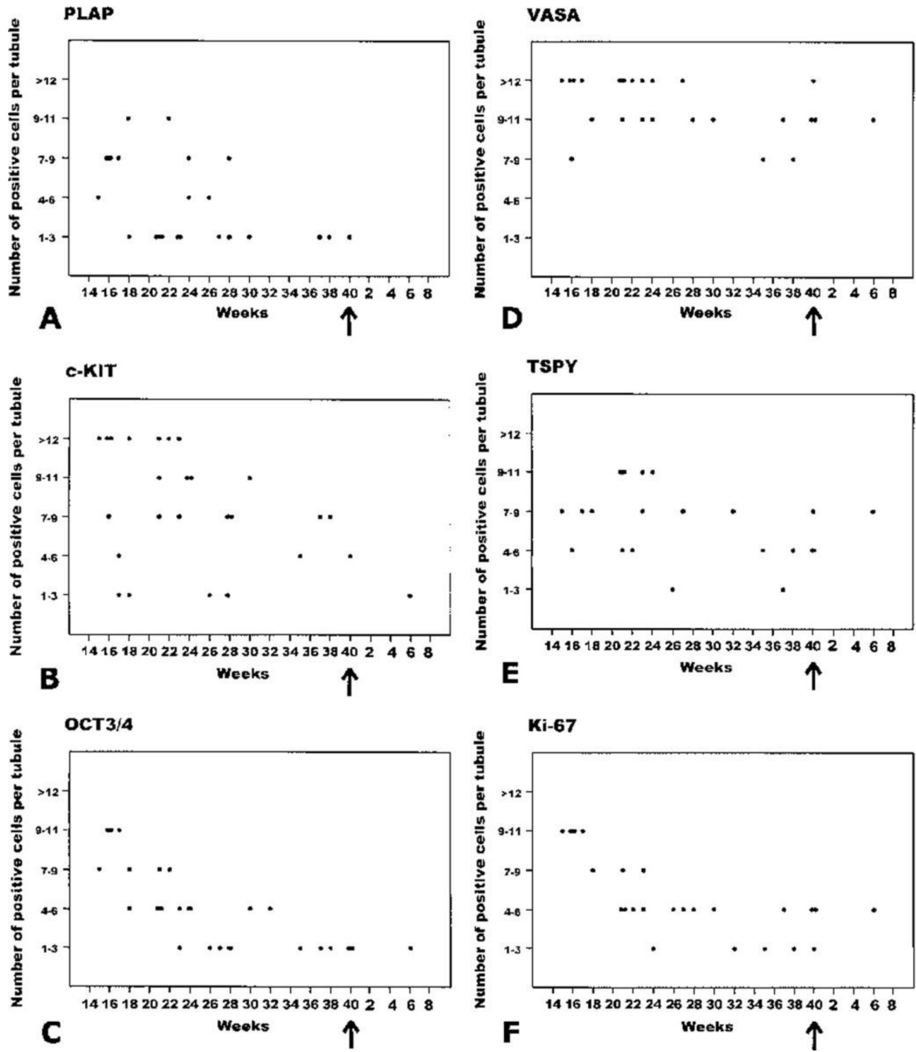

Figure 2. Absolute numbers of cells per tubule showing positive immunohistochemical staining for the markers at different gestational ages (weeks). The earliest case studied in this series was 15 weeks; the oldest was a neonate of 6 weeks. The arrow indicates the time of birth (median 40 ± 2 weeks). The left panels **(A–C)** show markers for CIS/ITGCNU; the right panels **(D–F)** illustrate factors associated with germ cell differentiation and proliferation. Each black spot represents one case

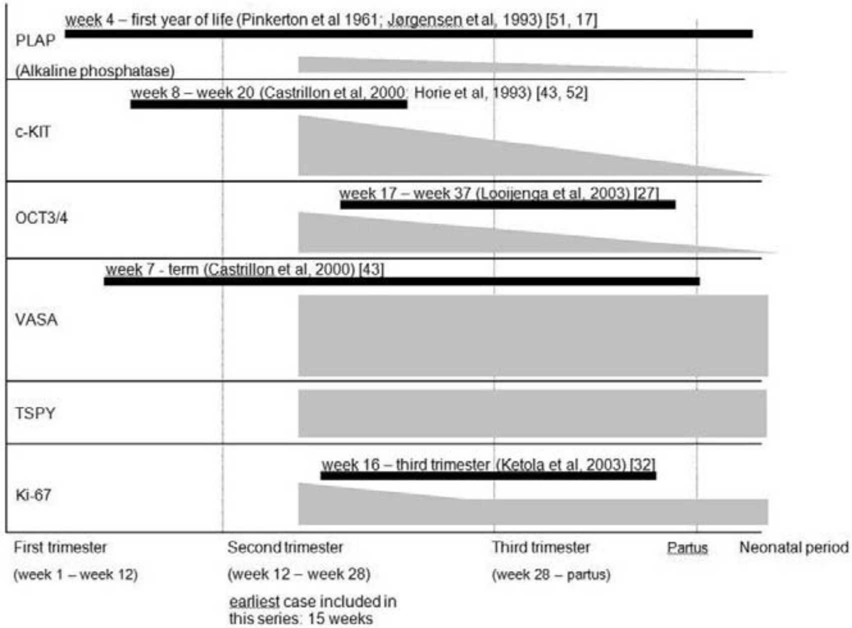

Figure 3. Overview of the data currently available from the literature on the expression of different antigens in germ cells during intrauterine development (black bars) compared with the findings of this study (grey bars). The sizes of the grey bars schematically represent the frequency of germ cells expressing the individual factor at different developmental ages.

In summary, our data indicate that during the second trimester, gonocytes are positive for a number of CIS/ITGCNU markers, such as OCT3/4, c-KIT, and PLAP. During the process of maturation towards pre-spermatogonia, these cellsgradually lose these early markers. During further development, there is a relative decrease in the number of gonocytes compared with more mature pre-spermatogonia. Interestingly, the time point at which more and more germ cells become attached to the basal lamina of the tubule (between weeks 20 and 24) coincides with an overall decrease in Ki-67 expression, down-regulation of the CIS/ITGCNU markers, and an increase in VASA staining intensity.

This suggests a preference for differentiation over proliferation at the transition from gonocytes to pre-spermatogonia during the second trimester and indicates that attachment to the basal membrane could be important for germ cell maturation. This model is supported by the results of the double-staining experiments for OCT3/4 and VASA, where gonocytes in the more central areas of developing tubules show expression of OCT3/4, but low staining intensity for VASA. Although dedifferentiation and consecutive re-expression of early markers cannot be ruled out as a mechanism for the development of CIS/ITGCNU, our data are in line with the model of a maturation arrest of immature germ cells as one of the first pathogenetic hits in the development of TGCTs.

Two observations from the study presented are particularly noteworthy. First, the presence of markers such as PLAP, c-KIT, and OCT3/4 is not restricted to the early stages of germ cell maturation, but extends well into the second and third trimesters and can, in fact, still be found in neonates. Therefore, in contrast to the situation in the testes of adolescents and adults, these markers can be unreliable for the detection of CIS/ITGCNU in very young children. This adds further evidence to the notion that testicular biopsy is of limited value in this age group [49,50]. Second, gonocytes that are positive for these markers at the later stages of normal development are hardly ever found on the basal membrane of the seminiferous tubules, but are localized more centrally. This distinguishes them from CIS/ITGCNU cells, which are always in contact with the basal membrane and phenotypically and ultrastructurally resemble pre-spermatogonia [11]. This indicates that CIS/ITGCNU cells, possibly due to a maturation arrest, show a certain marker profile that normal germ cells in this localization have already lost. However, while retaining features of immature germ cells, CIS/ITGCNU cells also show some potential to develop along the germ cell lineage, as is documented by the presence of VASA and TSPY in these cells.

Acknowledgements

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Chapter 4

Germ cell lineage differentiation in non-seminomatous germ cell tumours

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Abstract

Human germ cell tumours (GCTs) have long fascinated investigators for a number of reasons. Being pluripotential tumours, they can differentiate into both extra-embryonic and embryonic (somatic) tissues. However, it has never been shown convincingly that, in humans, these tumours are truly totipotent and can also give rise to the germ lineage, the third major differentiation lineage occurring early during embryonic life. Using a number of newly available, distinct, immunohistochemical markers, such as OCT3/4, VASA and TSPY, the occurrence of germ cells was investigated in a number of germ cell tumours. Development of germ cells was identified in three independent non-seminomas, including two pure yolk sac tumours and one mixed tumour composed of yolk sac tumour and immature teratoma. Our finding indicates a previously unknown totipotent potential of human GCTs and raises the question of whether, under certain culture conditions, primordial germ cells could be derived from human GCT cell lines.

Introduction

Germ cell tumours of adolescents and adults (GCTs) are at the cross-roads of tumour and developmental biology. They are pluripotent tumours with multiple possible fates. Two subgroups can be distinguished, seminomatous and non-seminomatous GCTs (for review, see [1]), and Figure 1 shows their developmental potential schematically. Seminomas resemble early germ cells (primordial germ cells/gonocytes). They show limited capacity to differentiate into somatic or extra-embryonic tissues, although they can switch to a non-seminomatous phenotype [2]. Non-seminomas mimic early embryonic development. Embryonal carcinoma (EC) cells, the stem cells of non-seminomas, are highly similar to embryonic stem (ES) cells [3] and can give rise to embryonic endo-, meso- and ectoderm and/or differentiate into extra-embryonal yolk sac and trophoblast. As they have never been shown to give rise to the germ line in humans, they seem to fall one step short of totipotency.

Recent studies show that germ cells can be derived from mouse and human ES cells [4–6]. So far, it has been impossible to identify the development of germ cells in human GCTs. Recently, antibodies against several markers present in early germ cells of both sexes (OCT3/4, a marker for pluripotency, which is also expressed in ES cells, and VASA, a germ line-specific protein) or exclusively in male germ cells (TSPY, the testis-specific protein, Y-encoded), have been described [7–9]. Our search for germ cell development in non-seminomas was significantly supported by recent studies on the presence of these proteins during normal testicular development [10].

Materials and methods

Research on human tumour samples has been performed according to the *Code for Proper Secondary Use of Human Tissue in The Netherlands*, as developed by the Dutch Federation of Medical Scientific Societies (FMWV) (version 2002), and has been approved by an institutional review board (MEC 02.981).

Formalin-fixed, paraffin-embedded tissue blocks from germ cell tumours that had been collected between 1991 and 2003, in close collaboration with urologists and pathologists in the south-western part of The Netherlands, were retrieved from our archive. In addition, tumour samples were obtained from patients treated according to protocols led by the Department of Hematology/Oncology, University of Tübingen. All cases were reviewed and diagnosed by J.W.O., according to the WHO classification.

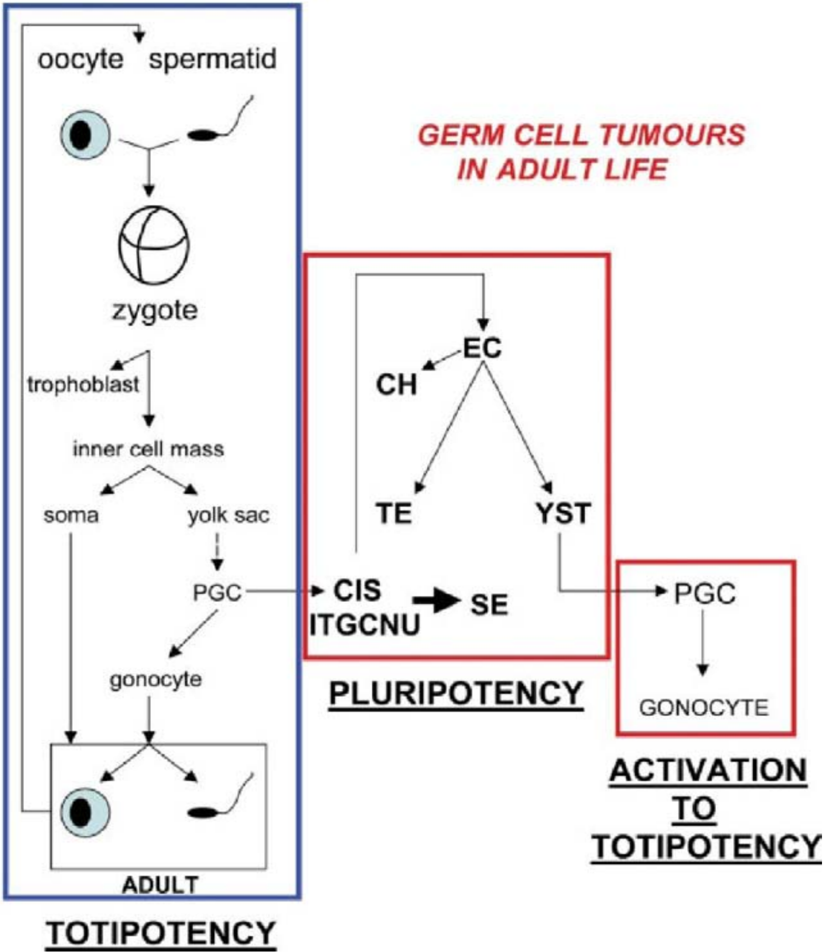

Figure 1. Developmental potential of normal and malignant germ cells.
Normal development after fertilization is depicted in the boxed area on the left. Both embryonic (soma) and extra-embryonic tissues (trophoblast and yolk sac) develop from the zygote. Cells from the proximal region of the epiblast contribute to the primordial germ cell (PGC) pool, the allantois and the extra-embryonic mesoderm. The fate of epiblast cells becoming PGCs is not predetermined, but is specified by response to localized signals. After puberty, the derivatives of PGCs, known as spermatogonia, will eventually give rise to mature germ cells that can be fertilized and restart the life cycle (oocyte in females and spermatid in males). Due to as-yet unknown pathogenetic hits during intrauterine life, developmentally arrested germ cells can give rise to germ cell tumours. In the post-pubertal testis, this is thought always to involve an intermediate stage, termed carcinoma *in situ*/intratubular germ cell neoplasia unclassified (CIS/ITGCNU). Seminomas resemble early germ cells and CIS/ITGCNU, whereas embryonal carcinoma (EC) cells that originate after reprogramming of CIS/ITGCNU are highly similar to embryonic stem cells from the inner cell mass and can mimic early intrauterine development after fertilization (see boxed area in the middle). EC can differentiate into choriocarcinoma (CH), teratoma (TE) or yolk sac tumour (YST). Here we describe for the first time that tumour cells from non-seminomas containing yolk sac histology can reactivate totipotency and show properties of immature and more mature germ cells (see boxed area to the right)

Histochemical and immunohistochemical staining

For immunohistochemistry, 3 µm sections were incubated with the primary antibody, followed by biotinylated secondary antibodies for 30 min and a biotinylated streptavidin horseradish peroxidase- or alkaline phosphatase coupled complex. The antibodies and conditions used are indicated in Table 1. All stains were counterstained with haematoxylin.

Table 1. Antibodies (source) and detection method used for immunohistochemistry

Antibody	Company	Code	Pre treatment	Dilution	Secondary antibody (1 : 200)(biotinylated) Code	Visualization
PLAP	Cell Marque	CMC203	HIAR*	1:200	Dako E0413	ABCplx-ap†
c-KIT	Dako	A4502	HIAR	1:500	Dako E0431	ABCplx-ap
OCT3/4	Santa Cruz	sc-8629	HIAR	1:1000	Vector BA9500	ABCplx-hrp‡
VASA	Provided by D Castrillon [43]		HIAR	1:2000	Dako E0431	ABCplx-ap
TSPY	Provided by Y Lau [39]		None	1:3000	Dako E0431	ABCplx-ap
BMP4	Novocastra	3H2	None	1:100	Dako E0431	ABCplx-ap
SCF	Santa Cruz	sc-1302	HIAR	1:400	Vector BA9500	ABCplx-ap
CD30	Dako	Ber-H2	HIAR	1:100	Dako E0413	ABCplx-hrp
CD34	Dako	Qbend/10 MS-363-S	None	1:20	Dako E0413	ABCplx-hrp
CD61	Immunotech	SZ21,2116	HIAR	1:300	Dako E0413	ABCplx-hrp
Glycophorin C	Dako	M0820	HIAR	1:100	Dako E0413	ABCplx-hrp
Lysozyme	Dako	A0099	Pronase	1:900	Dako E0431	ABCplx-hrp
Myeloperoxidase	Dako	A0398	HIAR	1:5000	Dako E0431	ABCplx-hrp

* Heat-induced antigen retrieval [42].
† ABCplx-ap, streptavidin-biotin- alkaline phosphatase complex.
‡ ABCplx-hrp streptavidin-biotin- horseradish peroxidase complex.

Double-staining was performed by using a combination of the same detection method but with different substrates: Fast Blue/Naphthol ASMX phosphate (F3378 and N500, Sigma, Steinheim, Germany) for blue staining and 3-amino-9-ethyl-carbazole (A5754 and D4254, Sigma, Steinheim, Germany)/H_2O_2 for red staining, without counterstaining. Endogenous peroxidase activity and/or endogenous biotin was blocked using 3% H_2O_2 (for 5 min) and/or a blocking kit for endogenous biotin (Vector Laboratories, Burlingame, CA, USA) to prevent background staining.

Results

Detection of cells with germ cell characteristics in non-seminomas

Markers for immature germ cells and other factors involved in fetal germ cell differentiation, e.g. germ cell/placental-like alkaline phosphatases (PLAP), stem cell factor receptor (c-KIT) and glycogen, were studied in a series of 34 GCTs of pure non-seminomatous histology (YSTs, TEs and ECs): in other words, none of these tumours contained a seminomatous component, as judged by morphology

and immunohistochemistry. In three cases, originating from the testis, the mediastinum and the pituitary gland, respectively, early germ cells were identified by morphology and marker expression (see Figure 2). In two cases, germ cells were found loosely distributed in clusters throughout the yolk sac component of the tumours (Figure 2A–E), whereas in the third case they were localized in tube-like structures (this tumour contained both yolk sac and immature teratoma components). The germ cells showed consistent staining for OCT3/4 (Figure 2C), PLAP (Figure 2D), c-KIT, TSPY (Figure 2E) and glycogen. CD30 was absent (Figure 2B insert), ruling out that these cells are EC cells rather than embryonic germ cells [11]. The presence of VASA (Figure 2F and lower insert), specific for late migratory and postmigratory germ cells [8], indicates that a number of these cells have progressed beyond the earliest stage of germ line commitment. While the majority of cells were positive for either OCT3/4 or VASA (Figure 2F), double staining revealed co-expression of both markers in only a minority of cells (lower insert), which was also the case for OCT3/4 and TSPY (Figure 2F, upper insert). The observation that the cells of interest are negative for OCT3/4 demonstrate that they are not seminoma cells.

Detection of cells with characteristics of early haematopoietic cells

Staining for c-KIT, CD34, lysozyme, myeloperoxidase, Glycophorin C and CD61 showed the presence of cells with characteristics of early haematopoiesis. In two cases, nests of cells resembling angiogenetic clusters (so-called haemangioblasts) could be identified by the presence of c-KIT, CD34 (Figure 2G insert), a marker for pluripotent haematopoietic stem cells, and glycophorin C, a marker for red cells and their precursors (Figure 2H insert). A number of other factors for haematopoietic differentiation (ie lysozyme, myeloperoxidase and CD61) were occasionally present. However, the cells lacked the morphological characteristics of haematopoietic differentiation, such as blast formation (data not shown).

All three cases showed the presence of bone morphogenetic protein 4 (BMP4) in the YST component (Figure 2G), mainly in areas showing hepatoid histology. BMP4 was not restricted to the areas containing germ cells. Moreover, BMP4 was also observed in non-seminomas (ie yolk sac tumours) without the presence of germ cell lineage differentiation (data not shown). Therefore it cannot be considered as a specific marker. Stem cell factor (SCF), the ligand for c-KIT, was found in the yolk sac component, most often in close association with the developing germ cells (Figure 2H).

Discussion

An extensive immunohistochemical analysis was performed to study the developmental potential and cell fate commitment of non-seminomas. We describe the novel finding of germ cell development in non-seminomas. In three cases, small groups of cells showing characteristics of fetal germ cell differentiation, ie loss of OCT3/4 and increased staining for VASA and TSPY, were found. The same pattern of OCT3/4 and VASA expression was recently described upon spontaneous differentiation of germ cells from human ES cells *in vitro* [6] and during normal germ cell development in humans [10]. Our findings indicate that germ cells can develop in non-seminomas, in particular YSTs showing expression of supporting factors, such as BMP4 and SCF. It is unknown whether the YST is the direct precursor of the germ cells or whether both lines co-develop from one common ancestor. This seems a rather semantic question; however, as all non-seminomas originate from a common stem cell, ie the EC cell (for review, see [1]; see also Figure 1).

Mammalian germ cells originate from the proximal region of the epiblast. In mouse experiments, ES cells can be stimulated to differentiate into germ cells and extra-embryonic mesoderm when grafted to the proximal epiblast [12]. This indicates that the potential to become a germ cell may not be restricted to predetermined progenitor cells but can be induced by extracellular factors [13]. The nature of these factors is largely unknown. Recent findings indicate that BMP4 might play a crucial role [4].

In two of the three cases, haematopoietic differentiation was found, underlining the close developmental relationship between germ cells and haematopoietic precursor cells. In fact, it has been hypothesized that primordial germ cells could be haematopoiesis-initiating cells [14], a process in which BMP4 could be an important signal [4,15]. Clinically, the relationship between mediastinal GCTs with yolk sac histology and acute myeloid leukemias is well established [16,17]. The haematopoietic precursor cells in these tumours are likely the origin of the leukaemias [18]. The clonal origin of mediastinal GCTs and haematological malignancies indicate a common malignant stem cell [19].

Failure to demonstrate germ cell development in non-seminomas so far might have been due to a number of reasons. It is probably a rare event (so far found in 3/34 non-seminomas), possibly due to the absence of crucial factors.

Alternatively, development of germ cells may be hampered by the fact that these cells are highly susceptible to apoptotic stimuli, leading to early death. Most important, however, could be the fact that so far the markers to identify germ lineage differentiation were lacking. Only the use of a combination of the

markers OCT3/4, VASA and TSPY has enabled us to identify germ cell development in non-seminomas. It is highly unlikely that the presence of these markers is due to aberrant expression, because of the consistency in loss of OCT3/4 and increase in VASA, as found during normal germ cell development. In addition, GCTs are known to show expression of various markers in accordance with their physiological pattern, dependent on their specific lineage of differentiation (for review, see [1]).

The fact that non-seminoma cells can differentiate into germ cells is exciting and should be exploited *in vitro* using pluripotent GCT cell lines, under culture conditions that enhance formation of germ cells [4,6,20]. The resulting germ cells could be traced using a specific reporter system [4,5] and studied for their expression profile [6]. Deriving germ cells from human GCTs would be more than a mere technical exercise. Even though the cells of origin show neoplastic properties (eg they are aneuploid), the system would clearly reflect many aspects of normal germ cell development. It could therefore provide a readily accessible tool for the investigation of mechanisms of human germ cell development, yet avoiding the ethical issues and legal restrictions associated with the use of normal primordial germ cells derived from human embryos or human ES cells.

Figure 2. Representative illustrations of the various immunohistochemical markers identified in the germ cell tumours showing germ cell differentiation. (A) Testicular yolk sac tumour, haematoxylin and eosin staining (magnification ×10; insert ×40). **(B)** Same case: α-fetoprotein (AFP) staining is seen as a red cytoplasmic signal. Note the stronger staining intensity in more solid yolk sac tumour areas, showing a hepatoid pattern (magnification ×10). Insert is staining for CD30, being negative (magnification $\times$20). **(C)** Same case: OCT3/4 (brown nuclear signal)-positive cells are scattered throughout the tumour (magnification ×10; insert ×40). **(D)** Same case: single cells show a red positive cytoplasmatic staining reaction for placental alkaline phosphatase (PLAP) (magnification ×10; insert ×40). **(E)** Same case: single cells show a red positive nuclear and cytoplasmic staining reaction for testis-specific protein on the Y chromosome (TSPY). Note also the weaker staining intensity in more solid yolk sac tumour areas, showing a hepatoid pattern (magnification ×10; insert 40×). **(F)** Same case: double staining for OCT3/4 (red nuclear signal) and VASA (blue cytoplasmic signal). Immature germ cells show strong signal intensity for OCT3/4, but are negative for VASA. This germ cell-specific marker is exclusively seen in more mature germ cells. The VASA-positive cells are negative for OCT3/4, and are therefore not seminoma cells (magnification ×40). Upper insert: double-staining for OCT3/4 (red nuclear signal) and TSPY (blue cytoplasmic signal). Note the double positive cells, and the single OCT3/4-positive cells (magnification ×40). Lower insert: double-staining for OCT3/4 (red nuclear signal) and VASA (blue cytoplasmic signal), showing some double positive cells for OCT3/4 and VASA. **(G)** Mediastinal yolk sac tumour, bone morphogenetic protein 4 (BMP4) staining (red cytoplasmic signal). Note the stronger staining intensity in more solid yolk sac tumour areas (magnification ×10). Insert: same case staining for CD34 (magnification $\times$20). **(H)** Testicular yolk sac tumour, same case as shown in A–F. Stem cell factor (SCF) is seen as a red cytoplasmic and membranous signal in the majority of yolk sac tumour cells (magnification ×10). Insert: same case, staining for glycophorin C (magnification ×40)

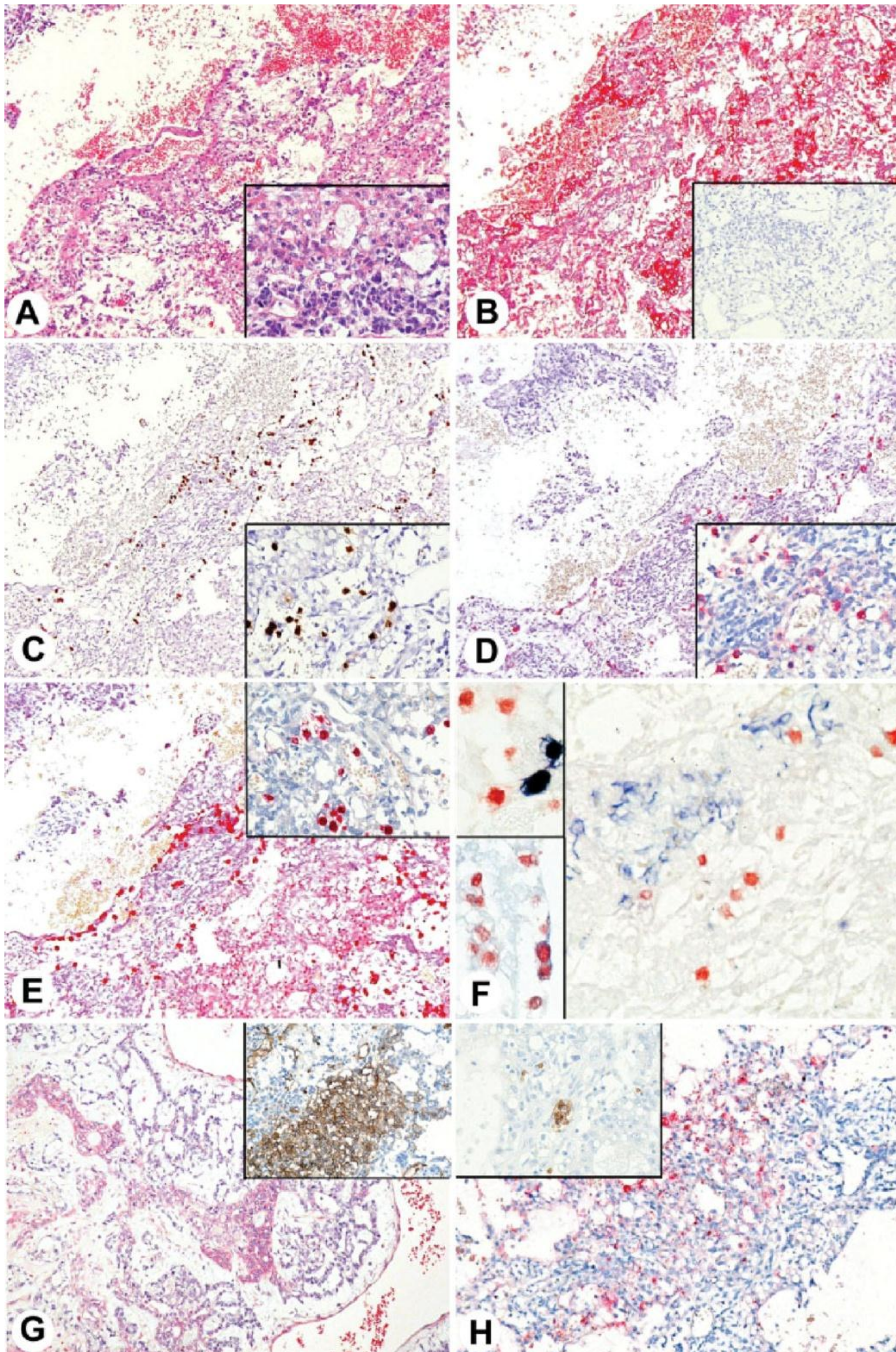

Figure 2.

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Chapter 5

POU5F1 (OCT3/4) identifies cells with pluripotent potential in human germ cell tumors

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Abstract

Human germ cell tumors (GCTs) may have variable histology and clinical behavior, depending on factors such as sex of the patient, age at clinical diagnosis, and anatomical site of the tumor. Some types of GCT, i.e., the seminomas/germinomas/dysgerminomas and embryonal carcinomas (the stem cell component of nonseminomas), have pluripotent potential, which is demonstrated by their capacity to differentiate into somatic and/or extraembryonic elements. Although embryonal carcinoma cells are intrinsically pluripotent, seminoma/germinoma/dysgerminoma cells, as well as their precursor *carcinoma in situ*/gonadoblastoma cells, have the phenotype of early germ cells that can be activated to pluripotency. The other types of GCT (teratomas and yolk sac tumors of infants and newborn, dermoid cyst of the ovary, and spermatocytic seminoma of elderly) are composed of (fully) differentiated tissues and lack the appearance of undifferentiated and pluripotent stem cells. OCT3/4, a transcription factor also known as OTF3 and POU5F1, is involved in regulation of pluripotency during normal development and is detectable in embryonic stem and germ cells. We analyzed the presence of POU5F1 in GCT and other tumor types using immunohistochemistry. The protein was consistently detected in carcinoma *in situ*/gonadoblastoma, seminomas/germinoma/dysgerminoma, and embryonal carcinoma but not in the various types of differentiated nonseminomas. Multitumor tissue microarray analysis covering >100 different tumor categories and 3600 individual cancers verified that POU5F1 expression is specific for particular subtypes of GCT of adults. No protein was observed in GCT of newborn and infants, spermatocytic seminomas, and the various tumors of nongerm cell origin. In addition, no difference in staining pattern was found in chemosensitive and chemoresistant GCT of adults. These results indicate preservation of the link between POU5F1 and pluripotency, as reported during normal development, after malignant transformation. Therefore, POU5F1 immunohistochemistry is an informative diagnostic tool for pluripotent GCT and offers new insights into the histological heterogeneity of this cancer.

Introduction

oct3/4, also known as *otf3* or *pou5f1*, is a member of the POU family of transcription factors, which is expressed in pluripotent mouse and human embryonic stem and germ cells, including PGCs (1– 6). Expression of this gene is downregulated during differentiation (7). Furthermore, knocking out the *pou5f1* gene in mice causes early lethality because of lack of inner cell mass formation (8) because pou5f1 is critical for self-renewal of embryonic stem cells (9). Interestingly, pou5f1 has been linked to the capacity of proper outgrowth of somatic cell clones (10). During human development, expression of *POU5F1* is found at least until the blastocyst stage (11) in which it is involved in gene expression regulation. The protein activates transcription via octamer motifs located distally or proximally from transcriptional start sites (12). POU5F1 binding sites have been identified in various genes, including *fibroblast growth factor 4* and the 1.5-kb alternative promoter of the *platelet-derived growth factor* α receptor (13). The data indicate that pou5f1/POU5F1 functions as a master switch in differentiation by regulating cells that have, or can develop, pluripotent potential.

We have previously demonstrated that *POU5F1* transcripts are found in a specific set of human testicular GCT of adolescents and young adults (TGCT): the seminomas and embryonal carcinomas (14). In addition, the precursor lesions of TGCT, known as CIS (15), also express *POU5F1* (14). These lesions are composed of cells that are considered to be the malignant counterpart of an embryonic germ cell, most likely a PGC (15–17). Interestingly, these cell types are in principle pluripotent or even multipotent (Ref. 18, for review). In contrast, no expression was found in the differentiated components of nonseminomas, i.e., teratomas, yolk sac tumors, and choriocarcinomas (14). Indeed, expression of *POU5F1* has been reported in embryonal carcinoma cells lines, and downregulation of expression is found upon differentiation (4, 13).

In contrast to our finding of a specific expression pattern of *POU5F1* in TGCT, expression of this gene has recently been reported in nonmalignant adult human tissues (19), as well as in a number of carcinoma cell lines (20). This latter finding was interpreted as the result of aberrant reactivation of embryonic genes during the process of malignant transformation. However, the conclusion that *POU5F1* is expressed in these cells was based solely on reverse transcription PCR results, which can be misleading because of the presence of multiple *POU5F1* pseudogenes (Refs. 14, 19, 21, own unpublished observations). We are not aware of any previous studies reporting analysis of POU5F1 protein expression in normal and malignant human tissues to clarify whether the mRNA is translated to functional POU5F1 protein. Therefore, an extensive immunohistochemical screening for POU5F1 protein expression was done in various types of GCT at

different sites and in a set of >3600 tumors of >100 different types using multitumor tissue microarrays. POU5F1 immunoreactivity was detected only in cells of CIS/gonadoblastoma, seminoma/ germinoma/dysgerminoma, and embryonal carcinoma. These results convincingly demonstrate that the presence of POU5F1 protein is related to pluripotent capacity of human GCT and that reactivation of its expression is not a common mechanism in cancer. Conclusively, POU5F1 is a distinctive immunohistochemical marker to identify tumor cells resembling embryonic/primordial germ and embryonic stem cells.

Materials and methods

Sample Handling and Characterization

The (T)GCT not included in the tissue arrays were collected in the southwestern part of the Netherlands in collaboration with urologists and pathologists. Representative parts of the tumor (and adjacent tissue, if available) were snap frozen in liquid nitrogen and were fixed in 10% formalin for paraffin embedding. Tumors were diagnosed according to the WHO classification (22), as previously described (23), supported by immunohistochemistry using antibodies directed against germ cell/ placental alkaline phosphatase, α-foeto protein, human chorionic gonadotropin, the stem cell factor receptor c-KIT, and cytokeratin (CAM5.2). The testicular tumors included 35 seminomas, 50 nonseminomas [with 14 embryonal carcinoma components, 21 teratoma components (6 mature, 5 immature, and 10 mixed), 18 yolk sac tumor components, and 5 choriocarcinoma components], and 10 spermatocytic seminomas. In addition, CIS containing testicular parenchyma (n = 16, including both adjacent to seminoma and nonseminoma) and embryonic testes of different developmental stages, *i.e.*, from 17 to 40 weeks and 28 weeks postpartum, were included. These latter samples have been reported before (24). Moreover, 3 gonadoblastomas, found in dysgenetic gonads, of which 2 also contained dysgerminoma, as well as 4 GCT of the midline of the brain of adults (including 1 germinoma, 1 embryonal carcinoma, and 2 mixed differentiated nonseminomas) were included. To investigate possible difference in presence of POU5F1 between chemotherapy-sensitive and resistant GCT, a series of 34 patients with known clinical course, including 12 high-risk patients that were relapse-free after high-dose chemotherapy and 22 refractory cases, were investigated. Part of this series has been reported before (25, 26). The clinical parameters are indicated in Table 1. Patients were considered refractory when progression or relapse occurred despite adequate initial and salvage treatment, including high-dose chemotherapy with autologous stem-cell transplantation.

Table 1. Clinical parameters of the patients with chemosensitive and chemoresistant GCT

	Sensitive	Resistant
Nos. of cases	12	22
Median age in years (range)	28 (20–47)	29 (17–56)
Histology[a]		
Seminoma	1	1
Nonseminoma	11	21
Stage at diagnosis (UICC[b])		
I	0	3
II	0	7
III	12	12
Initial treatment after surgery		
Surveillance	0	2
Chemotherapy	12	20
Follow-up (months)		
Median (range)	51 (14–69)	39 (11–180)
Relapse free survival (months)		
Median (range)	NR	7.1 (0–150)
Response to initial treatment		
Complete remission	8	5
Partial remission, marker -	4	8
Partial remission, marker +	0	3
Progressive disease	0	3
Unknown	0	3
No. of salvage regiments		
Median (range)	0	3 (1–9)

[a] For the POU5F1 immunostaining all seminoma, embryonal carcinoma, and CIS components of both the sensitive and resistant tumors (3 and 2; 6 and 8; and 4 and 6, respectively) are positive, whereas all differentiated nonseminoma components are negative.
[b] UICC, International Union Against Cancer; NR, not reached.

To expand the series of both GCT and non-GCT, three different multitumor tissue microarrays were investigated. One was generated at the Department of Pathology of the University of Basel (Basel, Switzerland), and included 3273 interpretable individual tumors of >100 different types, represented on seven different slides (Table 2 and Fig. 2). The second was generated at the Division of Pediatric Pathology of the Johns Hopkins Medical Institution (Baltimore, MD) and included 84 (T)GCT of newborn, infants, and adolescents (Table 3), represented on four different slides. Both arrays contained various positive and negative controls. The third array, generated at the Department of Pathology, Erasmus Medical Center (GE Rotterdam, the Netherlands), included 48 esophagus tumors and 100 prostate cancers (50 progressing and 50 nonprogressing), as well as benign prostatic hypertrophy (n = 18). The result of this latter array is included in Table 1.

Immunohistochemistry

Immunohistochemistry with anti-POU5F1 antibodies was performed on paraffin-embedded tissue sections of 3-μm thickness. The sections were incubated overnight at 4°C with a polyclonal goat anti-POU5F1 antibody (C20, sc-8629; Santa Cruz Biotechnology, Santa Cruz, CA), directed toward the COOH terminus of the protein, diluted 1:8000 (final concentration, 0.025 μg/ml). Subsequently, a biotinylated horse anti-goat secondary antibody was applied to the sections, and the bound antibody complex was visualized using the horseradish peroxidase avidin-biotin complex method. Double fluorescence staining was performed using the polyclonal goat-anti-POU5Fl antibody and a monoclonal mouse-anti-c-KIT antibody (final concentration 2 μg/ml; Neomarkers, Fremont, CA) on frozen tissue sections. c-KIT is a known marker for CIS and seminomas (27). Secondary antibodies were labeled with FITC and CY3 (Dako Diagnostics, Glostrup, Denmark, and Jackson Immunoresearch, West Grove, PA, with a final concentration of 5 μg/ml), respectively. The stainings were also performed separately. Every experiment was accompanied by appropriate positive and negative (without primary antibody) controls.

Cell Culture

The human cell lines Tera2 (28), 2102Ep (29), and NCCIT (30), all nonseminoma derived, were cultured and split under conventional conditions (37°C, 5% CO_2). Cell lines were cultured until 80% confluency, and differentiation was induced with retinoic acid as described previously (30). Immunohistochemistry was performed on cytospin preparations as described above.

Results

POU5F1 Immunohistochemistry on TGCT

On the basis of mRNA analysis (14), we expected that POU5F1 protein is present in specific different histological subtypes of TGCT. We first analyzed the presence of POU5F1 by immunohistochemistry on a series of TGCT of various histological types. Representative images of representative stainings are shown in Fig. 1. All tumor cells of seminomas and embryonal carcinomas showed a nuclear staining (Fig. 1, A and B), whereas all nontumor cells were negative. A similar pattern of staining was found in the gonadoblastomas, dysgerminomas and germinomas, and embryonal carcinomas of the brain (see also below). In contrast, all teratomas, both mature and immature, choriocarcinomas, and yolk sac tumors were negative (Fig. 1, C–E), as were the differentiated nonseminomas of the brain (data not shown). The staining intensity in the positive cases varied between moderate and high. None of the spermatocytic seminomas showed a positive staining (Fig. 1F). We also tested a series of CIS containing testicular parenchyma

samples, both adjacent to seminoma and nonseminoma. All CIS cells, identified by a double staining for c-KIT, irrespective of the histology of the invasive tumor, were positive for POU5F1 (Fig. 1G and inset). In contrast, no protein could be detected in any stage of spermatogenesis (see below). This makes POU5F1 one of the most informative immunohistochemical markers to identify CIS cells that our laboratory has tested.

Table 2. Results of tissue array immunohistochemistry of 3439 individual tumors of >100 different types for POU5F1
The organ (in alphabetic order), tumor type, number of cases studied, and positive findings are indicated

Organ	Tumor	Subgroup	*n*	Positive
Adrenal gland	Carcinoma	Adeno-	19	0
Anus	Carcinoma	Squamous	3	0
Brain		Gliobl. multif./astrocytoma/	46/35	0
		oligodendroglioma/meningioma/	24/46	0
		PNET/ependymoma/	17/9	0
		gangioneuroma/	7	0
		medulloblastoma/estioneuroblastoma/	4/2	0
		opticus glioma/cranioph.[a]	1/8	0
Carotid body	Glomus tumor		11	0
Cervix	Carcinoma	Squamous/CIS/adeno	37/7/4	0
Colon	Adenoma	Tubular and villous	134	0
	Carcinoma	Adeno-	47	0
Endometrium	Carcinoma	Adeno-	61	0
	Sarcoma	Endometrial stromal sarcoma	3	0
Esophagus	Carcinoma	Squamous/adeno	21/116	0
Gall bladder	Carcinoma	Adeno-	23	0
Glomus tumor			11	0
Kidney	Carcinoma	Clear cell/papillary	50/45	1/0
		Chromophobic	13	0
		Oncocytoma	7	0
Larynx	Carcinioma	Squamous	46	0
Liver	Carcinoma	Hepatocellular	41	0
Lung	Carcinoma	Adeno-/squamous/	48/50	0/1
		Large cell/small cell	47/41	1/0
Lymph nodes	Lymphoma	Hodgkin's	50	0
		Non-Hodgkin's high/low grade	69	0
		MALT	42	0
Breast	Carcinoma	Lobular/ductal	42/46	0
		Med./muc./tub.	27/23/26	0
		Cribriform/apocrine	5/3	0
		Phylloidees tumor	13	0
Mouth	Carcinoma	Squamous	57	0
Ovary	Carcinoma	Endometroid/serous mucinous	49/48/20	0
		Brenner/mixed Müllerian tumor	9/6	0
		Granulosa cell	7	0
		Dysgerminoma	2	2
		Gonadoblastoma	1	1
Pancreas	Carcinoma	Adeno-	47	0
Paraganglia	Paraganglioma		10	0
Parathyroid	Carcinoma	Adeno-	20	0
Parotid gland	Carcinoma	Mixed	15	0
		Acinic cell/mucoepithelial	5/6	0

Table 2: *continued*

Organ	Tumor	Subgroup	*n*	Positive
		Phaeochrom.	26	0
Penis	Carcinoma	Squamous	43	0
Prostate	Carcinoma	Adeno-, horm. (non-)refract.	200	0
		PIN/BPH	24/18	0
Salivary gland	Pleom. adenoma		49	0
	Lymphoep. lesion		5	0
	Carcinoma	Adenoid cystic	43	0
		Acinic cell	36	0
	Adenolymph.		30	0
Skin	Carcinoma	Squamous/basal	81/37	0
	Melanoma		44	0
	Merkel cell tumor		6	0
	Benign	Hemangioblastoma	8	0
		Nevus	38	0
		Appendix tumors	25	0
(Small) intestine	Carcinoma	Adeno-	17	0
	GIST		14	0
Stomach	Carcinoma	Intestinal/diffuse	44/23	0
Soft tissue	Benign	Lipoma/fibrous histiocytoma/Schwann/	15/58/35	0
		neurofibroma/leiomyoblastoma/	31/7	0
		giant cell tumors of tendon sheet/	29/	0
		hemangioma	17	0
	Intermediate	Haemangiopericytoma	8	0
		Dermatofibrosarcoma protuberans	2	0
	Malignant	Kaposi/liposarcoma	25/26	0
		Angiomyolipo./malign. Schwann.	1/8	0
		Rhabdomyosarcoma/synovial sarcoma	13/3	0
		Angios./fibros./alv. soft part sarc.	6-4-2001	0
		Epithelioid sarcoma	2	0
Testis		Seminoma/nonseminoma	49/47	49/33[b]
		Adenomatoid tumor	10	0
Thyroid	Carcinoma	Foll./papill./med.	42/48/36	0
Thymus	Thymoma		23	0
Urinary bladder	Carcinoma	TCC	101	0
		Small cell/squamous/adeno-	5-3-2004	0
	Sarcoma		7	0
Uterus	Myoma		107	0
Vagina	Carcinoma	Squamous	5	0
Vulva	Carcinoma	Squamous	37	0
Various	Carcinoid		48	0
	Adenomatoid tumor		10	0
	Mesothelioma		23	0
Controls [c]			30	0

[a] Gliob. multif., glioblastoma multiforme; PNET, primitive neuro-ectodermal tumor; cranioph, craniopharyngioma; CIS, carcinoma in situ; MALT, mucosa associated lymphoid tissue; med, medullar; muc, mucinous; tub, tubular; horm (non-)refract, hormonal (non)refractory; angiomyolipo., angiomyoliposarcoma; malign. Schwann., malignant Schwannoma; anglo., angiosarcoma; fibros., fibrosarcoma; alv. soft part sarc., alveolar soft part sarcoma; foll., follical.

[b] If containing seminoma in case of combined tumors or embryonal carcinoma elements.

[c] Included in all seven slides: skin ($n = 4$); lymph node ($n = 2$); heart ($n = 2$); skeletal muscle ($n = 2$); smooth muscle ($n = 2$); myometrium ($n = 2$); thyroid gland ($n = 2$); liver ($n = 2$); kidney cortex ($n = 2$); prostate ($n = 2$); testis ($n = 2$); submand. gland ($n = 2$); proliferative endometrium ($n = 2$); gray matter cerebrum ($n = 2$).

To investigate whether a difference in presence of POU5F1 exists between chemosensitive and chemoresistant GCT, immunohistochemistry was done on a well-characterized series of GCT with known clinical outcome (Refs. 25, 26 and Table 2). The results indicate that the staining pattern is determined by histology, i.e., positive in CIS, seminoma and nonseminoma, irrespective of sensitivity to chemotherapy.

Table 3. Results of the multitissue microarray immunohistochemistry on 84 germ cell tumors of predominantly newborn, infants, and adolescents for POU5F1
The histology and anatomical localization of the tumor, sex of the patient, number of studied cases (n), age at clinical presentation and number of positive findings are indicated.

Histology	Sex[a]	Anat. Local.	*n*	Age (yr)	Positive
Teratoma		*Total*:	25		0
Mature	M	Sacral	2	0/1	0
	F	"	2	0/0	0
	M	Nervous system	1	6	0
	F	Gonad	2	15/34	0
	M	"	1	0,3	0
Immature	F	Sacral	3	0/0/0	0
	M	"	1	0,3	0
	F	Gonad	11	5/6/8/10/10/11/ 12/12/13/15/?[b]	0
	M	"	1	0,8	0
	?	?	1	?	0
Yolk sac tumor		*Total:*	33		
	F	Sacral	6	0.2/1/1/2/2.6/5	0
	M	"	1	1,5	0
	?	"	6	0/0.5/?/?/?/?	0
	M	Gonad	10	0.7/0.7/1.3/1.3/ 1.9/2/2/2.8/3/4	0
	F	"	9	7/8/9/10/10/13/ 18/20/24	0
Gonadoblastoma/ Dysgerminoma	D	Gonad	1	?	1
Dysgerminoma	F	Gonad	11	12/12/12/17/18/ 14/14/?/?/?/?	1
Dysgerminoma/ Nonseminoma	F	Gonad	1	12	1
Seminoma	M	Gonad	4	27/30/41/?	4
Mixed GCT[c]	M	Gonad	4	27/28/29/?	4[d]
Nonseminoma	M	Gonad	5	23/?/?/?	5[e]
Controls*f*	M/F		30		0

a M, male; F, female; ?, unknown; D, dysgenetic gonad.
b ?, unknown.
c Germ cell tumor.
d When containing either a seminoma of embryonal carcinoma component.
e When containing an embryonal carcinoma component.
f POC (n = 9); fetal kidney (n = 4), fetal ovary (n = 1), fetal uterus (n = 2), Wilms' tumor (n = 2), hepatoblastoma (n = 3), embryonal neuroblastoma (n = 5), testis (n = 3), MMMT (n = 1).

Immunohistochemistry for POU5F1 in Normal Spermatogenesis and Embryonic Germ Cells.

Although POU5F1 could be detected by immunohistochemistry in CIS cells and cells of seminoma and embryonal carcinoma (see above), no staining was observed in seminiferous tubules containing normal spermatogenesis (Fig. 1G), neither on frozen nor formalin-fixed, paraffin-embedded tissue sections. In contrast, embryonic germ cells in embryos and fetuses were occasionally positive. The highest number and most intensely stained germ cells were found in gonads between week 17 and 24 of gestational age (Fig. 1H and see below). Earlier stages of development were not available for investigation. Only sporadic positive cells were identified in the samples obtained from developmental stages weeks 28, 35 and 37, whereas no staining was found in samples of 38 and 40 weeks gestational age and after birth. Germ cells are present in these samples, as identified by staining for various markers, including c-KIT and VASA (24, 31). These data indicate that POU5F1 is present in germ cells at least from the 17th to 37th week of gestational age, after which the protein is no longer detectable by immunohistochemistry.

POU5F1 in Undifferentiated and Differentiated Cells of TGCT-derived Cell Lines

In the nonseminomatous TGCT-derived cell line Tera2, expression of the POU5F1 is only observed in undifferentiated cells, whereas induction of differentiation of embryonal carcinoma cells with retinoic acid results in down-regulation of *POU5F1* expression. The potential of Tera2 cells to differentiate has been described before (4, 14). Here, we confirm this finding on the protein level by immunohistochemistry (Fig. 1*I, left and right panel*). In contrast, both the cell lines NCCIT and 2102Ep showed *POU5F1* expression and protein before and induction with retinoic acid differentiation (data not shown). This is in accordance to the finding that these cell lines, in contrast to Tera2, do not show complete differentiation after exposure to retinoic acid (28, 30).

POU5F1 Immunohistochemistry on Multitumor Tissue Microarrays

To investigate whether POU5F1 protein can be detected in other tumors than GCT, we performed immunohistochemistry using the same antibody on three different multitumor tissue microarrays. One contained a large series of tumors of different origin (Table 1), one of the other contained specifically prostate and esophageal carcinomas (of which the results are also included in Table 1), and one contained mainly GCT of newborn and infants (Table 3). None of the control tissues, both adult and embryonal (with one exception, see below), included in the arrays showed positive staining for POU5F1. Of the 3439 tumors (from the first arrays), all testicular seminomas and nonseminomas (containing either a

seminoma or an embryonal carcinoma component), as well as two dysgerminomas and one gonadoblastoma were positive, confirming the results presented above. Representative examples of the first arrays are shown in Fig. 2. In addition, one clear cell carcinoma of the kidney and two lung carcinomas (one squamous cell carcinoma and one undifferentiated large cell carcinoma) were positive, suggesting that POU5F1 protein can be found in a small percentage (1–2%) of other tumors.

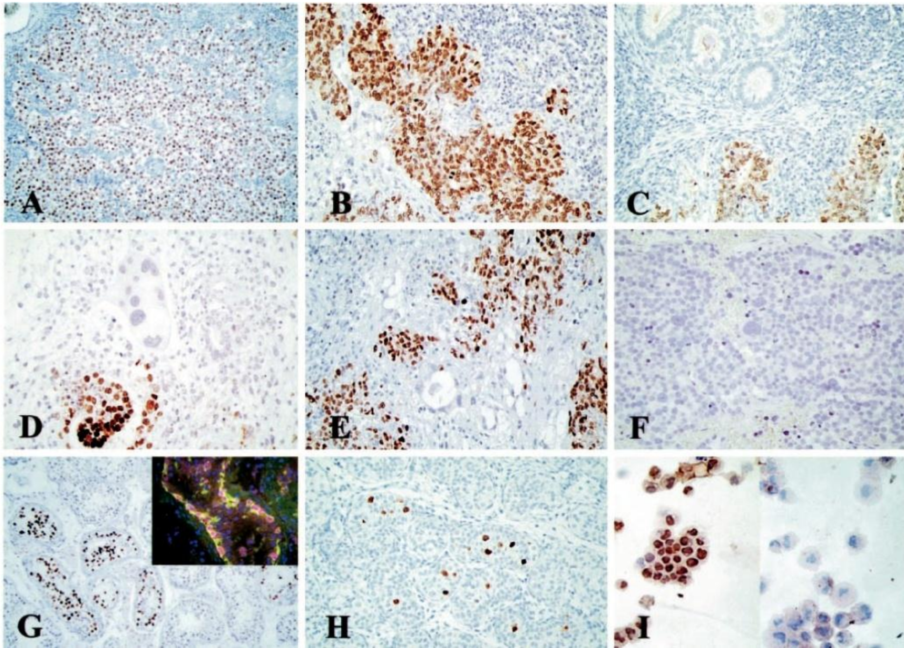

Fig. 1. Visualization of POU5F1 protein by immunohistochemistry on formalin-fixed, paraffin-embedded tissue sections of a seminoma (A-B); pure embryonal carcinoma **(C)**; mixed embryonal carcinoma and teratoma **(D)**; mixed embryonal carcinoma and choriocarcinoma **(E)**; mixed embryonal carcinoma and yolk sac tumor **(F)**; spermatocytic seminoma **(G)**; testicular parenchyma containing seminiferous tubules with normal spermatogenesis and CIS cells. Inset shows a seminiferous tubules with CIS cells double stained for c-KIT (green) and POU5F1 (red) **(H)**; an embryonic testis of 21 weeks of gestation **(I)**; cytospins of Tera2 cells before (left panel) and after (right panel) exposure to retinoic acid, inducing differentiation, and resulting in loss of POU5F1.

The multitumor tissue microarray of the (T)GCT confirmed the specificity of the POU5F1 immunohistochemistry for the histological types CIS/gonadoblastoma, seminoma/dysgerminoma, and embryonal carcinoma, as described above (Table 3). None of the immature and mature teratomas and yolk sac tumors showed a positive staining. Again, embryonic germ cells, included as arrays in the array, were also positive (data not shown). These data establish POU5F1 as highly specific marker for normal and malignant embryonic germ cells and embryonic stem cells, both in precursor (CIS) lesions and invasive tumors.

Discussion

Human GCTs comprise a heterogeneous group of neoplasms, which are predominantly found along the midline of the body. This localization can be explained by the migration route of the PGC during embryogenesis from the yolk sac to the genital ridge (32). The histology and clinical behavior of the various types of GCT depends on the sex of the patient, the age at clinical diagnosis, the anatomical location, and histology of the tumor (Ref. 33 for review). Overall, four different entities can be distinguished: (a) the teratomas and yolk sac tumors of newborn and infants; (b) the seminomas/dysgerminomas/ germinomas and nonseminomas of the gonads (both ovary and testis, and dysgenetic gonad), anterior mediastinum, and midline of the brain; (c) the dermoid cyst (mature cystic teratoma) of the ovary; and (d) the spermatocytic seminoma of the elderly testis. From a biological point of view, the pluripotent tumors (entity b) are the most intriguing. Histologically, they are subdivided into seminomas (known as dysgerminoma when identified in the ovary and dysgenetic gonad, and germinoma when arising in extragonadal sites) and non-seminomas (Ref. 34 for review). The seminoma-like tumors are composed of cells with a similar morphology as CIS cells. The nonseminomas can contain different histological elements, including embryonal carcinoma as the stem cell component, teratoma (representing somatic differentiation), yolk sac tumor, and choriocarcinoma (representing extraembryonic differentiation). The occurrence of embryoid bodies in nonseminomas (22), as well as certain patterns of gene expression, including POU5F1 (4, 13, 14, 35–38), illustrates the similarities between developmental patterns in the embryo and non-seminomas. This study aimed at a further understanding of the nature of the pluripotent (T)GCT compared with the other types of GCT and tumors of nongerm cell origin.

Here, we convincingly show that testicular CIS, seminoma, and embryonal carcinoma cells are consistently positive for POU5F1, irrespective of chemosensitivity or chemoresistance. Gonadoblastomas and associated dysgerminomas, as well as germinomas and embryonal carcinomas of the midline of the brain, showed a similar pattern. In contrast, none of the differentiated nonseminomatous derivatives (teratoma, yolk sac tumor, and choriocarcinoma) stained positive, irrespective of anatomical localization. This suggests that loss of protein expression is because of down-regulation of gene expression upon differentiation/maturation. These data are in accordance with earlier findings in mice, indicating that *pou5f1* is indeed a marker for embryonic stem cells and germ cells (1–3) and that expression is lost upon additional differentiation and maturation (7). The fact that we observed a homogeneously positive staining for POU5F1 in the embryonal carcinoma components of both pure and mixed nonseminomas (and representative cell lines) supports the

model that the encoded protein is crucial but not sufficient for pluripotency. This has also been implied by the recent finding that *POU5F1* overexpression had no effect on HeLa cells (39). Apparently, particular auxiliary proteins are needed. In accordance with the histology-dependent pattern of POU5F1 staining, both mature and immature teratomas, as well as the yolk sac tumors of newborn and infants, are negative. This is also true for the spermatocytic seminomas. Both tumor types have a different pathogenesis from TGCT (22, 24, 40 – 47). The absence of POU5F1 in these tumors is in agreement with their inability to generate pluripotent stem cells.

Our findings within *in vivo* tumor samples and representative cell lines are concordant with previous findings in mice (8, 9), showing that *pou5f1* is expressed in pluripotent cells and is down-regulated upon differentiation. Therefore, we conclude that POU5F1 is involved in regulation of differentiation of human cells in line with a report on human embryos (6). No POU5F1 was detectable in adult testicular samples with normal spermatogenesis by immunohistochemistry, which is consistent with findings in mice using fluorescent protein-tagged pou5f1 expression analysis (48). However, a positive staining by immunohistochemistry has been reported in mouse spermatogonia A (7). This difference might be because of sensitivity differences of the methods applied and/or differences in protein level. The finding of POU5F1 in human embryonic germ cells and the down-regulation of expression before spermatogonia A formation supports an embryonic initiation of the pathogenesis of TGCT (15, 16, 27, 49 –52).

We did not find POU5F1 protein in any of the differentiated components of nonseminomas or in the majority (>99.9%) of 3340 nongerm cell tumors. This indicates that reactivation of *POU5F1* expression and translation, as previously suggested based on cell line data (20), is not a frequent finding in cancer. The positive tumors were one kidney carcinoma (of 50 clear cell carcinomas) and two lung carcinomas (1 of 50 squamous and 1 of 47 large cell carcinomas analyzed). We do not currently have any plausible biological explanation for POU5F1 expression in these tumor types. Nevertheless, rare occurrence of POU5F1 immunoreactivity in kidney and lung must be kept in mind when performing immunohistochemistry for POU5F1 to demonstrate a pluripotent GCT in these organs. We think that the discrepancy between our study and the previous report suggesting reactivation of *POU5F1* occurring as part of the malignant transformation process (20) can be attributable to the different techniques used. The mRNA expression analyses by Monk and Holding (20) were performed using reverse transcription-PCR with primers that may amplify both *POU5F1* and different intron-less *POU5F1*-related sequences (unpublished observations).

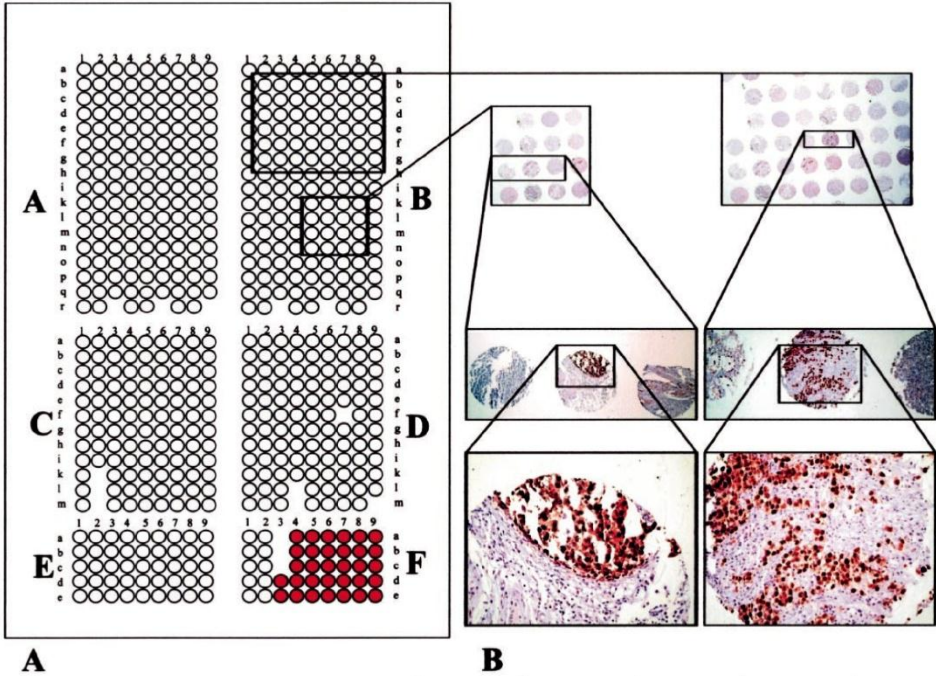

Fig. 2. A. schematic representation of one of the multitumor tissue microarrays used for the immunohistochemical detection of POU5F1, of which higher magnification examples are indicated in **B.** including a testicular seminoma (left panel) and an ovarian dysgerminoma (right panel).

In conclusion, this study demonstrates the usefulness of POU5F1 immunohistochemistry to support that TGCT originate from PGC and have pluripotent potential. However, POU5F1 is also present in nullipotent embryonal carcinoma and thus does not predict whether an embryonal carcinoma is able to generate various lineages of differentiation. Overall, POU5F1 is a highly specific immunohistochemical marker to confirm the diagnosis of CIS/gonadoblastoma, seminoma/dysgerminoma, and embryonal carcinoma. In addition, these data support the hypothesis that (T)GCT are excellent models to study the molecular mechanisms of pluripotency and differentiation, and therefore, a deeper understanding of these mechanism might also reflect into the rapidly evolving field of stem cell biology. Conversely, advances in the rapid evolving field of stem cell biology regarding the control of differentiation might also provide innovative therapeutic alternatives in (T)GCT in the future.

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Chapter 6

Diagnostic value of OCT3/4 for pre-invasive and invasive testicular germ cell tumours

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Abstract

Human testicular germ cell tumours of adolescents and adults (TGCTs), the seminomatous and non-seminomatous germ cell tumours, show morphological and biological similarities to normal embryonic development, presumably determined by their supposed cell of origin, the primordial germ cell/gonocyte. Based on this knowledge, OCT3/4, also known as POU5F1, was recently defined as a diagnostic marker for these tumour types. In the adult testis, positive immunohistochemistry for OCT3/4 is an absolute indicator for the presence of the TGCT precursor carcinoma *in situ*/intratubular germ cell neoplasia undifferentiated (CIS/ITGCNU), seminoma, and/or embryonal carcinoma. Several studies have confirmed this observation, using the same polyclonal antibody. The present study demonstrates the usefulness of OCT3/4 immunohistochemistry in a diagnostic setting of a consecutively collected series of more than 200 testicular tumours and over 80 testicular biopsies. Moreover, it is shown that a monoclonal antibody directed against OCT3/4 is as informative as the polyclonal antibody, both in immunohistochemistry and in western blot analysis. The antibodies are robust and applicable with different methods of pretreatment and storage of tissue. This allows routine application of this diagnostic marker.

Introduction

Testicular germ cell tumours of adolescents and adults (TGCTs) seminomas and non-seminomas are the most common malignancies in Caucasian men in the second to fourth decades of life [1]. These tumours can be treated effectively using surgery, irradiation, and chemotherapy, resulting in 5-and 10-year survivals in the range of 90–95%. Despite this good prognosis, a major concern is the annual increase in incidence of 2–5% [2].

Invasive TGCTs exhibit a wide spectrum of histological types, ranging from seminoma to various non-seminomatous elements: embryonal carcinoma, teratoma, choriocarcinoma, and yolk sac tumour [3]. Seminoma cells are similar to embryonic germ cells, most likely primordial germ cells (PGCs) or gonocytes. Embryonal carcinoma is the malignant counterpart of embryonic stem cells, in principle pluripotent, and thus able to differentiate into all three germ lineages. This histological spectrum mimics embryonal development to a certain extent (see ref 4 for a review).

Despite the palette of histological appearances of TGCTs, they all originate from a common precursor, known as carcinoma *in situ* (CIS) [5] or intratubular germ cell neoplasia unclassified (ITGCNU). Recent findings regarding the expression of embryonic traits, and the high expression of the cell cycle regulators that are typical of embryonic mitotic germ cells in CIS cells, support the hypothesis that these cells originate from PGCs/gonocytes (see ref 6 for a review). So far, all known markers of CIS cells are also found in immature germ cells [7]. This hampers the applicability of these markers at an early age, particularly if a delay or block of maturation of germ cells is expected, for example in the case of cryptorchidism or gonadal dysgenesis [8]. The presence of immature germ cells may result in overdiagnosis of CIS, and therefore possibly overtreatment.

To detect CIS by means of immunohistochemistry, the markers c-KIT [9] and PLAP [10] have been used for more than a decade. One of the most recent markers is OCT3/4, also known as POU5F1. This transcriptional regulator is expressed in undifferentiated, pluripotent cells including embryonic stem cells and germ cells, both mouse [11–13] and human [14,15]. Knock-out mice are non-viable, because they fail to form a pluripotent inner cell mass, but instead consist primarily of cells committed to the trophoblast lineage [16]. Inhibition of expression of this gene in mouse and human embryonic stem cells results in a similar type of differentiation induction to trophectoderm [17,18]. In contrast, recent data show that PGCs without OCT3/4 do not differentiate, but undergo apoptosis [19]. This indicates that the role of OCT3/4 in embryonic stem cells and PGCs is different. We showed that expression of OCT3/4 in TGCTs is highly similar to the pattern found during normal development. In fact, testicular parenchyma containing CIS,

as well as seminoma and embryonal carcinoma cells, contained mRNA of this gene, while it was absent in differentiated non-seminomas [20]. Subsequently, we demonstrated, by using a polyclonal antibody, that OCT3/4 protein is detectable in the cell types also positive for the mRNAs [21]. We applied immunohistochemistry to a large series of solid tumours (organized in tissue microarrays, TMAs) and found that OCT3/4 is a specific marker for CIS, seminoma, and embryonal carcinoma. In addition, its expression was also found in gonadoblastoma (the CIS counterpart of dysgenetic gonads), dysgerminoma, and germinoma (the seminomatous tumours of the ovary and midline of the brain, respectively). No correlation between treatment response and OCT3/4 staining was observed. None of the non-germ cell tumours investigated expressed the protein, except for three isolated cases [21]. Subsequently, these findings were confirmed by different studies [8,22–27] (see Table 1). In addition, Baker and Oliva recently reviewed the value of diagnostic markers in ovarian tumours, in which again OCT3/4 was concluded to be suitable for identifying dysgerminomas and ovarian embryonal carcinomas [28].

Table 1.
Chronological summary of published data on OCT3/4 in germ cell tumours at the mRNA and protein levels

			Germ cell tumours												
			Pre-invasive		Invasive									Metastatic	
					Seminomatous				Nonseminomatous						
Reference	**Method**	**SpA**	**CIS**	**GB**	**Se**	**GE**	**DG**	**SpSE**	**EC**	**YST**	**MTE**	**ITE**	**ChC**	**EC**	**SE**
Looijenga et al [21]	IHC	-	+	+	+	+	+	-	+	-	-	-	-		
Gidekel et al [24]	IHC	+	+		+				+						
Sperger et al [22]	Microarray				+				+	-					
Rajpert-De Meyts et al [8]	IHC	-	+	+	+			-	+						
Jones et al [25]	IHC				+			-	+	-	-	-	-		
Almstrup et al [23]	Microarray		+		+				+						
Cheng et al [26]	IHC			+			+			-	-	-			
Cheng [34]	IHC													+	+
Jones et al [27]	IHC		+												

All immunohistochemical studies were performed with a polyclonal goat anti-human OCT3/4 Antibody (C-20, sc 8629; Santa Cruz Biotechnology, Santa Cruz, CA, USA), except for Gidekel et al, where a self-made rabbit anti-mouse OCT3/4 antibody was used.
IHC = immunohistochemistry; SpA = spermatogonium A; CIS = carcinoma *in situ*; GB = gonadoblastoma; Se = seminoma; GE = germinoma; DG = dysgerminoma; SpSE = spermatocytic seminoma; EC = embryonal carcinoma; YST = yolk sac tumour; MTE = mature teratoma; ITE = immature teratoma; ChC = choriocarcinoma.

Here we report on our diagnostic experience of using immunohistochemistry for OCT3/4 on a consecutive series of more than 200 testicular cancers. Moreover, the applicability for the diagnosis of CIS in testicular biopsies is shown. We demonstrate that, in addition to the polyclonal antibody, which has been used in various studies, a suitable monoclonal antibody is now available that is

informative for both immunohistochemistry and western blotting. The monoclonal antibody assures infinite availability of OCT3/4 for clinical pathology and research. The diagnostic value of OCT3/4 immunohistochemistry is exemplified by the presentation of two patients. One presented with an unusual tumour in the brain and the other with a highly necrotic tumour in the retroperitoneal region. The positive staining of the neoplasms for OCT3/4 led to their recognition as germ cell tumours and the patients were treated successfully.

Materials and methods

Sample handling and characterization

Use of tissues for scientific purposes was approved by the institutional review board (MEC 02.981). The samples were used according to the 'Code for proper secondary use of human tissue in The Netherlands', as developed by the Dutch Federation of Medical Scientific Societies (FMWV) (version 2002).

A total of 223 testicular tumours were consecutively collected in the south-western part of The Netherlands, in collaboration with urologists and pathologists. Representative parts of the tumour (and adjacent tissue, if available) were snapfrozen in liquid nitrogen or fixed in 10% formalin overnight for paraffin wax embedding. Tumours were diagnosed according to the WHO classification, supported by immunohistochemistry using antibodies directed against germ cell/placental alkaline phosphatase (PLAP), α-fetoprotein (AFP), human chorionic gonadotropin (hCG), the stem cell factor receptor c-KIT, and cytokeratin 8 (CAM5.2). CD30 was used on a selected series. In addition, 81 open surgical biopsies from 56 adults with primary infertility or a history of TGCT were evaluated for Johnson score and the presence of CIS.

Immunohistochemistry

Immunohistochemistry with anti-OCT3/4 antibodies was performed on paraffin wax-embedded tissue sections of 3 µm thickness. Endogenous peroxidase activity was inactivated by incubation in 3% H_2O_2 for 5 min. Antigen retrieval was carried out by heating sections in 0.01 M sodium citrate (pH 6.0) under high pressure up to 1.2 bar. Endogenous biotin was blocked using an avidin/biotin blocking kit (SP-2001; Vector Laboratories, Burlingame, CA, USA). The sections were incubated for 2 h at room temperature with a polyclonal goat anti-human OCT3/4 antibody (sc-8629; Santa Cruz Biotechnology, Santa Cruz, CA, USA) directed against the COOH terminus of the protein. This antibody is used in almost all studies of OCT3/4 and germ cell tumours (Table 1). The antibody was diluted 1 : 1000. Subsequently, a biotinylated horse anti-goat secondary antibody

(BA-9500; Vector) diluted 1 : 200 was applied and bound antibody complex was visualized using the horseradish peroxidase (HRP) avidin–biotin complex method. Sections were counterstained with haematoxylin.

A monoclonal antibody against the N-terminus of human OCT3/4 (sc-5279; Santa Cruz Biotechnology) was compared with the polyclonal antibody using a 1 : 1000 dilution in the same assay as described above. As a secondary antibody, a biotinylated rabbit anti-mouse antibody was used (E0413; Dako, Glostrup, Denmark). Detection and counterstaining were as described above. To compare the staining pattern of the monoclonal and polyclonal anti-OCT3/4 antibodies, immunohistochemistry was performed on parallel paraffin wax sections. In addition, both antibodies were used at a dilution of 1 : 4000 on parallel, 4 µm thick and acetone-fixed, frozen tissue sections.

Initial OCT3/4 staining of the brain tumour from patient 2 was performed on limited paraffin wax-embedded material that was left after a large panel of markers was tested. Therefore, only a small fragment containing tumour cells was present. To improve this result, a negative control slide was reused for OCT3/4 immunohistochemistry using the monoclonal antibody.

Immunofluorescence

Fluorescent immunohistochemistry was performed on acetone-fixed frozen tissue sections of 4 µm thickness and cytospins of cell lines. The monoclonal anti-OCT3/4 antibody was incubated 1 : 2000 for 1 h at room temperature. Subsequently, a secondary FITC-labeled goat anti-mouse antibody (1090-02; Southern Biotechnology Associates, Birmingham, AL, USA) was incubated for 30 min. Counterstaining was done with Vectashield (Vector)/DAPI (Sigma Chemical Co, St Louis, MO, USA).

Cell culture

The human cell lines Tera1 [29], 2102Ep [30], NT2 [31], and NCCIT [32], all of which were derived from embryonal carcinomas, were grown in a 5% humidified incubator until 80% confluency was reached. Cells were incubated with trypsin–EDTA, harvested, and cytospins were prepared.

Protein isolation

Tissue from frozen sections containing normal testis, embryonal carcinoma, yolk sac tumour, mature teratoma and seminoma, and pellets from cell lines Tera1, 2102Ep, NT2, and NCCIT were used to extract protein. Samples were washed in cold PBS and centrifuged at 0 ^{o}C for 5 min at 1500 rpm. Lysis buffer (1× PBS, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS) including protease inhibitors (1 836 153 complete mini cocktail; Roche Diagnostics, Mannheim, Germany) was added. Samples were sonicated and centrifuged at 0 ^{o}C for 10 min at 14 000 rpm. Protein lysates were stored at - 80 ^{o}C.

Western blot

Samples of 20 µg protein were run on a 10% SDS-polyacrylamide gel, blotted on a PVDF membrane for 1 h, and incubated with a primary antibody overnight at 4 ^{o}C (anti-Oct3/4 monoclonal and polyclonal anti-body both diluted 1 : 1000). HRP-labelled secondary antibody was incubated for 1 h at room temperature. After washing, ECL western blotting detection reagents (RPN2109; Amersham Biosciences, UK) were added for 5 min and the blots exposed. After scanning, the blots were stripped and reprobed with anti- β -actin (1 : 5000) as a loading control.

Results

Immunohistochemistry

To test the diagnostic use of OCT3/4, immunohistochemistry was performed using the polyclonal antibody on a series of consecutively collected cases. In total, 209 testicular germ cell tumours were investigated after radical orchidectomy: three spermatocytic seminomas and 206 TGCTs (110 pure seminomas, 50 pure non-seminomas, and 46 combined tumours, the latter containing both a seminoma and a non-seminoma component). Mean age and range in major tumour groups were according to the literature. None of the three spermatocytic seminomas or 14 non-germ cell tumours [five B-cell lymphomas, three Leydig cell tumours, three embryonal rhabdomyosarcomas, one sarcoma, one carcinoid, and one metastatic renal carcinoma (clear cell type)] stained for OCT3/4. In contrast, all seminoma and embryonal carcinoma components were positive. CIS cells in the adjacent parenchyma of seminomas, non-seminomas, and combined tumours were all consistently positive. This was also true for intratubular seminoma (n = 29) and embryonal carcinoma (n = 18). There was a sharp transition between positive and negative cells, coinciding with the border of CIS, seminoma, and embryonal carcinoma with adjacent tissue. The CIS and seminoma cells showed highly consistent and strong nuclear staining, whereas in embryonal carcinoma cells, diffuse cytoplasmic staining was seen in addition to

the nuclear staining. Representative examples are shown in Figures 1A and 1B (seminoma in Figure 1A; seminoma and embryonal carcinoma in Figure 1B). Fluorescent detection confirmed this cytoplasmic staining to be absent in seminoma (Figure 1C) and CIS, and specific for embryonal carcinoma (Figure 1D). Because cytoplasmic staining was never seen in the negative controls, and was only found together with nuclear presence of the protein, we regard cytoplasmic staining to be an intrinsic characteristic of embryonal carcinoma. Based on morphological and immunohistochemical data, no CIS, seminoma or embryonal carcinoma cell was found to be negative. This pattern of staining is in agreement with our previous observations, in line with the published data of others (Table 1).

Besides the different tumour samples, four embryonal carcinoma-derived cell lines were investigated. All cell lines showed strong nuclear and faint cytoplasmic staining with both enzyme-based and fluorescent immunohistochemistry. Remarkably, all cell lines contained a number of cells without positive nuclear OCT3/4 staining. This heterogeneity was clearly greater in the pluripotent NT2 cell line (41% OCT3/4-negative) than in nullipotent cell lines [2102Ep (9%), NCCIT (18%), and Tera1 (14%)], possibly due to spontaneous differentiation (Figures 1E and 1F). OCT3/4-negative cells were predominantly located at the borders of cell aggregates, which is consistent with differentiation [33].

Besides the analysis of orchidectomy specimens (see above), a series of consecutively collected testicular biopsies was included. These were taken because of infertility or a history of TGCT. In total, 81 biopsies from 56 patients were included. Based on both morphology and immunohistochemistry for OCT3/4, CIS was found in open surgical biopsies in four patients: two had a prior TGCT of the contralateral testis and two presented with infertility. A clinically occult seminoma was diagnosed in the biopsy from another patient with prior TGCT. In addition, in two patients with a palpable testicular node, the excision biopsy showed seminoma in both cases.

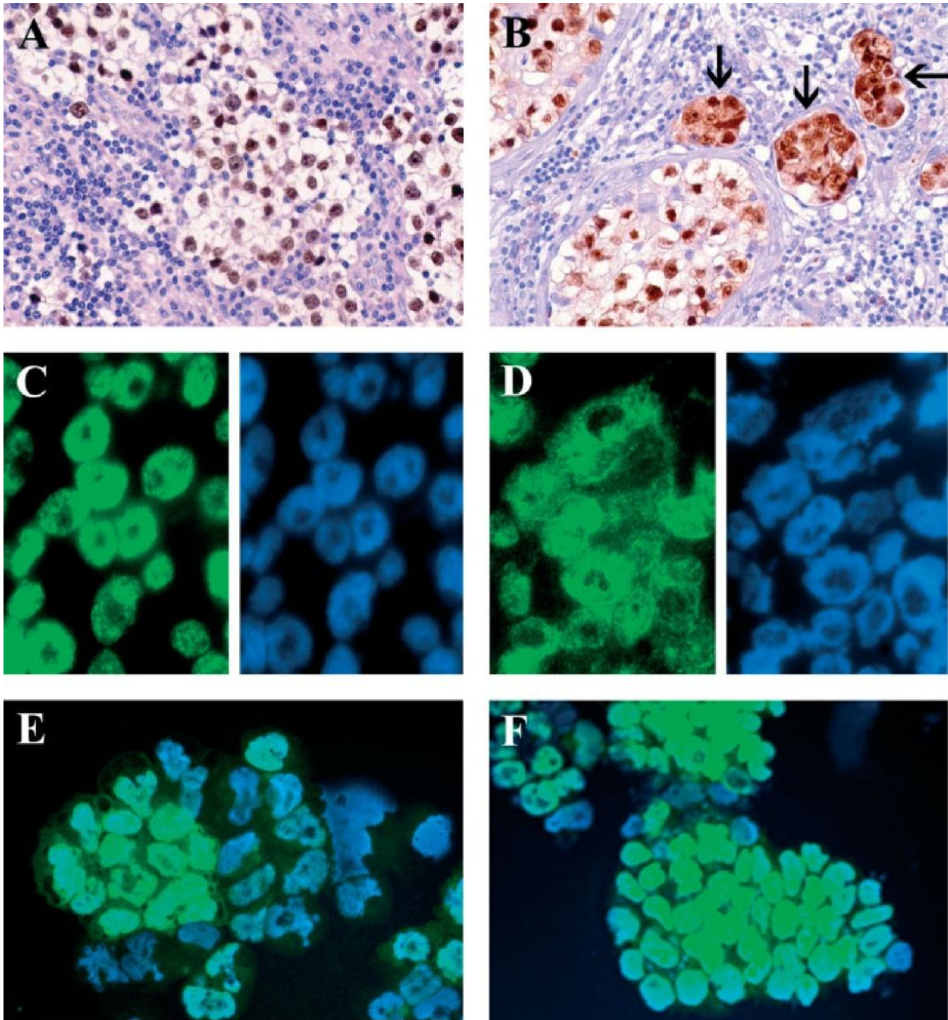

Figure 1. OCT3/4 immunohistochemistry using different embedding, fixation, and detection methods. (A) Nuclear staining (brown) of seminoma cells with a monoclonal antibody. **(B)** Embryonal carcinoma and seminoma incubated with a polyclonal antibody. Note the cytoplasmic staining in the angio-invasive embryonal carcinoma (arrows) compared with the clear cytoplasm of intratubular seminoma cells. Both slides are from formalin-fixed and paraffin wax-embedded tissue. Images **C–F** show FITC-labelled fluorescence with the monoclonal antibody as the primary step. Nuclear staining is in blue (DAPI) and OCT3/4 localization in green (FITC). **(C)** Frozen tissue from a seminoma. **(D)** Frozen tissue from an embryonal carcinoma. Note the difference in cytoplasmic staining pattern between seminoma and embryonal carcinoma cells. Cytospin preparations of **(E)** pluripotent embryonal carcinoma-derived cell line NT2 and **(F)** nullipotent 2102Ep. Note the OCT3/4-negative cells located at the periphery of the aggregates

Comparison of the monoclonal and polyclonal antibodies

Because of the, by definition, finite availability of a polyclonal antibody, and possible differences between batches, we studied the suitability of a commercially available monoclonal antibody directed against OCT3/4. In parallel, this monoclonal and the polyclonal antibody used to date (also see above) were compared using immunohistochemistry on both frozen and formalin-fixed, paraffin wax-embedded tissue, and western blot analysis. Both antibodies showed a similar pattern in both approaches, without background staining (data not shown). Western blot analysis was performed on total protein extracted from normal testis, seminoma, and the various histological types of non-seminoma, as well as the TGCT-derived cell lines (Figure 2A). These results are in accordance with the immunohistochemical data. The presence of the two bands around the expected size of 43 kD, corresponding to the longer OCT3/4 variant [13], is currently under investigation.

Robustness of the polyclonal and monoclonal antibodies for immunohistochemistry

To check the stability of the polyclonal and monoclonal antibodies for immunohistochemistry, the following procedure was performed repeatedly (n = 4). Undiluted antibody was frozen at -20^{0}C, thawed, used for immunohistochemistry, and again stored at -20^{0}C. Staining results did not decrease after multiple cycles. Immunohistochemistry results for frozen acetone-fixed, paraffin wax-embedded, formalin-, formaldehyde- and Bouin (Figure 2B) -fixed material and pretreated by microwave or high pressure were all successful, and suitable for diagnostic purposes.

Demonstration of the clinical value of OCT3/4 immunohistochemistry

The clinical value of immunohistochemistry for OCT3/4 in the diagnosis of solid tumours is demonstrated by the following two cases.

Case 1

A 37-year-old male presented with pain in the left flank for 1 year, related to an abdominal mass extending from the diaphragm to the aortic bifurcation with involvement of the left kidney and retroperitoneal lymphadenopathy. The patient showed small testicles on ultrasound. Serum LDH was strongly elevated up to 3568 U/l (normal: 0–449), while hCG levels were elevated to 29.5 IU/l (normal: 0–1.9). Guided biopsies showed large areas of necrosis and fibrosis with fields and strands of closely packed neoplastic cells with intermingling of lymphocytes. The malignant cells had round nuclei with prominent nucleoli and intermediate to large amounts of clear cytoplasm.

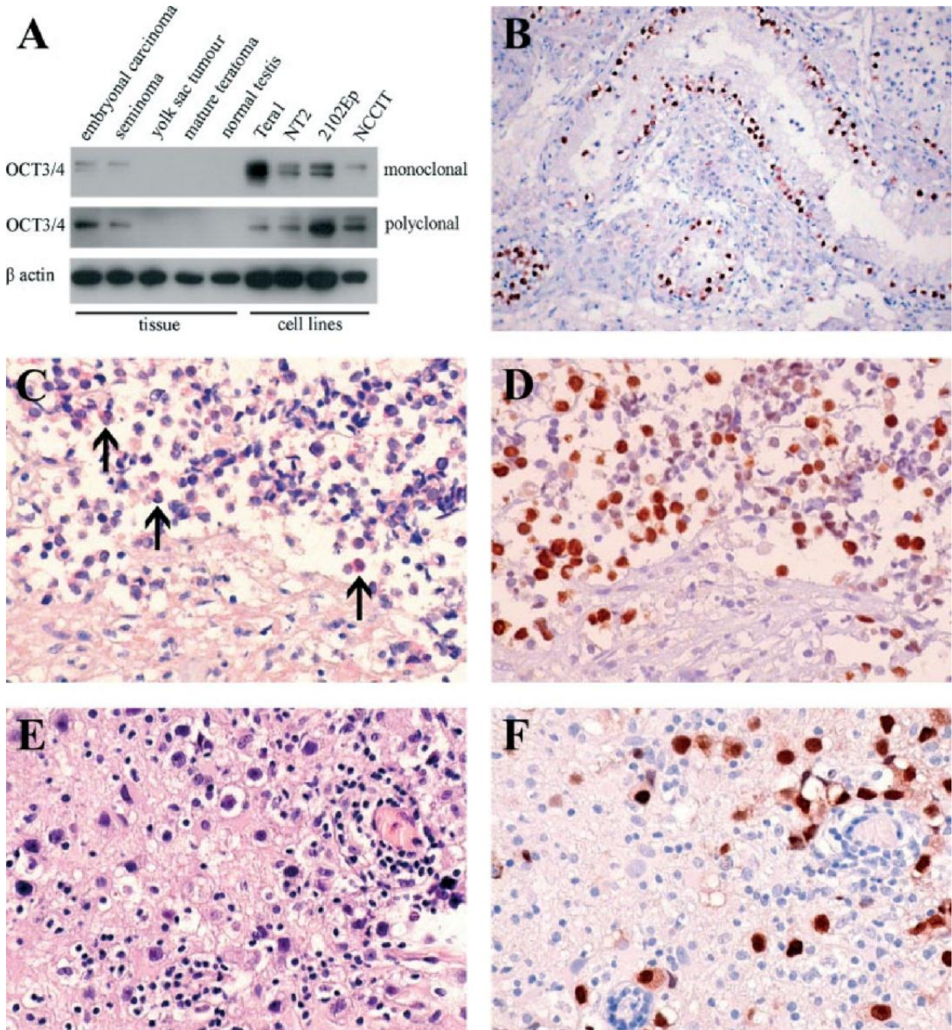

Figure 2. (A) Western blot analysis for OCT3/4 in different testicular germ cell tumours, derived cell lines, and normal testis. Note the specificity for seminoma and embryonal carcinoma. **(B)** OCT3/4 immunohistochemistry on Bouin-fixed tissue showing positive staining of CIS. **(C, D)** Retroperitoneal tumour with extensive necrosis from patient 1 showing weak cytoplasmic, focally membranous staining for c-KIT in red (C, positive cells indicated with arrows) and distinct brown nuclear staining for OCT3/4 **(D)**. **(E, F)** Tumour localized in the brain of patient 2, with scattered cells with clear cytoplasm and prominent nucleoli **(E)** with positive staining for OCT3/4 **(F)**

The morphological differential diagnosis was ‘seminoma, Ewing/small blue round cell tumour and lymphoma’. Immunohistochemically, the tumour cells showed very weak cytoplasmic and focally membranous staining for c-KIT (Figure 2C), while scattered cells were positive for keratin 8 (not shown). No staining was found in tumour cells for PLAP, hCG, CD30, AFP, CD99, desmin, CD21, CD45, EMA,

and TDT. The lymphocytes were positive for CD45. The tumour cells were consistently positive for OCT3/4 (Figure 2D), from which the diagnosis of seminoma was made and the patient was accordingly treated with chemotherapy. During standard chemotherapy, there was a rise in AFP levels, unusual for a seminoma. A non-seminomatous component was therefore suspected and the patient underwent retroperitoneal lymph node dissection (RPLND) and nephrectomy of the involved left kidney. In addition, his left testis, which showed microlithiasis on ultrasound, was removed. AFP levels normalized and no viable tumour tissue was found in the RPLND, kidney or testis specimens. After a rise in hCG level to 5.8 IU/l suggesting residual tumour, his right testis was removed; again, no tumour tissue was found and hCG normalized. Two and a half years after the initial diagnosis, the patient has no signs of recurrent disease.

Case 2

A 40-year-old female presented with visual disturbances, followed by slowly progressive left-sided motor and sensory disturbances. She also suffered from memory disturbances and was mildly spatially and temporally disorientated. A T2 weighted MRI scan showed a large area of high signal intensity in the periventricular white matter of the right more than the left occipital region and the corpus callosum, without enhancement on T1 weighted images after gadolinium administration and without mass effect. A tumour was considered unlikely and an acute demyelinating encephalomyelopathy was suspected. Repeated cerebrospinal fluid examination showed mononuclear pleiocytosis (10 cells per cubic mm; normal value: <3 cells), without tumour cells. Flow cytometry showed predominantly reactive T-lymphocytes, without evidence of tumour cells. A CT scan of the thorax and abdomen did not reveal any abnormalities. The CSF-serum IgG index was normal and oligoclonal bands were absent. No evidence of vasculitis or an inflammatory disease was found, and treatment with steroids was ineffective. A stereotactic biopsy of the intracerebral lesion showed blastlike neoplastic cells within a mononuclear infiltrate (Figure 2E). A wide panel of markers including those for a primary central nervous system lymphoma was applied: CD3, CD4, CD5, ALK-1, CD19, CD20, CD79a, CD45, CD30, S-100, MELAN-A, HB45, CD68, CD43, PLAP, hCG, AFP, and CD56 all showed no staining; there was some punctate keratin positivity and no final diagnosis was made. At first, her condition stabilized, but after a few months it deteriorated further and an MRI scan showed increasing focal areas of enhancement. Immunohistochemistry for OCT3/4 (Figure 2F) revealed the tumour to be a germinoma and she was treated with whole brain irradiation. Follow-up MR imaging showed disappearance of the enhancement and her condition improved, although focal deficits remained present. One and a half year after treatment, her condition is stable.

Discussion

After our initial publication [21] showing the sensitivity and specificity of OCT3/4 immunohistochemistry for the detection of a number of specific histological variants of human germ cell tumours, the results were largely confirmed in subsequent studies, both by immunohistochemistry with the same polyclonal antibody and at the mRNA level (Table 1). The first study included an extensive multi-tumour tissue microarray covering more than 100 different tumour categories and 3600 individual cancers. Strong nuclear expression was found in seminomatous tumours, irrespective of anatomical localization (like germinoma and dysgerminoma), and embryonal carcinoma, and their common precursor cell CIS (as well as gonadoblastoma), whereas the differentiated components of non-seminomas (yolk sac tumour, teratoma, and choriocarcinoma) were consistently negative. No correlation with chemotherapy sensitivity or resistance was found. Since then, it has been demonstrated that OCT3/4 immunohistochemistry is of value to diagnose metastatic seminoma and embryonal carcinoma [34]. The antibody applied in the different studies reported so far is of polyclonal origin, generated in goat. Therefore, availability of this antibody is finite. Here we have demonstrated for the first time that similar results can be obtained using a mouse monoclonal antibody, the availability of which is unlimited. These antibodies give the same results in immunohistochemistry and western blot analysis. Both antibodies appear to be robust and highly stable, and the immunohistochemical detection method is relatively insensitive to pretreatment and poor quality of the tissue samples.

That OCT3/4 is indeed of additional value in clinical pathology compared with the other available markers is demonstrated by the two cases reported in this paper. One of these patients had an unusual presentation of a germinoma (seminoma of the brain) and the other patient had a highly necrotic retroperitoneal seminoma, and both were successfully treated as such. OCT3/4 was the only positive marker found, even when applied on a slide used previously as a negative control. The negativity of the other markers could be due to the absence of these markers in the tissue under investigation and/or poor quality of the tissue.

The value of using immunohistochemistry for OCT3/4 to screen for CIS in testicular parenchyma biopsies taken in the context of an outpatient andrology clinic is also presented. The data again demonstrate the usefulness of OCT3/4 immunohistochemistry for the diagnosis of these pre-invasive cancer cells. It must be kept in mind that the presence of OCT3/4-positive cells in testicular parenchyma of patients below the age of 1 year does not justify the diagnosis of

CIS [7], in particular when a germ cell maturation delay or block is expected. For this specific application, additional criteria have to be developed.

Recently, Tai et al [35] reported OCT3/4 protein expression in some cells in the basal layer of human skin, corresponding in their hypothesis to the presence of adult stem cells. To verify these results, we incubated a total of 20 skin sections from five different individuals with the same monoclonal OCT3/4 antibody; no positive cells were found and this discrepancy remains to be resolved.

The data on OCT3/4 are also highly interesting from the biological point of view. An oncogenic driving force of OCT3/4 in the pathogenesis of TGCTs has been suggested based on mouse studies [24]. This conclusion was drawn from results obtained with experiments using mouse embryonic stem cells, which have various expression levels of OCT3/4. These cells give rise to so-called teratocarcinomas, which are not the proper animal model for TGCTs (see ref 36 for a review). In addition, it has been shown that the effect of OCT3/4 on a PGC and an embryonic stem cell is indeed different [19]. The fact that, in TGCTs, OCT3/4 expression mimics the pattern found during normal development (ie no OCT3/4 expression is detected in the differentiated non-seminomatous components) supports the model that OCT3/4 is under the control of physiological expression machinery and is not an oncogene in its classical sense. Our findings and those of others are in line with OCT3/4 expression being influenced by neither ploidy nor parental genome composition [37]. In fact, TGCTs originate from an erased PGC/gonocyte [38] and are consistently aneuploid [39]. It remains to be established whether epigenetic modifications have a regulatory role in the expression of OCT3/4 in TGCTs, as indicated in mouse cells [40].

In conclusion, we have demonstrated the diagnostic value of OCT3/4 immunohistochemistry for CIS, seminoma, and embryonal carcinoma in a clinical set-ting. Use of the monoclonal antibody is preferable for this application.

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Chapter 7

Non-invasive detection of testicular carcinoma *in situ* in semen using OCT3/4

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Abstract

Objective: Carcinoma *in situ* (CIS) is accepted as the precursor of the germ cell tumors of the adult testis. CIS cells are located within the seminiferous tubules and can be exfoliated into semen. We performed a study to detect CIS cells in semen using the highly specific immunohistochemical marker OCT3/4, potentially a method for non-invasive diagnosis.

Material and methods: In 2006, 41 men at risk for CIS of the testis were found eligible for this study. Indications for inclusions were a suspicious lesion on scrotal ultrasound investigation (*n* = 14), patients on surveillance after a history of a testicular tumor (*n* = 14), and 13 patients with bilateral testicular microlithiasis (TM).

Results: Three of the 13 men (23%) who underwent testicular biopsies for bilateral TM were histologically diagnosed with CIS (two bilateral), and their semen showed OCT3/4-positive cells in all cases. Twelve of the 14 patients (86%) with a solid mass were diagnosed with a TGCT with adjacent CIS in the parenchyma, and in 9 cases (75%) OCT3/4-positive cells were present in the semen. No OCT3/4-positive cells were found in patients with biopsies who did not show any evidence of malignancy.

Conclusion: This study demonstrates that OCT3/4-positive cells can be found in semen from the majority of patients with CIS. The observations indicate that there is probably a time window in which the CIS cells are exfoliated, which gives an opportunity for early detection of CIS cells in semen of men at risk for TGCT.

Introduction

Testicular germ cell tumors (TGCTs) of adults, seminomas and nonseminomas (also known as type II GCTs) [1], account for 1–2% of all malignancies in men. Although the disease is uncommon, an annual rise in incidence is observed in most Western countries [1–4]. In the Netherlands a 5% annual increase was found between 1990 and 2005 [5]. Carcinoma *in situ* (CIS) is accepted as the precursor of TGCT [6,7]. CIS cells originate from primordial stem cells or gonocytes that escape normal development at an early point during intrauterine development [8]. These CIS cells start to proliferate during puberty, presumably after a rise in sex hormone levels. Patients with CIS of the testis will develop testicular cancer within 5 yr in 50%, and probably all patients will develop testicular cancer ultimately [9].

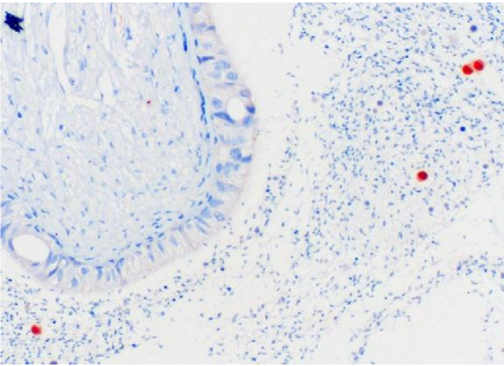

Fig. 1. Postorchidectomy specimen showing distinct OCT3/4-positive cells (stained red) in the epididymal lumen.

CIS is frequently found in the adjacent parenchyma of TGCTs [10]. CIS cells are located inside the seminiferous tubules at the basal membrane, in the niche of the spermatogonia, but they can leave their original location and spill over into the lumen. In addition, CIS cells can show a pagetoid spread to neighboring tubules, the rete testis [11] and even epididymus (Fig. 1).

In view of this, it is plausible that CIS cells are exfoliated in semen, as are spermatozoa. CIS is present in the testis long before a tumor develops and may be used for screening purposes in men at risk for TGCT, specifically in men with fertility problems (0.6%) and testicular microlithiasis (20%), men who were treated for TGCT, and men at risk for a contralateral tumor (5%) [12–14]. Type II TGCTs are highly sensitive to treatment, which is also true for CIS [9,15]. This sensitivity allows early local treatment, preserving hormonal function in most cases [16].

Ever since it was established that CIS is the precursor of TGCTs, efforts have been made to detect CIS cells in semen. The use of semen for detection of neoplastic cells in patients with testicular cancer was already suggested by Czaplicki in 1987

[17] and Giwercman in 1988 [18]. Different methods such as fluorescent in situ hybridization [19], immunohistochemistry using Ap-2 gamma and PLAP [20–22], and immunohistochemistry with magnetic beads using the M2A antibody [23] proved to be unsuccessful or too laborious. In contrast to the markers mentioned above, OCT3/4 is a very robust nuclear marker that has proven to be an absolute and specific marker for CIS, seminoma, and embryonal carcinoma in testicular tissue, and is now used as a standard marker in diagnosing CIS and TGCTs [24–26] (Fig. 2A). Our goal of this pilot study was to develop a reliable staining method. Moreover, we tried to evaluate the use of the OCT3/4 marker for early detection of CIS cells in semen of patients with known risk factors for CIS or TGCT.

Patients and methods

Patient selection

The Institutional Ethics Committee approved this study and all participants gave written informed consent (code MEC-2005-282).

In this pilot study we selected, on the basis of ultrasound evaluation, patients who were at risk of harboring CIS in their testicles. Between January 2006 and January 2007, a total of 42 patients and 15 controls were included (see Table 1 for group characteristics). Fourteen patients were diagnosed with a testicular mass or ultrasound-detected lesion suspected for malignancy; all except 1 underwent unilateral inguinal orchidectomy. This latter patient underwent an open testicular biopsy with frozen-section analysis. No malignancy was found on frozen section; therefore; no orchidectomy was performed. All orchidectomy patients were counseled for semen cryopreservation and were asked to donate a semen sample for this study. In 6 of the 14 orchidectomy patients, we were able to obtain a complete semen sample before orchidectomy. Eight of the 14 orchidectomy patients donated only a residual part of at least 0.3 ml after the majority was cryopreserved for future fertility treatments. Also, patients who were diagnosed with testicular microcalcifications (TMs) during analysis for male infertility, candidates for testicular biopsies were asked to participate. A total of 13 men with bilateral TMs and bilateral testicular biopsy were included and all donated a complete semen sample. Fourteen postorchidectomy patients were included. These patients were either diagnosed with a marker relapse (n = 12) during their active surveillance protocol or were informed about this study by their physician who requested their participation (n = 2). None of these patients had testicular abnormalities on clinical examination or ultrasound. Moreover, 15 normospermic patients from the andrology clinic with no known risk factors for TGCT were used as negative controls. All semen samples were produced by masturbation.

Methods

The testicular biopsies and the orchidectomy specimens were examined with the use of standard protocols for the detection of TGCT and CIS. The semen samples were allowed to liquefy after production and thereafter dissolved in 10% phosphate-buffered formalin for 1 h. In one patient who underwent a bilateral orchidectomy, the left testicle showed a spermatocele, whose content was aspirated and used for the detection of OCT3/4-positive cells. At least 0.3 ml semen was obtained if the patient participated. After fixation the samples were centrifuged for 20 min at 1600g; then the pellet was resuspended in phosphate-buffered saline and vibrated with the use of an automatic shaker to make a single-cell solution. Cytospins of this suspension were made on a strong adhesive microscope slide (Starfrost©) and were dried overnight. Immunohistochemistry with monoclonal anti-OCT3/4 (POU5F1, Santa Cruz sc-10, sc-5279) antibodies was performed on the formalin-fixed semen samples as described earlier [25]. After the first 15 patients, we modified our last staining step by converting from a 3-diaminobenzidine tetrahydrochloride (DAB, brown) to a 3-amino-9-ethylcarbazole (AEC, red) reaction to increase the contrast between the OCT3/4-positive cells and the spermatozoa, and to lower the background staining. No differences in cell morphology were seen between these techniques. As a positive control, microscope slides were used with OCT3/4-positive cells from an established TGCT cell line (NT2) [27]. The microscope slides were blinded and evaluated separately by two individuals and were scored positive if a distinct nuclear staining was seen in large cells with large nuclei and clearly recognizable cytoplasm. Doubtful staining was scored negative.

Results

Three of the 13 men (23%) who underwent testicular biopsies for TM were diagnosed with CIS, which in 2 of the cases was bilateral. The 14 orchidectomy specimens showed a seminoma in 4 patients (of which 1 was bilateral), nonseminoma in 4 patients, and a combined tumor (containing both a seminoma and a nonseminoma component in a single tumor) in 2 patients. In 2 testicles CIS was found next to a burned-out tumor. In 1 patient who had a suspicious intratesticular hypoechogenic lesion, an open testicular biopsy was performed and the specimen immediately stained by a direct alkaline phosphatase staining method [28]. Histopathological evaluation of this sample showed a Sertoli cell only (SCO) pattern. No CIS or malignant component was found and therefore no orchidectomy was performed. One orchidectomy specimen showed a Leydig cell tumor.

Table 1 – Oct3/4 staining results, listed by diagnosis

Group	No.	Diagnosis	Mean age at time of diagnosis (yr)	OCT3/4-positive staining
Suspicion of TGCT	14	4 seminoma[a]	33,8	2/4 (50%)
		4 nonseminomas[a]	23,7	4/4 (100%)
		2 combined tumors[a]	26,6	2/2 (100%)
		1 Leydig cell tumor	26	0
		2 testis-containing CIS with a burned-out tumor	28,9	1/2 (50%)
		1 Sertoli cell only	28	0
Postorchidectomy	14	11 nonseminomas	26,3	0
		3 combined tumors	24,8	0
Bilateral testicular microcalcifications	13	3 CIS (2 bilateral)	34,4	3 (100%)
		10 no malignancy	34	0
Control group	15	Not available	—	0
Total	56	15 testis-containing CIS		12/15 (80%)

TCGT, testicular germ cell tumor; CIS, carcinoma *in situ*.
[a]: All TGCT tumors had CIS in the adjacent parenchyma.

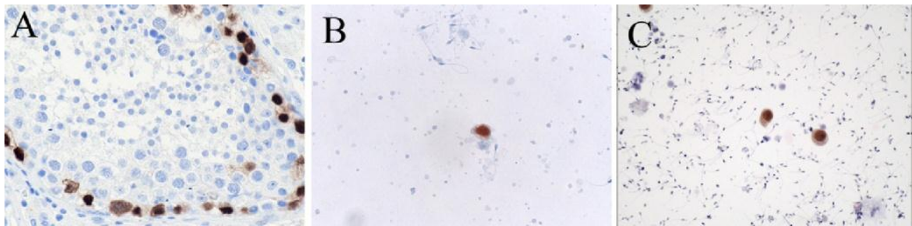

Fig. 2. (A) Seminiferous tubule with carcinoma *in situ* stained with the OCT3/4 marker (brown). (**B** and **C**) Microscopic slides of semen showing spermatozoa and OCT3/4-positive cells from two patients diagnosed with a testicular germ cell tumor.

All orchidectomy specimens containing a TGCT showed CIS in the tissue adjacent to the tumor. In 12 of the 15 patients (80%) harboring CIS at the time of semen donation, OCT3/4-positive cells were found in the semen sample (Table 1). Two of the 3 negative patients were diagnosed with a seminoma. OCT3/4-positive cells were seen with distinct CIS morphology as shown in Fig. 2B and C. The number of OCT3/ 4-positive cells ranged from 1 to >10 per sample. No correlation was found between the extensiveness of CIS in the testis or ejaculate volume and the number of exfoliated CIS cells. In patients with bilateral CIS who donated at least two total ejaculates, OCT3/4-positive cells could be found in most successive samples. Patient characteristics and a comparison between the OCT3/4 antibody staining of semen and the histopathological findings are shown in Table 1.

In the patient with the bilateral seminoma only, OCT3/4-positive cells could be found in the aspirated spermatocele but not in the semen sample. No OCT3/4 positive cells were found in the patients who had no histologically proven abnormalities of the testis, nor did we find OCT3/4-positive cells in the semen samples from the postorchidectomy group. Mean age of the three patients in which no OCT3/4-positive cell were found in the semen, although proven TGCT was higher compared with the patients in which OCT3/4-positive cells were seen, was 35.8 versus 28.4 yr, respectively ($p = 0.067$). Mean age in the seminoma patients with or without OCT3/4-positive cells was 26.8 versus 40.9 yr, respectively ($p = 0.12$).

Discussion

CIS is an asymptomatic condition that can be found in testicular biopsies in infertile patient with risk factors for TGCT or in patients with abnormal findings on ultrasound examination [9]. A testicular biopsy is still the gold standard in diagnosing CIS, with a false-negative percentage of 0.5%; recently it was even suggested to take a two-site biopsy to increase the diagnostic yield [29]. Although biopsy is a very sensitive diagnostic test, it is reported that CIS cells are not always randomly distributed, explaining false negative test-results in some cases [30]. Since the discovery of exfoliated CIS cells in semen in 1988 [18], a great deal of effort has been put in finding a method to detect these cells in patients at risk for TGCTs [23,31]. A non-invasive detection method would be helpful to a certain extent [25].

In this study we were able to detect OCT3/4-positive cells in semen from all CIS-bearing patients. This specific patient group may benefit from early detection. Although other studies as well as our own study have shown that CIS cells can be detected in semen, there are still a number of pitfalls. Malignant cells from the invasive tumor are not likely to be exfoliated owing to the predominant intact architecture of the seminiferous tubules. The primary location of the CIS cells beneath the tight junctions of the Sertoli cells may prevent spread in the first stage before proliferation has started. There is probably a time window between the early stage of CIS and the time the tumor obstructs the tubules in which the CIS cells are being exfoliated. Also the impact of the change in environment on cell morphology of CIS cells passing through the male genital tract may prevent proper detection. In the third place, with this technique, the natural surrounding in which the CIS cells are easily recognizable is lacking, making a positive identification more difficult and totally dependent on immunohistochemistry and cell morphology. In our study all patients showed recognizable CIS cells stained with the OCT3/4 marker (Fig. 2B and C). The use of immunohistochemistry to detect the CIS cells, although it is highly specific,

should be performed by using a standardized method. Expertise to per-form these staining techniques accurately to avoid false-negative results is, therefore, of major importance. Moreover, the specificity of the antibodies used must be confirmed, and a standardized method for immunohistochemical detection must be applied, including proper positive and negative controls. Hoei-Hansen et al [22] reported a diagnostic rate for the detection of CIS cells in semen from men diagnosed with CIS only of the testis, using AP2-gamma of 50% and 0% for OCT3/4. They were, however, not able to compare these techniques because they had not stained the same slides for AP2-gamma as well as OCT3/4. Also possible sub-optimal immunohistochemical detection of OCT3/4 might be related to the published limitation. These authors also described that three false-positive results were seminal fluid that showed borderline AP2-gamma–stained cells. A subsequently performed testicular biopsy failed to confirm this diagnosis. We did not encounter any false-positive results using the OCT3/4 marker. Although we did not have pathological examination of the controls, no abnormalities were seen on ultrasound or clinical investigation, making the change of an existing TGCT very small [32]. All three cases in which no OCT3/4 cells were found in the ejaculate showed severe fibrotic tubules potentially hampering exfoliation of CIS cells. The incidence of pagetoid spread of CIS cells in the rete testis differs between patients with seminoma and nonseminoma, with less pagetoid CIS involvement in the seminoma group [11]. Patients with a seminoma often have an extensive host response, less CIS, and more atrophic tubules [10]. This lymphocytic infiltration may even result in a complete eradication of CIS in seminoma patients [10,33]. Seminoma patients generally are older than nonseminoma patients, and the risk of any of these factors inhibiting exfoliation is increased. In our study semen from two patients (50%) with seminomas did not show any OCT3/4-positive cells, whereas in all patients with non-seminomas, OCT3/4-positive cells were seen. A similar tendency was seen in the study of Hoei-Hansen et al [22], in which they saw a significant difference in positive cells exfoliated between seminoma (17.4%) and nonseminoma patients (56.6%). Age (and histology of the tumor) might therefore play an important role in the exfoliation of CIS cells, with a higher chance of finding these cells in patients at a young age.

The purpose of this study was to try to detect CIS cells in semen especially to diagnose CIS before an overt tumor has developed. Although TGCT is a highly curable disease, the potential long-term side effects of the treatment protocols used are numerous and severe. Long-term side effects will occur in approximately 20-30% and consist mainly of nephrotoxicity, ototoxicity, neurotoxicity, and gonadal damage [34]. CIS can be treated with orchidectomy or local radiotherapy with a curability rate reaching 100%, thus preventing potential hazardous chemotherapy in case CIS is left untreated and a testicular

tumor develops. In this study, two patients had bilateral CIS and were advised to undergo bilateral radiation therapy. However, in case of infertility, couples may choose to have artificial reproductive techniques performed before radiation treatment is performed. The third patient with unilateral CIS underwent an inguinal orchidectomy to prevent the potential scatter radiation on the contralateral testicle in case of radiation therapy.

The combination of the peak-incidence of TGCTs, the long-term presence and exfoliation of CIS cells, and well-known risk factors may provide a setting in which screening could be useful. Also the relative high incidence (5%) of patients with TGCT occurring in the contralateral testis underscores the need for close follow-up and early detection in this specific group [35]. Patients visiting a fertility clinic have a 20-fold greater incidence of testicular germ cell cancer than fertile men, and CIS is found in approximately 0.6%; therefore, this might also be a population that could benefit from screening [36]. In addition, infertile males with bilateral TM, who have approximately a 20% chance of harboring CIS, might specifically profit from this technique [12]. Owing to the relative low incidence, the high number of patients at risk, and the overall good survival in case of TGCT, this test should be easy to use, cheap, highly specific, and sensitive, making it cost-effective. We are aware that the numbers in our study are relatively small; therefore, the results presented here are preliminary. Future research will focus on a more specific enrichment of the CIS cells, integration of an automatic screening tool for the detection of OCT3/4-positive cells, an increase in the numbers to substantiate our results, performance of a cost-benefit analysis, and, perhaps, the discovery of treatments that eradicate CIS while preserving remaining spermatogenesis.

Conflicts of interest

The authors do not have any commercial relation-ships related to this article.

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Letter to the Editor

Re: Niels J. van Casteren, Hans Stoop, Gert R. Dohle, Ronald de Wit, J. Wolter Oosterhuis, Leendert H.J. Looijenga. Non-invasive Detection of Testicular Carcinoma *In Situ* in Semen Using OCT3/4. Eur Urol 2008;54:153–60

van Casteren et al set out to establish a method for detecting precursor cells of testicular germ cell cancer (CIS: carcinoma *in situ*, or TIN: testicular intraepithelial neoplasia) in semen with the aim of replacing testicular biopsy as the current diagnostic standard. By employing immunocytochemical staining with OCT3/4, they identified exfoliated TIN-cells in seminal fluid. This result is novel and exciting. Nonetheless, I have some concerns with this method.

1. If the patient examined possesses two testicles, then a positive finding in semen does not disclose the afflicted side because, clearly, semen is composed of excretions from both sides. Even shortly after unilateral orchidectomy, semen analysis will still reflect the pooled production of both testicles. As is known from post-vasectomy evaluations, the seminal vesicles can store exfoliated spermatozoa for months. With respect to searching for contralateral TIN, semen analysis will not be meaningful for several months.

2. We do not know the sensitivity of semen analysis for TIN. There appear to be unsolved technical problems because OCT 3/4 staining of semen cells had apparently failed in the hands of another experienced group [1].

3. The standard method for diagnosis of TIN rests, first, on morphological features and, second, on histochemical criteria, as found in tissue specimens featuring intact seminiferous tubules. Accordingly, conservation of specimens by proper fixation had been urged [2] and mechanical damage to specimens had been strongly cautioned [3].

Searching for exfoliated TIN-cells in semen represents a change of paradigm with respect to diagnostics of TIN because this new technology solely rests on immunocytochemical staining. Some of the distinct morphological features of TIN-cells as found in the tissue compound of seminiferous tubules are not detectable in single exfoliated cells in semen. In light of the problems many pathologists still have in diagnosing TIN in biopsies [4], one might speculate that sporadic TIN-cells in seminal fluid might easily escape detection. Generally, if a diagnostic technique is to replace current standards, then this new technique should involve clear-cut advantages over the standard; for example, higher accuracy, less costs, or better handling. Frankly, at this point, the advantages of semen analysis are still elusive.

4. Searching for contralateral TIN in patients with testis cancer is the most frequent condition of TIN-diagnostics. Testicular biopsy is usually performed under general anaesthesia simultaneously with orchidectomy for the cancer. Although the potential for psychological distress imposed by such a biopsy has not been formally evaluated, it appears obvious that it is minimal. Nor has the emotional burden secondary to the quest to produce an ejaculate been formally evaluated. Yet conceivably, the latter procedure could involve an unpleasant task for a young man left with the problem of coping with cancer.

In all, van Casteren and coworkers present an appealing novel approach for detecting TIN. However, in light of a well-established current standard with high accuracy [2] and low surgical morbidity [5], open questions exist with the new technique regarding its clinical usefulness, methodological safety, diagnostic sensitivity, and patient inconvenience.

Conflicts of interest: The author has nothing to disclose.

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Letter to the Editor

Reply to Klaus-Peter Dieckmann's Letter to the Editor re: Niels J. van Casteren, Hans Stoop, Gert R. Dohle, Ronald de Wit, J. Wolter Oosterhuis, Leendert H.J. Looijenga. Non-invasive Detection of Testicular Carcinoma *In Situ* in Semen Using OCT3/4. Eur Urol 2008;54:153–60

We thank Dr Dieckmann for his valuable comments on our paper in which we described the observation that precursor cells of type II testicular germ cell tumours (TGCTs) can be found in semen, based on immunohistochemical detection of OCT3/4 (POU5F1) [1].

There are a number of issues to clarify. First, the primary objective of our study was to evaluate if carcinoma *in situ*/testicular intraepithelial neoplasia (CIS/TIN) cells could be detected in semen using OCT3/4 immunohistochemistry. This is a relevant question to answer because until now, this was not convincingly shown. The use of OCT3/4 is the optimal target for identification of CIS/TIN cells; because of its specificity, as discussed in the manuscript, OCT3/4 is the best marker for CIS/TIN cells in the adult testis as compared to other markers. If OCT3/4 is unable to detect CIS/TIN in semen, it is unlikely that another marker will.

However, there are additional issues to keep in mind. In general, success of immunohistochemistry depends on many factors, including the antibody and protocol used as well as fixation method and possible antigen retrieval. Considering these points, we believe that if in the study of Hoei-Hansen and colleagues the slides with Ap2-gamma positive cells were stained with OCT3/4, CIS cells would be found [2]. However, as we noted, because of their study design, no comparison can be made between Ap2-gamma and OCT3/4.

After succeeding in our primary objective, ie, feasibility to detect testicular tumour cells in semen, the question arises of how to implement this method in clinical practice. We are aware that the achievements made are only part of the first step and that many aspects must be clarified before implementation in clinical practice is achieved. Dieckmann discussed a number of those aspects. Indeed, an ejaculate is composed of germ cells and fluids from both testicles, and if OCT3/4-positive cells are found in an individual with two testes, the origin of the CIS/TIN cells is not known. In these cases, a surgical testicular biopsy is still required for CIS diagnosis. In addition, for testicular biopsies immunohisto-chemistry, preferentially using OCT3/ 4-specific antibodies, is mandatory to allow accurate diagnosis. Although generally CIS/TIN cells can be identified using

morphological criteria alone, they can be missed in testicular biopsies without immunohistochemistry.

Because of the low incidence of TGCT and the invasiveness of the procedure, a testicular biopsy for patients at risk for TGCT, such as those with infertility, cryptorchidism, testicular atrophy, and a unilateral TGCT, is not the preferred screening tool. In fact, country-specific differences are present, eg, a contralateral testicular biopsy in the case of a unilateral TGCT is obtained in every patient in Germany and Denmark, whereas such practice is uncommon in the Netherlands. However, we believe that in men with signs of testicular dysgenesis such as microlithiasis, small testicular volume, and an inhomogeneous ultrasound pattern, a diagnostic procedure to detect CIS/TIN should be considered. Detecting CIS cells in semen could be the ideal screening tool for men at risk for TGCTs.

An interesting and easily accessible group for CIS/ TIN screening are males who visit a fertility clinic. These patients always provide semen samples to assess fertility capacity, part of which can be used for screening for the presence of CIS/TIN. The extra burden lies only in explaining to the patient that he may have a higher risk for type II TGCTs, especially if signs of testicular dysgenesis exist, or in cases of testicular atrophy. This may also apply to men with a history of TGCT and in men with testicular microlitiasis found in scrotal ultrasound. If CIS cells are detected for validation, a surgical testicular biopsy is still required.

In conclusion, we believe that our approach might result in a future screening tool; however, various issues must be clarified before it can be implemented into clinical use. Therefore, prospective studies are needed to assess the sensitivity of the test. Our current study was a proof of concept, which will hopefully stimulate further development of an accurate and early diagnostic semen test that can be used for screening men at risk for TGCT.

Conflicts of interest: The authors have nothing to disclose.

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We have with interest read the paper by van Casteren et al [1], the subsequent letter by Dieckmann [2], and the author's reply [3], and consider this line of research commendable. The study is a continuation of many years of research aiming at non-invasive detection of testicular carcinoma *in situ* (CIS) [4].

The primary objective of van Casteren et al was to evaluate the suitability of OCT-3/4 for immunocytochemical detection of CIS cells in 56 selected participants. The study is a confirmatory study of our earlier study [5], where we tested another solid marker for CIS, AP-2*γ*, in semen from 503 individuals, and, in parallel, examined OCT-3/4 in 84 participants [5]. Our conclusion was that AP-2*γ* and OCT-3/4 are equally suitable for CIS detection in semen [5]. In patients with a testicular germ cell tumour before orchidectomy we detected OCT-3/4-positive/borderline-positive cells in 35.3%, similar to AP-2*γ* (33.9%). With OCT-3/4 we analysed one patient with CIS-only and did not detect any positive cells. In van Casteren et al [1] three patients with CIS-only were examined and in all cases positive cells were found. If one considers the number of patients (3/3 vs 0/1), there is no scientific or statistical basis to state that the OCT-3/4 assay "failed" in our hands [2]. Nor is there, based on the data presented by Van Casteren et al [1], any scientific basis for their statement that OCT-3/4 is the "optimal target" for CIS detection. Van Casteren et al. state in the paper that their data are preliminary, but still draw extensive conclusions.

We believe that the two studies basically are not comparable, as their designs and inclusion criteria were completely different. The van Casteren et al [1] study was performed on a selected group of patients where CIS or an overt tumour was suspected, whereas we performed a screening of a total of 503 andrologic patients, only a quarter of which had germ cell neoplasia [5]. Van Casteren et al obviously had a much higher likelihood of identifying positive samples, when the study group consisted of 15/56 shown to have CIS (26.8%) compared to our study consisting of 73/503 (14.5%). The larger group examined by us also explains why we detected three false-positive cases, whereas van Casteren et al detected none, as we included 292 controls and they 15. We believe that it cannot from such a small study be concluded that exfoliated CIS cells can be detected in 80–100% of cases [1], especially if no statistical analysis of predictive values or confidence intervals are given.

We agree with Dieckmann [2] that there are several instances where a semen-based analysis is not applicable, that is, for detection of contralateral CIS. However, a semen-based method would be of great use in screening where CIS is suspected, and semen analysis could aid in selection of patients for biopsy, thereby decreasing the number of unnecessary surgeries. We agree here with van Casteren and colleagues that there is a large potential for a non-invasive detection method, best illustrated by the detection of CIS by the AP-2*γ*-based method in a young subfertile man, where testicular neoplasia was detected at the non-invasive stage due to his inclusion as a control subject in our studies [5].

Conflicts of interest: The studies of the Copenhagen group on various aspects of testicular cancer are supported by the Danish Medical Research Council, Vissing Foundation, Danish Cancer Society, Carla Thiel Kraghs Foundation, Kirsten & Freddy Johansen's Foundation, Danish National Advanced Technology Foundation, and Copenhagen University Hospital. A patent application covering the use of novel CIS markers for diagnosis of germ cell neoplasia is pending.

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Chapter 8

A 40 year-old woman with a progressive periventricular white matter lesion

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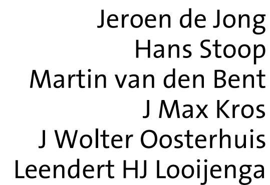

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Martin van den Bent
J Max Kros
J Wolter Oosterhuis
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Clinical history

A 40-year-old woman presented with blurred vision and diplopia, followed by slowly progressive left-sided motor and sensory disturbances. She also suffered from memory loss and mild spatial and temporal disorientation. A T2-weighted MRI (Figure 1A) showed a large area of high signal intensity (indicated by arrows) in the periventricular white matter of the right more than the left occipital region and the corpus callosum, without enhancement on T1-weighted images (Figure 1B), There was no mass effect. A tumor was considered unlikely, and acute demyelinating encephalomyelopathy was suspected. Repeated cerebrospinal fluid (CSF) examinations showed a mononuclear pleocytosis (10 cells per cubic mm, normal value: <3cells), without immunohistochemical evidence of tumor cells; tumor markers HCG, AFP and CEA were not elevated. Flow cytometry showed predominantly reactive T-lymphocytes, but again no evidence of tumor cells. CT scan of thorax and abdomen did not reveal any abnormalities. The CSF-serum IgG index was normal, and oligoclonal bands were absent. No evidence of a vasculitis or an inflammatory disease was found. Treatment with steroids proved ineffective.

Microscopic description

A stereotactic biopsy of the intracerebral lesion showed blast-like neoplastic cells within a mononuclear infiltrate (Figure 2A). The rounded tumor cells contained large centrally located nuclei. A wide panel of markers including those for carcinoma, melanoma and primary central nervous system lymphoma was applied: CD3, CD4, CD5, ALK-1, CD19, CD20, CD79a, CD45, CD30, S-100, MELAN-A, HMB45, CD68, CD43, placental alkaline phosphatase (PLAP), hCG, AFP and CD56, all found to be negative; there was some punctuated NCL5D3 (low molecular weight keratins 8 and 18) positivity (Figure 2B). No final diagnosis could be made.

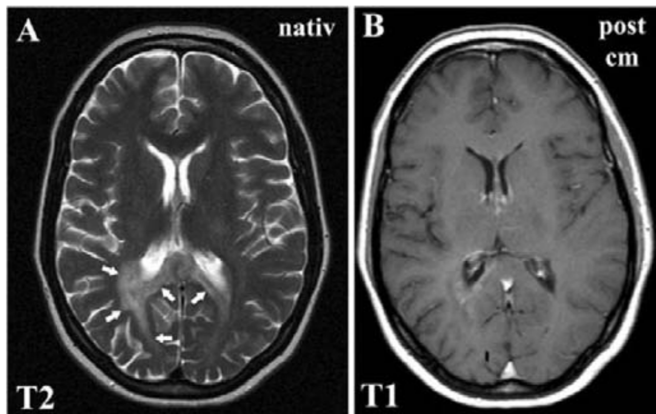

Figure 1. (A) T2-weighted MRI. Large area of high signal intensity (indicated by arrows). **(B)** T1-weighted MRI.

Subsequently, the marker OCT3/4 became available, which has proven to be specific for certain histological types of germ cell tumors (6), including seminomatous tumors and embryonal carcinoma. This has been confirmed in multiple independent studies (1) (for review). One hundred per cent of tumor cell nuclei present in the biopsy of the above-mentioned patient clearly stained positive for OCT3/4 (Figure 2C). Because only a small number of tumor cells were present in this slide and no biopsy material was left anymore, OCT3/4 was also applied to a slide previously found to be negative by immunohistochemistry for another unrelated marker. Again all tumor nuclei stained positive for OCT3/4 without any background. The stem cell factor receptor c-KIT was also applied to a previously negative slide and the cytoplasm of some tumor cells was stained positive (Figure 2D).

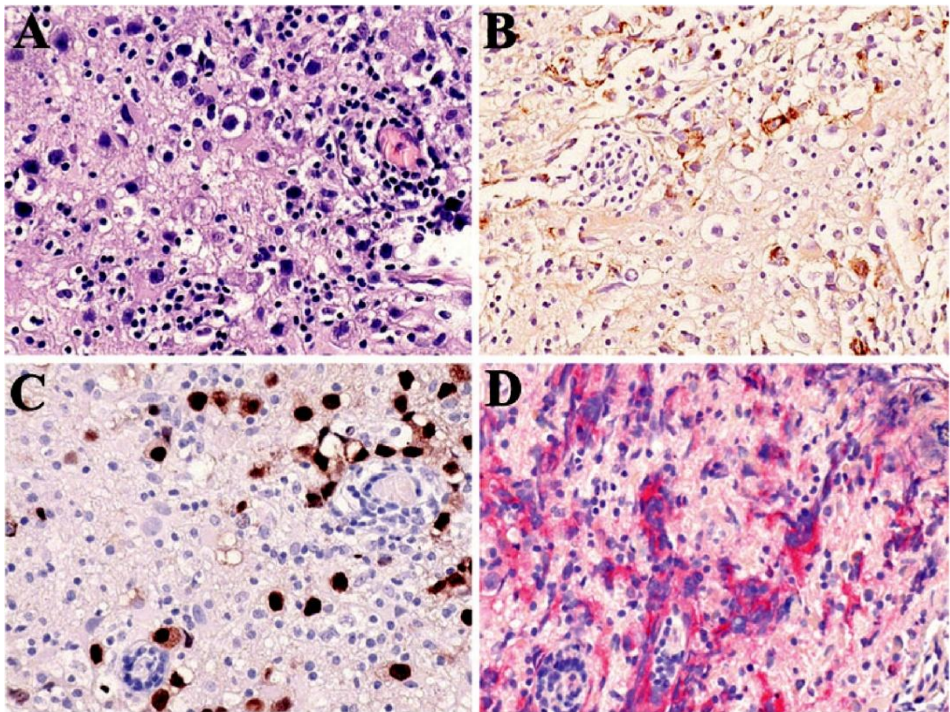

Figure 2. Tumour localized in the brain. (A) H&E staining. Blast-like neoplastic cells within a mononuclear infiltrate. **(B)** some punctuated NCL5D3 positivity (brown). **(C)** OCT3/4 positive nuclei (brown). **(D)** c-KIT positive cytoplasm (red).

Diagnosis

Diffusely infiltrating germinoma.

Additional treatment and clinical progress

After the lesion was diagnosed as a germinoma, the patient was treated with whole brain irradiation in fractions of 1.6 Gy, to a total dose of 44.8 Gy. Follow-up MRI showed disappearance of the enhancement and her condition improved, although focal deficits remained present. Three years after treatment she had a relapse which was treated with chemotherapy (bleomycin-etoposide-cisplatin) with good response. One year later a second relapse occurred.

Discussion

Germinomas are tumors of germ cell origin and represent the brain counterpart of testicular seminoma. They are relatively rare and represent 0.4%–3.4% of all primary intracranial tumors, although in Northeast Asia this is significantly higher, up to 9% (4). Like other extragonadal germ cell tumors, germinomas occur primarily in the midline, with the region of the pineal gland being the most common site, followed by the suprasellar compartment (8). They occur predominantly in children with a peak incidence around 12 years of age (4).

Depending on anatomical localization, patients with germinoma may present with a range of neurological, endocrine and psychiatric symptoms. Pineal region tumors often compress the cerebral aqueduct, resulting in progressive hydrocephalus, and invade the tectal plate, producing a paralysis of upward gaze and convergence known as Parinaud syndrome. Suprasellar germ cell tumors compress the optic chiasm, resulting in visual loss, and present with endocrinopathy caused by pituitary failure with diabetes insipidus, retarded growth and sexual maturation (8). Cure rates for this tumor exceed 90% at 10 years, and limitation of treatment-related late morbidity is therefore essential (7).

In this case, the characteristic radiological features of a germinoma were absent (3, 5). There was no mass effect, that is, compression of pre-existent anatomical structures by growth of a solid tumor, and no enhancement after gadolinium administration. Instead, the tumor was diffusely infiltrating the surrounding tissue and followed the contours of the ventricular system. In addition, the marker PLAP usually present in germinomas (2) was negative and diagnosis was made on nuclear OCT3/4 staining, which has proven to be a valuable marker in diagnosis of germ cell tumors (1, 6).

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Chapter 9

Differential expression of SOX17 and SOX2 in germ cells and stem cells has biological and clinical implications

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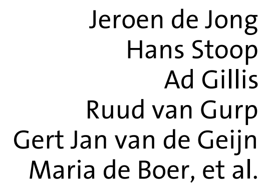

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Abstract

Combined action of SOX and POU families of transcription factors plays major roles in embryonic development. In embryonic stem cells, the combination of SOX2 and POU5F1 (OCT3/4) is essential for maintaining the undifferentiated state by activating pluripotency-linked genes, and inhibition of genes involved in differentiation. Besides embryonic stem cells, POU5F1 is also present in early germ cells, primordial germ cells, and gonocytes, where it has a role in suppression of apoptosis. Here we demonstrate that SOX2 is absent in germ cells of human fetal gonads, and as expected carcinoma *in situ* (CIS), ie the precursor lesion of testicular germ cell tumours of adolescents and adults (TGCTs), and seminoma. Based on genome-wide expression profiling, SOX17 was found to be present, instead of SOX2, in early germ cells and their malignant counterparts, CIS and seminoma. Immunohistochemistry, western blot analysis, and quantitative RT-PCR showed that SOX17 is a suitable marker to distinguish seminoma from embryonal carcinoma, confirmed in representative cell lines. Aberrant SOX2 expression can be present in Sertoli cells when associated with CIS, which can be misdiagnosed as embryonal carcinoma. In conclusion, this study demonstrates the absence of SOX2 in human embryonic and malignant germ cells, which express SOX17 in conjunction with POU5F1. This finding has both diagnostic and developmental biological implications. It allows the identification of seminoma-like cells from embryonal carcinoma based on a positive marker and might be the explanation for the different function of POU5F1 in normal and malignant germ cells versus embryonic stem cells.

Introduction

The SOX family of transcription factors is involved in orchestrating development from the early cells in the embryo to differentiated lineages of specialized cells. Currently, 20 SOX proteins have been identified in humans [1]. SOX proteins have similarity to the HMG domain of SRY, the testis-determining factor in mammals excluding monotremes [2], and are therefore named SRY-related HMG box (SOX) proteins. Although different SOX proteins can bind highly similar DNA sequences *in vitro*, they are expressed in a cell-type-specific manner by which they regulate their target genes [3]. This regulation is achieved by the collaboration of SOX proteins in association with other proteins [4]. Abundantly represented among the protein partners are the POU homeodomain proteins [5,6]. These transcription factors are also expressed in a cell-specific way and interact simultaneously with the DNA and stabilizing partner proteins [7,8]. The cooperative activity of SOX and POU proteins has been confirmed in different systems, such as lens placode formation [9], myelination of Schwann cells [10], and in oligodendrocytes [11].

The most extensively studied SOX–POU partnership is that of SOX2 and POU5F1 (also known as OCT3/4 and OCT4) in the inner cell mass of early embryonic development and the *in vitro* counterpart, ie embryonic stem (ES) cells [12,13–15]. Both factors are in the centre of an intricate network required to maintain pluripotency (see refs 16–18 for a review), where feedback mechanisms control the appropriate level of the transcription factors [19], and deviation will promote differentiation [20,21]. Genome-wide screens in human and mouse ES cells have identified a large number of target genes involved in the regulation of pluripotency and differentiation [22,23]. Besides being present in ES cells, POU5F1 is also present in primordial germ cells (PGCs), the cell of origin of gametogenesis, which arise from the proximal epiblast cells of the bi-layered embryo [24,25]. During the migration of PGCs, *POU5F1* remains highly expressed [26] and after arrival at the gonadal ridge (the PGCs are now called gonocytes), these cells progressively lose POU5F1 upon maturation during gametogenesis. Interestingly, knock-out of *POU5F1* in mouse PGCs results in induction of apoptosis, instead of differentiation as in ES cells [27]. It is not known so far how this functional difference between POU5F1 in ES and PGC cells, which might be related to a different interaction with a SOX member (see below), is regulated. Normally, 1 year after birth, no POU5F1-positive cells are present either in the gonads or in any other organs and tissues. In humans, prolonged expression of *POU5F1* is strongly associated with the development of malignant testicular germ cell tumours of adolescents and young adults (TGCTs), which clinically manifest after puberty. These so-called type II TGCTs, ie seminomas and non-seminomas, arise from a malignantly transformed PGC/gonocyte and this

dormant precursor lesion is named carcinoma *in situ* (CIS) or intratubular germ cell neoplasia unclassified (IGCNU) (see ref 28 for a review). The precursor lesion of dysgenetic gonads is known as gonadoblastoma (GB) [29,30]. After puberty, proliferation of these precursor cells will give rise to invasive seminoma (known as dysgerminoma in the ovary and dysgenetic gonad), the default pathway for CIS cells, or - by acquiring additional expression of ES cell markers - to invasive embryonal carcinoma (EC), the undifferentiated cells of non-seminoma, representing the inner cell mass during normal embryogenesis. Furthermore, EC cells can differentiate into teratoma (the somatic lineage) and the different extra-embryonal lineages, ie yolk sac tumour and choriocarcinoma, as well as the germ cell lineage [31]. POU5F1 appears to be a specific marker in TGCT diagnostics with 100% of CIS, seminoma, and EC cells, showing profound nuclear staining [32–36].

The role of SOX2 interaction with POU5F1 in maintaining pluripotency and preventing differentiation is well established [13]; however, how POU5F1 expression is involved in apoptosis control in PGCs (see above) is not clear. This cannot be due to NANOG, for example, because this protein is expressed in PGCs/gonocytes and ES, as well as in their malignant counterparts CIS/GB, seminomas, and EC [37]. The presence of both NANOG and POU5F1, and the absence of SOX2 have been proposed to be informative for distinguishing seminoma-like cells from EC. However, a specific positive marker for the seminoma-like cells would be of interest in a diagnostic setting, like we previously reported for spermatocytic seminoma compared with (classic) seminoma [38]. We show here that SOX2 is not present in germ cells of early embryonic gonads to adult testis and ovary, or in pre-invasive and invasive TGCTs by immunohistochemistry, but we have confirmed the presence in EC, as well as heterogeneously in different derivatives. SOX2 can also be present in Sertoli cells associated with CIS; this observation is novel and significant, because it may result in overdiagnosis of intratubular EC, as done recently [39]. Moreover, this study shows that SOX17, instead of SOX2, is present in CIS and seminoma cells. Although a differential pattern of expression of SOX17 has been reported before [40,41], this study for the first time investigates SOX17 in more detail, especially on the protein level, in the context of both normal germ cell development and germ cell tumour diagnosis. SOX17 has multiple functions [42–44], including a specific role in embryonic compared with adult haematopoietic stem cells [45]. Immunohistochemistry of SOX17 demonstrated nuclear co-expression with POU5F1 in fetal gonocytes, CIS, and seminoma cells, as well as in the recently characterized seminoma cell line TCam-2, but not in EC and derived cell lines. This finding allows distinction between seminoma and EC in a diagnostic setting, for which so far no absolute positive marker for seminoma is available. In addition,

this knowledge will improve our understanding of the progression of TGCTs from CIS to either seminoma or EC.

Materials and methods

Research on human gonadal and tumour samples has been performed according to the *Code for Proper Secondary Use of Human Tissue in The Netherlands*, as developed by the Dutch Federation of Medical Scientific Societies (FMWV) (version 2002), and has been approved by an institutional review board (MEC 02.981). All samples were collected in close collaboration with (paediatric) urologists and pathologists in the southwestern part of The Netherlands. The following normal gonadal samples were used: 16 samples of fetal testis (15–40 weeks' gestational age), five fetal ovaries (18–41 weeks' gestational age), 42 adult testes, and four adult ovaries. Type II TGCTs were as follows: 39 CIS samples, 50 seminomas, 42 ECs, eight yolk sac tumours, 13 choriocarcinomas, and 26 teratomas. Nine dysgenetic gonads - some containing gonadoblastoma or dysgerminoma - were used for SOX2 immunohistochemistry (see below).

Immunohistochemistry

The presence of SOX2 in normal and pathological gonadal development was studied by immunohistochemistry using a monoclonal antibody (AF2018; R&D Systems, Abingdon, UK) in a 1 : 750 dilution incubated for 2 h at room temperature. As a secondary antibody, a biotinylated rabbit anti-mouse antibody was applied (1 : 200; E0413; Dako, Glostrup, Denmark) and bound antibody complex was visualized using the horseradish peroxidase avidin–biotin complex (HRP-ABC) method. The samples examined are listed above. In addition, double staining was performed using a monoclonal antibody directed against the N-terminus of the human POU5F1 (sc-5279; 1 : 1000; sc-Santa Cruz Biotechnology, Santa Cruz, CA, USA) and SOX2, respectively, with 3-amino-9-ethyl-carbazole (A5754; Sigma, Steinheim, Germany)/H_2O_2 for red staining and Fast Blue/naphthol AS-MX phosphate (F3378 and N500; Sigma) for blue staining. Detection of SOX17 in gonadal development, normal spermatogenesis, and TGCTs was carried out with a goat polyclonal antibody (GT15094; Neuromics, Edina, MN, USA) in a 1 : 3000 dilution for 2 h at room temperature. Subsequently, a biotinylated horse anti-goat secondary antibody (1 : 200; BA-9500; Vector) was used and detection was carried out using the HRP-ABC method. Antibodies against SOX17 from SantaCruz (sc-17 355) and R&D Systems (MAB1924) were also tested, but these gave non-specific and cytoplasmic staining, tested by western blotting and immunohistochemistry, respectively. These antibodies were therefore not used for further experiments.

In addition to the fetal gonads indicated above, a series of early fetal gonads (57–63 days' gestational age) was obtained following medical termination of pregnancy [46] and fixed in Bouin's. Extensive testing using an appropriate positive control showed that the SOX2 antibody (AF-2018; R&D Systems) did not work on Bouin-fixed material and therefore only SOX17 (GT15094; Neuromics) immunohistochemistry could be performed on these samples.

Cell culture

The cell line TCam-2, recently confirmed to be a seminoma cell line [47], was obtained from Sohei Kitazawa (Division of Molecular Pathology, Kobe University, Japan). The cells were cultured in RPMI 1640 (Invitrogen, Breda, The Netherlands) supplemented with 10% fetal calf serum (Hyclone, Thermo Fisher Scientific, Etten-Leur, The Netherlands) and penicillin/streptomycin (Invitrogen, Breda, The Netherlands) at 37°C in a humidified cell culture incubator with 5% carbon dioxide. The EC cell lines Tera-1 [48], NTera-2 [49], 2102Ep [50], 833KE [51], and NCCIT [52] were cultured as described before [53]. The cell line JKT-1, recently demonstrated not to be a seminoma cell line, was cultured as described before [54]. The cells likely represent a later stage of germ cell development. Cells were trypsinized and harvested for the different experiments. Cytospins were prepared and RNA was isolated (see below).

Protein isolation and western blot analysis

Isolation of protein and western blot analysis were essentially performed as described previously [33]. The antibodies used were the same as those used for immunohistochemistry. In addition, mouse monoclonal beta-actin (clone AC-15; Sigma Aldrich, St Louis, MO, USA) was used. Binding of the primary antibodies was visualized by using IRDye donkey anti-mouse or donkey anti-goat secondary antibodies and the blots were scanned on the Odyssey infrared imaging system (all from LI COR Biosciences, NE, USA).

Quantitative RT-PCR for SOX2 and SOX17 in normal testis and TGCTs

High-quality total RNA was extracted from normal testis (n = 4) and patient-derived TGCT samples con-taining either CIS (n = 4) or seminoma (n = 12) or EC (n = 11) using an RNAqueous-4PCR kit (Ambion Europe, Huntingdon, UK) according to the manufacturer's instructions. In addition, RNA from the cell lines JKT-1, TCam-2, and the EC cell lines Tera-1, NTera-2, 2102Ep, and NCCIT was used. Samples were treated with DNase and checked for residual DNA contamination by PCR. Quantitative PCR was performed using the real-time PCR ABI PRISM 7700 sequence detector system (Applied Biosystems, Foster City, CA, USA). For the detection of SOX2 expression, the following primer set was used: SOX2-F 5' -ATGGAGACGGACCTGAAGC-3' and SOX2-R 5'-GCAGCGTGTACTTATCCTTC-3'. Quantitative values were obtained from the Ct. SOX2 mRNA was quantified

relative to HPRT [SOX2 mRNA value = $2^{(\text{mean Ct HPRT} - \text{mean Ct SOX2})}$] [38]. For the detection of SOX17, the following primers were used: SOX17-F (exon 1) 5'-GATGCGGGAT ACGCCAGTGAC-3' and SOX17-R (exon 2) 5'-GCTCTGCCTCCTCCACGAAG-3' and SOX17 mRNA was quantified by the formula described above.

Micro-array expression profiling

The expression profiles of frozen TGCTs (five spermatocytic seminomas, three dysgerminomas, being the ovarian counterpart of seminoma [38], four seminomas, and five ECs), the cell line JKT-1, and the seminomatous TCam-2 cell line were determined using the Affymetrix GeneChip Human Genome U133 plus 2.0 array as described before [38]. Analysis was done using Omniviz and SAM software as reported in the same paper. Expression levels of each SOX family member were analysed for all samples and plotted as a graph.

Results

SOX2 is not expressed in normal human gonadogenesis

To determine whether SOX2 may play a role in normal germ cell development and gonadogenesis, immunohistochemistry was performed on gonads from early fetal life (age: 15 weeks' gestational age and older) to adult ovary and testis. The SOX2 protein was not expressed in germ cells, regardless of their state of maturation (data not shown). Occasionally, SOX2-positive Sertoli cells were present in normal adult testis; however, the expression level of SOX2 mRNA was very low (Figure 1A).

SOX2 in testicular germ cell tumours

The differential expression of SOX2 between seminoma and EC cells is used as an argument to explain the differences in pluripotency between these cells [41,55]. In contrast to SOX2, the other markers related to pluripotency, ie POU5F1 and NANOG, show a similar pattern of expression in these histological subtypes. The relative overexpression of several core 'stemness' genes, such as *EBAF*, *TDGF1*, and *SOX2*, reflects the undifferentiated state of EC cells and is the starting point of their differentiation potential, analogous to the ES cells derived from the inner cell mass of early embryonic development.

To study SOX2 expression in the development of TGCTs, tissue micro-arrays (TMAs) of the various invasive histological subtypes and the precursor lesion CIS were used. As expected, also based on previous studies [56], all EC cells were positive for SOX2, while CIS, seminoma, yolk sac tumour, and choriocarcinoma were consistently negative. In teratoma, a small number of SOX2-positive cuboidal or columnar epithelium cells of endodermal origin were present, as was also recently reported in teratomas of the central nervous system [57]. However,

in the seminiferous tubules containing CIS cells (always POU5F1-positive), a large number of SOX2-positive cells were seen to be more luminally situated than the CIS cells (Figure 1B). This observation has not been reported before and deserves further investigation. Detailed morphological study of these SOX2-positive cells and double staining for SOX2 and vimentin showed that they are in fact Sertoli cells, and not tumour cells (Figure 1C). Indeed, quantitative RT-PCR confirmed the elevated expression of SOX2 in samples containing CIS cells, compared with normal testis (Figure 1A). This presence of SOX2 in the context of germ cell tumours might be overdiagnosed as EC, when per-formed as single staining. However, when it is combined with staining for POU5F1, it must be investigated whether SOX2 and POU5f1 are present within the same cells, indicating EC, preferentially using a double-staining approach.

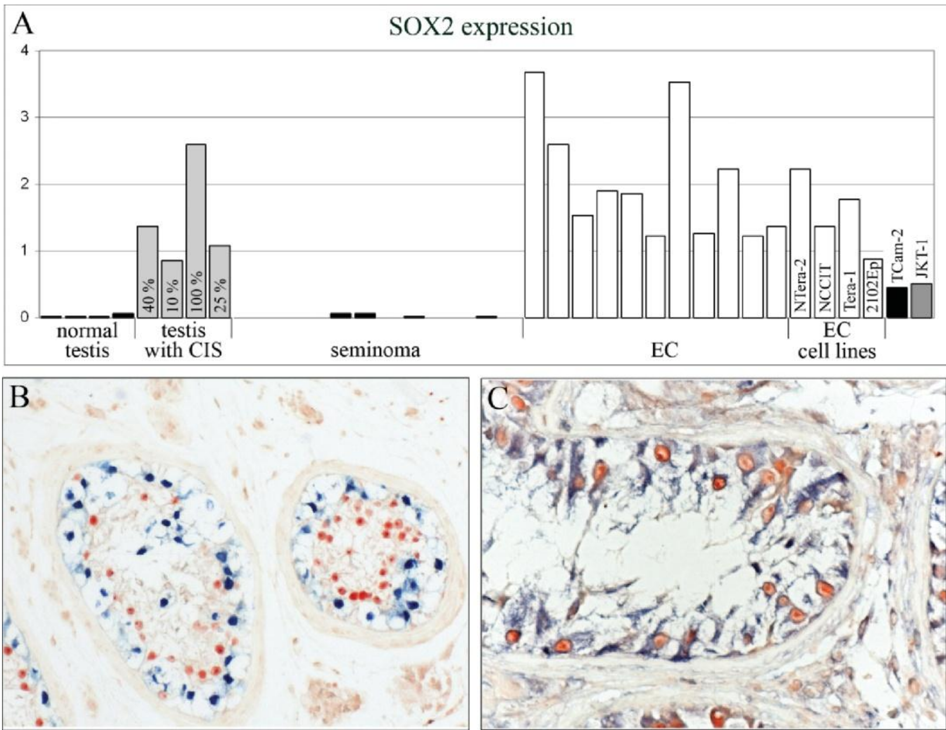

Figure 1. (A) Quantitative RT-PCR for SOX2 on TGCT samples. The cell line JKT-1, the seminomatous cell line TCam-2, and the EC cell lines (NTera-2, NCCIT, Tera-1, and 2102Ep) were tested. High expression of SOX2 was observed in the EC samples and cell lines. In testis parenchyma containing CIS (the percentage is indicated in the bars), SOX2 mRNA expression was elevated compared with normal testis; the level of expression parallels the amount of CIS-containing seminiferous tubules. TCam-2 and JKT-1 show a level of expression in between normal testis/seminoma and EC samples and cell lines.

(**B) Double immunohistochemistry for SOX2 (red) and POU5F1 (blue).** SOX2 is not expressed in the POU5F1-positive CIS cells, but in a cell type more luminally located, being Sertoli cells. **(C)** Double staining for vimentin (blue) and SOX2 (red) combined with morphology and the position in the seminiferous tubule shows that the SOX2-positive cells are Sertoli cells

SOX17 is expressed in gonocytes, CIS/gonadoblastoma, and seminoma/dysgerminoma cells

With the absence of SOX2 expression in germ cells and their malignant counterparts, CIS and seminoma cells, the question arises as to whether another SOX member will be specifically expressed in these cells. This might be related to the different regulatory function of POU5F1 in the germ cells versus the stem cells, ie apoptosis and differentiation. To test this, we screened the expression profiles of all SOX family members in TGCT samples and the cell lines JKT-1 and TCam-2 (Figure 2). The expression pattern of SOX2 was confirmed on the mRNA level (being specifically expressed in EC). Most of the *SOX* members gave a non-specific pattern for the different histological elements investigated, including the cell lines. Possible exceptions are SOX15 and SOX17. For this study, we focused on SOX17, although SOX15 is of interest as well. *SOX17* was highly expressed in all seminoma/dysgerminoma samples, as well as the seminoma cell line TCam-2, and virtually absent in EC cells (absolute value for specific probe sets below 30). The mRNA expression profile of SOX17 in POU5F1-positive TGCTs (seminoma /dysgerminoma and EC) was exactly complementary to that of SOX2. Quantitative RT-PCR confirmed the elevated SOX17 expression in seminoma samples and the seminoma cell line TCam-2, compared with EC samples and cell lines (Figure 3A). Immunohistochemistry using a specific antibody showed the nuclear expression of SOX17 in all CIS/gonadoblastoma and seminoma/ dysgerminoma cells, being co-expressed with POU5F1 (Figures 3B and 3C). In addition, the seminoma cell line TCam-2 expressed SOX17 protein (Figures 3D and 3E), while no SOX17 was detected on cytospins of EC cell lines (data not shown) or by western blot analysis (Figure 3E). No staining was identified in either Sertoli cells (testis) or granulosa cells (ovary).

The specific nuclear staining for SOX17 is of diagnostic value in discriminating seminoma/dysgerminoma from EC. The differential mRNA expression of SOX17 between seminoma/dysgerminoma and spermatocytic seminoma, as shown in Figure 2, is in line with earlier findings [38]. In spite of its value in distinguishing seminoma from embryonal carcinoma based on a positive marker in the first, SOX17 is not a suitable marker for the diagnosis of CIS, because it is found in different maturation stages of spermatogenesis (Figure 3F). In addition, SOX17 expression is present in fetal male and female gonocytes (the earliest sample tested was a 57 days old gestational age gonad) and remains present in these cells throughout fetal life (Figure 3G). In contrast to male spermatogenesis, SOX17 is not detected in oogenesis beyond the stage of the gonocyte. However, SOX17 protein is detected in the epithelium of the Fallopian tube and rete testis (data not shown). Therefore POU5F1 is still the best marker for the detection of CIS in the adult testis [33]. However, SOX17 can be helpful in distinguishing

between the invasive components seminoma and EC, especially when performed together with analysis of POU5F1.

Discussion

Transcription factors act as pathway switches in embryonic development and commitment to the differentiation lineages. Specific combinations of transcription factors belonging to the SOX and POU families are known to establish tissue and cell-specific expression of target genes. In this study, we focused on possible protein partners of POU5F1 which are subsequently expressed during embryonic development in cells of the inner cell mass, the epiblast and PGCs. In ES cells, the intimate relationship between POU5F1 and SOX2 has been most extensively studied and the binding of both proteins to adjacent sites on the DNA with simultaneous physical protein interaction controls a large number of target genes involved in pluripotency and differentiation. However, in PGCs and gonocytes, SOX2 is lacking (this paper), and POU5F1 has a regulatory role in apoptosis, for which the mechanism is unknown so far.

Besides ES cells, SOX2 is abundantly expressed in differentiating and differentiated cells and is found in endodermal cells of the primitive foregut [58], the developing inner ear [59], and the central nervous system [60]. However, in these cells, it is never detected in combination with POU5F1, which is likely related to the absence of pluripotency. It has been reported that *SOX2* is expressed in mouse fetal germ cells and neonatal ovine gonads [61,62], but the actual data were not shown. These reports were confirmed by RNA in situ detecting SOX2 in male E12.5 to E15.5 gonad/mesonephros tissues; however, no protein detection was done [63]. Recently, the expression of SOX2 in the establishment and early migration of mouse PGCs was identified by quantitative single-cell gene expression profiling [64]. These results indicated that SOX2 expression is not immediately lost upon commitment to the germ cell lineage. However, the timing of SOX2 down-regulation in gametogenesis remains to be clarified. Our results show that SOX2 is not expressed in human gonadogenesis from 15 weeks of gestation onwards, or in normal adult testis and ovary.

The study in early mouse PGCs also investigated the expression pattern of *SOX3* and *SOX17* [64]. Both genes were transiently expressed in migrating PGCs at developmental stage E7.25 dpc. Here we demonstrated for the first time that *SOX17* is expressed and the protein is present in CIS and seminoma cells, but not in EC cells, either *in vivo* tumours or cell lines. This is in line with previous studies, which also demonstrated *SOX17* as differentially expressed between seminoma and embryonal carcinoma at the mRNA level, although no follow-up studies were performed [40,41]. The absence of *SOX3* expression in seminoma cells (Figure 2)

could be related to the timing of the maturation block that has occurred in the precursor cells of TGCTs, or be the result of species-specific differences. So far, the existing evidence for a role of SOX17 in gametogenesis is fragmented. Heterozygous *SOX17* knock-out mice are normal and fertile, while homozygous knock-out mice are deficient in gut endoderm and after 10.5 dpc, no homozygous mutants are found [65]. This lethality due to endoderm deficiency is prior to gonad development and therefore the role of SOX17 absence during gametogenesis can be studied only in conditional knock-outs. Furthermore, two isoforms (one without an HMG domain) have been isolated from an adult mouse testis library. Analysis of these isoforms suggests that SOX17 may function as a transcriptional activator in pre-meiotic germ cells, and that a splicing switch into a truncated SOX17 protein may lead to loss of its function in post-meiotic germ cells [43]. So far, no human SOX17 splice variants have been identified. The co-expression of SOX17 and POU5F1 in CIS and seminoma cells in the absence of SOX2 qualifies SOX17 as a potential protein partner in these cells, which is currently under investigation. This observation initiates a new hypothesis in the regulation of the function of POU5F1 (see below). In addition, *SOX17* is a repressed target gene of the POU5F1 protein and not of SOX2 in human ES cells [22]. It remains to be investigated whether this is also the case in PGCs.

The co-expression of SOX17 and POU5F1 in the seminoma cell line TCam-2 compared with established EC cell lines, being SOX2-positive and SOX17-negative, is an informative experimental model for studying this hypothesis, and for identifying common target genes in CIS and seminoma. One of the interesting hypotheses to test is whether SOX17 is the determinant factor resulting in the regulatory role of POU5F1 in apoptosis in normal and malignant germ cells, while the co-presence with SOX2 regulates differentiation in normal and malignant embryonic stem cells. The WNT pathway is possibly a regulatory factor involved in the transcriptional activity of POU5F1 and SOX17 in normal and malignant germ cells. This has been shown in ES cells [66] and embryonic endoderm [67] in which the proteins physically interact with β-catenin. A recent observation is that SOX17 is essential for fetal and neonatal haematopoietic stem cells (HSCs), but not for adult HSCs [45]. Knock-out studies and expression data of SOX17 in primitive endoderm, fetal HSCs, and early germ cells indicate a crucial role for this transcription factor in a number of lineages during embryonic and fetal development. The origin of PGCs from the primitive endoderm, like fetal HSCs, is of high interest in this context.

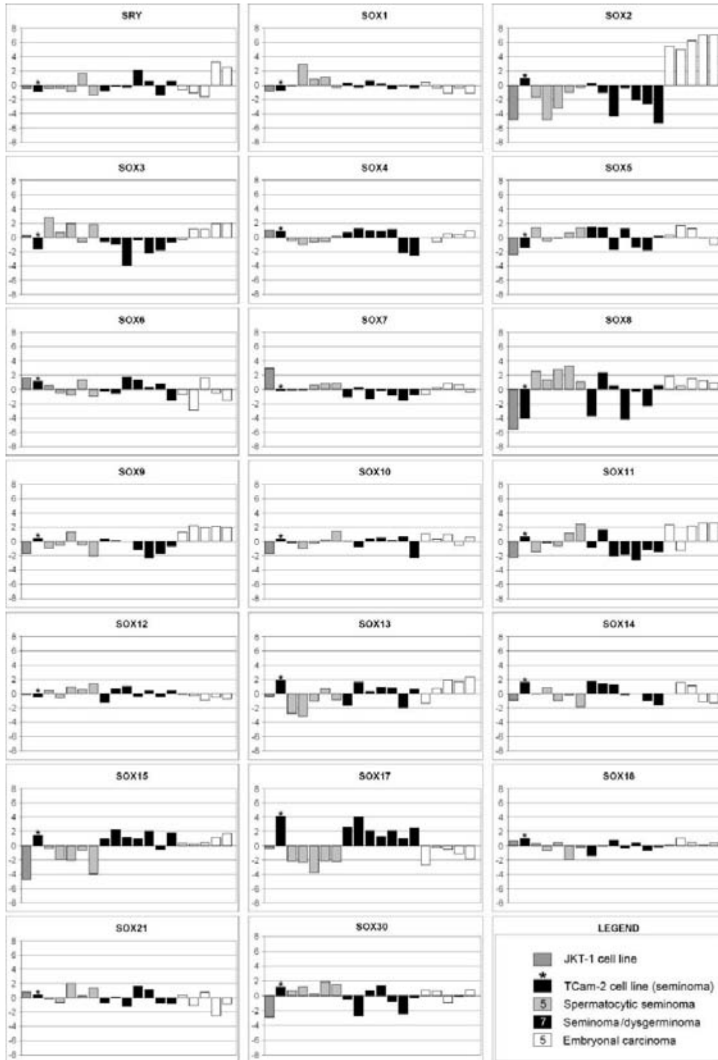

Figure 2. Expression results of all 20 human SOX family members in TGCT samples and cell lines JKT-1 and TCam-2 based on genome-wide (Affymetrix) expression profiling (log2 transformed data). The following samples were used: five spermatocytic seminomas, four seminomas, three dysgerminomas, and five ECs (also indicated in the legend in the bottom right corner of the figure explaining the different bar colours). The main findings were the well-known high SOX2 expression in EC and low expression in seminoma/dysgerminoma. The profile of SOX17 shows an inverse relationship with low expression in EC and high expression in seminoma/dysgerminoma samples and the seminoma cell line TCam-2 (indicated by an asterisk)

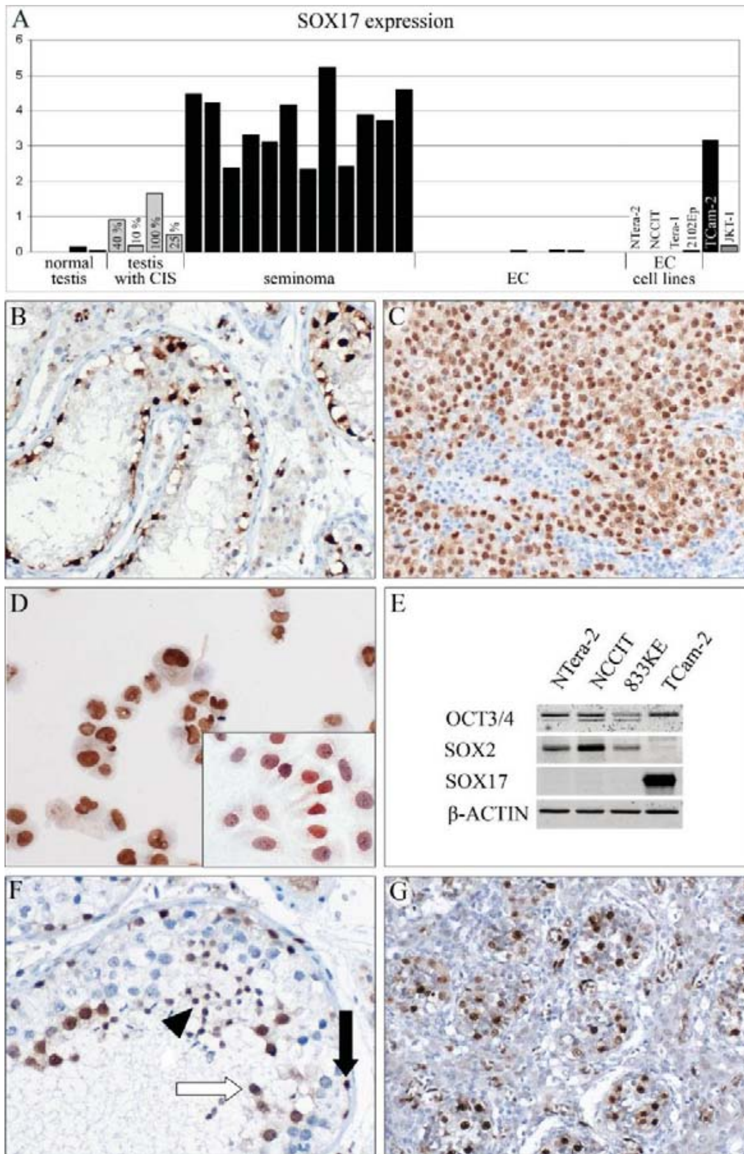

Figure 3. (A) Quantitative RT-PCR for SOX17 on TGCT samples, the cell line JKT-1, the seminomatous cell line TCam-2, and EC cell lines (NTera-2, NCCIT, Tera-1, and 2102Ep) demonstrates high expression of SOX17 in the seminoma samples and cell line TCam-2, whereas the expression is low in all the other samples. Nuclear SOX17 signal (brown) in **(B)** CIS, **(C)** seminoma, and **(D)** the seminomatous cell line TCam-2. Inset: nuclear POU5F1 staining in TCam-2 cells. **(E)** Western blotting shows a complementary expression pattern for SOX2 and SOX17 in the TCam-2 and EC cell lines with high SOX17 expression in TCam-2 and no SOX17 expression in the EC cell lines NTera-2, NCCIT, and 833KE. SOX2 is highly expressed in the EC cell lines but not in TCam-2. The embryonic stem cell marker OCT3/4 is expressed in all samples. ß -actin was used as a loading control. **(F)** SOX17 protein is present in various maturation stages of spermatogenesis: spermatogonium (black arrow), secondary spermatocyte (white arrow), and spermatid (black arrowhead), while primary spermatocytes, spermatozoa, and Sertoli cells are negative. **(G)** Nuclear SOX17 in gonocytes of an 18-week-old fetus

As suggested, the presence of expression of SOX17 in CIS could indicate the timing of the first step in the development of TGCTs. Whether these cells are already malignantly transformed at the time of this maturation arrest or acquire malignant potential later in development as a result of an unfavourable micro-environment for immature germ cells is not yet clear. Therefore analysis of these markers in patients with disorders of sex development (DSD) will be informative, because some of them show a significant increased risk for type II GCTs [30]. Our study of SOX2 expression in normal gonadogenesis and TGCTs demonstrated that SOX2 can be present in Sertoli cells in CIS-containing seminiferous tubules, whereas this was rarely observed in tubules without CIS. SOX2 is not expressed in fetal gonads from 15 weeks of gestation onwards, indicating either aberrant expression of SOX2 in Sertoli cells in CIS-positive tubules or possibly persistent expression assuming that SOX2 is expressed in Sertoli cells before 15 weeks. Either way, SOX2 expression in CIS-positive tubules reflects an abnormal micro-environment which might have contributed to the aetiology of TGCT initiation or has a later origin under the influence of the aberrant germ cells. An important implication of this result is that SOX2 expression in seminiferous tubules should not be mistaken for intratubular EC known to be SOX2-positive. This misinterpretation has indeed been made recently, leading to overdiagnosis, and possibly overtreatment [39].

Overall, this study shows the significant value of combined investigation of normal germ cell development in shedding light on the pathogenesis of type II GCTs and vice versa. It adds SOX17 as a positive marker for seminoma-like cells to the spectrum of diagnostic markers, which will initiate a new line of research on the regulation of apoptosis and differentiation in the malignant counterparts of PGC and ES, which is so characteristic of this type of cancer.

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Chapter 10

Stem cell factor as a novel diagnostic marker for early malignant germ cells

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Abstract

Carcinoma *in situ* (CIS) of the testis is the pre-invasive stage of type II testicular germ cell tumours (TGCTs) of adolescents and adults. These tumours are the most frequently diagnosed cancer in Caucasian adolescents and young adults. In dysgenetic gonads, the precursor of type II GCTs can be either CIS or a lesion known as gonadoblastoma (GB). CIS/GB originates from a primordial germ cell (PGC)/gonocyte, ie an embryonic cell. CIS can be cured by local low-dose irradiation, with limited side effects on hormonal function. Therefore, strategies for early diagnosis of CIS are essential. Various markers are informative to diagnose CIS in adult testis by immunohistochemistry, including c-KIT, PLAP, AP-2*y* , NANOG, and POU5F1 (OCT3/4). OCT3/4 is the most informative and consistent in presence and expression level, resulting in intense nuclear staining. In the case of maturational delay of germ cells, frequently present in gonads of individuals at risk for type II (T)GCTs, use of these markers can result in overdiagnosis of malignant germ cells. This demonstrates the need for a more specific diagnostic marker to distinguish malignant germ cells from germ cells showing maturation delay. Here we report the novel finding that immunohistochemical detection of stem cell factor (SCF), the c-KIT ligand, is informative in this context. This was demonstrated in over 400 cases of normal (fetal, neonatal, infantile, and adult) and pathological gonads, as well as TGCT-derived cell lines, specifically in cases of CIS and GB. Both membrane-bound and soluble SCF were expressed, suggestive of an autocrine loop. SCF immunohistochemistry can be a valuable diagnostic tool, in addition to OCT3/4, to screen for precursor lesions of TGCTs, especially in patients with germ cell maturation delay.

Introduction

Type II testicular germ cell tumours (TGCTs) are the most frequent malignancy among Caucasian men aged 20–45 years [1,2], with rising incidence in most Western countries [3,4]. TGCTs originate from a common precursor, known as carcinoma *in situ* (CIS) or intratubular germ cell neoplasia unclassified (ITGCNU) [2,5]. Dysgenetic gonads show a higher incidence of these tumours [2], of which gonadoblastoma (GB), or CIS, is the precursor [6–8]. CIS/GB cells resemble fetal germ cells [primordial germ cells (PGCs) or gonocytes] by morphology [9] and an erased pattern of genomic imprinting [10]. They also express embryonic markers, including PLAP [11,12], POU5F1 (OCT3/4) [13], NANOG [14], AP-2*y* [15,16], and c-KIT [17]. These are found in normal fetal germ cells and are lost upon further maturation [11,12,14,16]. Germ cell maturation delay can lead to overdiagnosis and overtreatment [18–20], as in cases of cryptorchidism and disorders of sex development (DSD), ie hypovirilization or gonadal dysgenesis [21–25] (for reviews see refs 18 and 26). [Correction made here after initial online publication]. Although criteria have been developed to distinguish malignant germ cells from cells showing delayed maturation only in cases of hypovirilization [27], reliable diagnostic tools are lacking. Therefore a specific marker for malignant germ cells would be informative.

Normal germ cell lineage development depends on the c-KIT–stem cell factor (SCF) pathway [28]. In mice, c-KIT (the receptor) is present in PGCs, gonocytes, spermatogonia, spermatocytes, round spermatids, and Leydig cells [29–33]. c-KIT signalling is also important for human spermatogenesis, demonstrated by defects in this pathway (for a review see ref 34). However, the pattern of expression is different between mice and humans. In the latter, c-KIT expression is low and is not detected in adult testis, unless frozen tissue and a sensitive detection method are used [17,35–37]. This makes c-KIT a diagnostic marker for CIS in adults on formalin-fixed, paraffin-embedded (FFPE) material [11,17]. However, false-negative findings have been reported [38,39].In adult testes, the c-KIT ligand, stem cell factor (SCF), is produced by Sertoli cells [34,40–42], under the control of follicle-stimulating hormone (FSH) [43]. Due to alternative RNA splicing and post-translational processing, two forms of SCF exist: a membrane-bound and a soluble protein [44,45]. Membrane-bound SCF is the most efficient in establishing and maintaining germ cells [46–50], while soluble SCF activates c-KIT on Leydig cells to induce testosterone production [51].

We investigated the presence of SCF in a series of normal and pathological gonads. SCF is virtually absent in normal testis, while all cases of CIS and GB were positive. No SCF was found to be related to germ cells with proven maturational delay, unlike the other known CIS/GB markers. This demonstrates the additional

value of immunohistochemical detection of SCF to identify malignant germ cells, especially in the case of germ cell maturation delay.

Materials and methods

Use of tissues for scientific purposes was approved by an institutional review board (MEC 02.981 and CCR2041). Samples were used according to the '*Code for Proper Secondary Use of Human Tissue in The Netherlands*', from the Dutch Federation of Medical Scientific Societies (FMWV) (version 2002).

Pathological tissues

Tissue samples were collected as described before [52] and tumours were diagnosed according to WHO standards [5]. Male gonads, obtained after spontaneous or induced abortions (gestational age 15–40 weeks) or from premature and term neonates that died shortly after birth, were included as previously described [11].

Gonadal tissues	n	Positive	%
Fetal and infantile testicular tissue			
Fetal gonads	26	2	8
Infantile biopsies	7	0	0
Infantile gonads	20	0	0
Hypovirilization patients	39	5	13
Testicular tissue adults			
Biopsies	23	3	13
Total tissue sections TGCT			
CIS	81	81	100
Seminoma	68	64	94
Non-seminoma	72	47	65
Embryonal carcinoma	25	11	44
Yolk sac tumour	23	20	87
Choriocarcinoma	5	4	80
Teratoma	19	12	63
TMA			
CIS	43	43	100
Seminoma	50	50	100
Non-seminoma	121	54	45
Embryonal carcinoma	47	14	30
Yolk sac tumour	30	22	73
Choriocarcinoma	17	6	35
Teratoma	27	12	44
Gonadoblastoma	13	13	100

Table 1.
SCF immunohistochemical staining data of FFPE material of gonads of fetal, infantile, and hypovirilized patients, as well as CIS, TGCTs, and gonadoblastomas. Note the higher percentage of SCF-positive cases when comparing total tissue sections with TMA, due to heterogeneity

Furthermore, gonads of complete (CAIS) or partial androgen insensitivity syndrome (PAIS), dysgenetic gonads [27], and open surgical testicular biopsies from adult infertile patients as well as gonadal biopsies and complete orchidectomy samples from routine paediatric urological practice were included (Table 1).

For a quick scan, tissue microarrays (TMAs) were used, including seminoma, CIS, embryonal carcinoma, yolk sac tumour, choriocarcinoma, and teratoma (see Table 1).

Cell lines

Cytospins were prepared from type II (T)GCT-derived cell lines, cultured according to standard procedures, including the embryonal carcinoma cell lines Tera1 [53], 2102Ep [54], NT2 [55], NCCIT [56], and the seminoma-derived cell line TCam-2 [57–59].

Immunohistochemistry

The antibodies used have been described before [11,12]. The SCF antibody is a goat polyclonal (N19; Santa Cruz Biotechnology; 1 : 500 to 1 : 350), detecting both membrane-bound and soluble human SCF. Its specificity has been demonstrated previously [60]. Staining was performed as described previously [61]: tissue sections were incubated, after antigen retrieval [62], with primary antibody overnight at 4^{0}C (PLAP, c-KIT, SCF) or for 2 h at room temperature (OCT3/4). Frozen sections and cytospins of cell lines were incubated for 1 h at room temperature with the antibody for SCF and c-KIT after acetone fixation. For negative controls, the primary antibody was omitted, resulting in complete absence of signal.

Double staining was performed as described previously [27]. Immunofluorescence was carried out on acetone-fixed and paraformaldehyde-treated frozen sections of normal testicular parenchyma by using the secondary biotinylated antibody before visualization with avidin-CY3 and counterstaining with Vectashield (Vector)/DAPI (Sigma Chemical Co, St Louis, MO, USA).

Quantitative RT-PCR for c-KIT and SCF

RNA isolation, cDNA synthesis, and quantitative RT-PCR for c-KIT and SCF were performed essentially as described previously [63–65]. The primers, amplifying both membrane-bound and soluble SCF, were SCF-f: 5’-AGCCAAGTCTTACAAGGGCA-3’ and SCF-r: 5’-TAAATGAGACCCAAGTCCCG-3’. Mastermix from Finnzyme was used (Bioke, The Netherlands). In total, 79 seminomas, 43 non-seminomas, 19 normal testes, 14 CIS-containing testicular parenchyma cases, and 7 peripheral blood samples were analysed. Statistical analysis was performed using SPSS v11.5 software (SPSS Inc, Chicago, IL, USA).

RT-PCR for mRNA analysis of the membrane-bound and soluble SCF

RT-PCR was performed to analyse the presence of membrane-bound and soluble SCF, using SCF-f: 5'-ATGACCTTGTGGAGTGCGTC-3' and SCF-r2: 5' - ACTTGGCTGTCTCTTCTTCCAG-3' (Figure 6A). Amplification was performed using Platinum *Taq* DNA polymerase High Fidelity (Invitrogen) for 2 min at 95°C, followed by 38 cycles of 94°C for 30 s, 58°C for 30 s, and 68°C for 45 s. A boost PCR, identical to the first, was done for 20 additional cycles.

miRNA expression analysis

RNA isolation and analysis for the expression of known miRNAs with the *mir*Vana miRNA Isolation Kit (Ambion, Austin, TX, USA) and TaqMan MicroRNA Assays Human Panel (Applied Biosystems, Foster City, CA, USA) were carried out according to the manufacturers' protocol, as previously reported [52].

Results

To study the suitability of immunohistochemical detection of SCF, a series of well-characterized histological samples was evaluated. Characteristics of the cases are summarized in Table 1 and the observations are discussed below for the different subgroups.

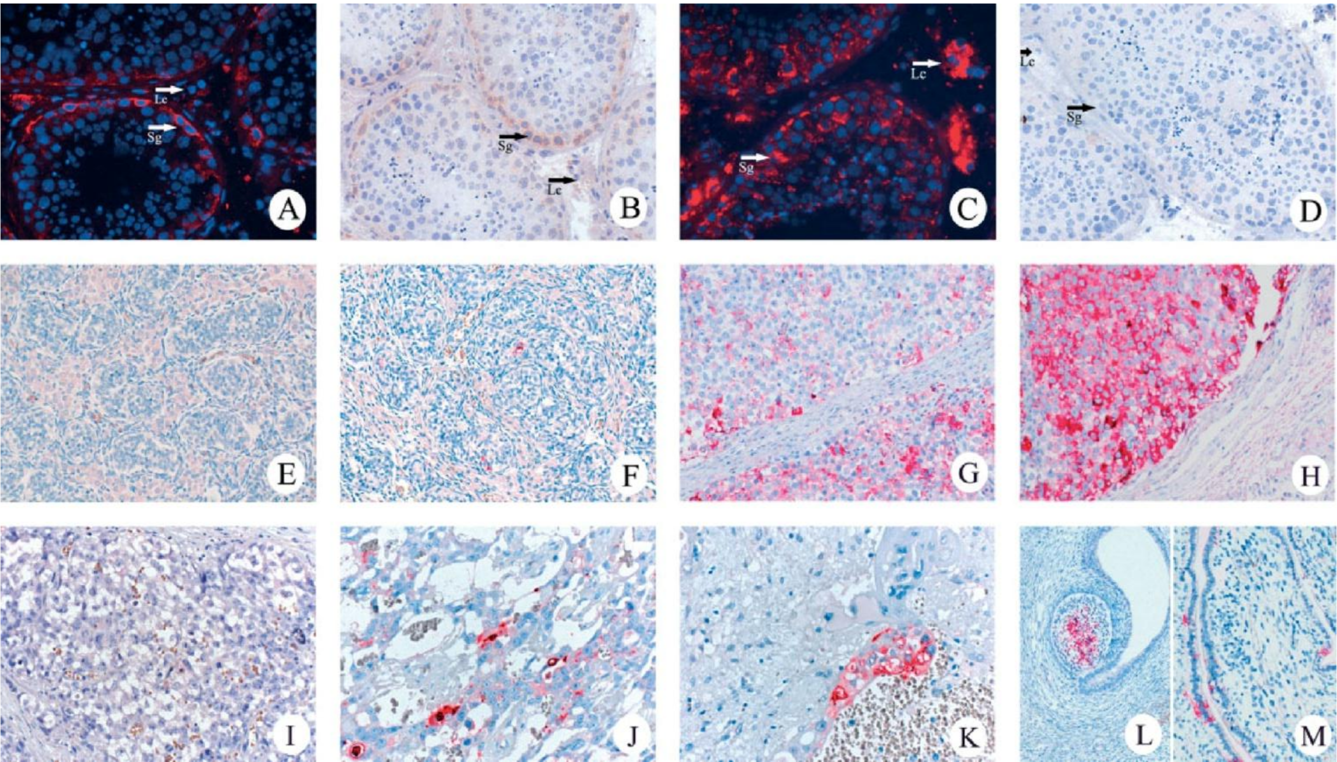
Normal adult testes

Fluorescent and non-fluorescent detection of immunostaining on frozen normal adult testicular tissue (n = 3) showed positive staining for c-KIT in spermatogonia and Leydig cells (Figures 1A and 1B), as reported earlier [35]. Fluorescent visualization of SCF showed diffuse staining in the seminiferous tubules and Leydig cells (Figure 1C), while SCF was not seen with non-fluorescent detection (Figure 1D). c-KIT and SCF were not detectable on FFPE tissue of the same samples, irrespective of pretreatment and visualization protocols (data not shown). All the immunohistochemistry presented in the following sections is on FFPE material, representative for routine pathology.

Fetal gonads

SCF immunohistochemistry showed that only two out of 26 fetal gonads had a few positive cells [see Figures 1E (negative) and 1F (positive)]; these were from an 18-week-old male trisomy 21 fetus without further abnormalities and a 16-week-old male fetus with bicephaly. Trisomy 21 is indeed a risk factor for type II TGCTs [66], associated with maturational delay of germ cells [19]. All the others were negative for SCF, including cases of ages similar to those of the positive cases.

Figure 1. Immunohistochemistry for c-KIT **(A, B)** and SCF **(C, D)** on frozen normal adult testicular tissue: **(A)** fluorescent detection and **(B)** bright field detection of c-KIT. Arrows indicate positive spermatogonia (Sg) and Leydig cells (Lc). SCF immunostaining with **(C)** fluorescent detection (showing diffusely positive staining in the tubules and Leydig cells) and **(D)** non-fluorescent detection of SCF, being negative in the latter. SCF staining is **(E)** negative in a fetal male gonad of 15 weeks' gestational age and **(F)** reveals a few SCF-positive cells in a gonad of an 18-week-old male fetus with trisomy 21. Immunohistochemistry for SCF of invasive TGCTs **(G–M)** demonstrates heterogeneity in the different histological subtypes: **(G, H)** seminoma; **(I)** embryonal carcinoma; **(J)** yolk sac tumour; **(K)** choriocarcinoma; **(L, M)** two elements of mature teratoma



Invasive TGCTs

All TGCTs represented in TMA showed a heterogeneous staining pattern for SCF (Table 1), confirmed on total tissue section (see Figures 1G-1M and Table 1). The majority showed some positive staining; seminomas heterogeneously, both in number of positive cells and in signal intensity (Figures 1G and 1H). Non-seminomas showed different patterns in histological subgroups (Figures 1I–1M) (positive: 44% of embryonal carcinoma, 87% of yolk sac tumours, and 80% of choriocarcinomas). In 63% of the teratomas, positive cells were found in various types of immature and mature tissue (eg stratified epithelium, cytoplasm of cylindrical cells, and subepithelial cells with cylindrical differentiation, Figures 1L and 1M).

Pre-invasive lesions: CIS and GB

All CIS samples, both adjacent to seminoma and adjacent to non-seminoma, represented in TMA and total sections, were positive for SCF (see Table 1 and Figure 2A). This was also found for CIS before the development of invasiveness (*n* = 3) (Figure 2B). Double staining (OCT3/4 and SCF) confirmed the identity of CIS cells (Figures 2C and 2D). Like CIS, all GBs (*n* = 13) and intratubular seminomas were positive for SCF (Figures 2E and 2F).

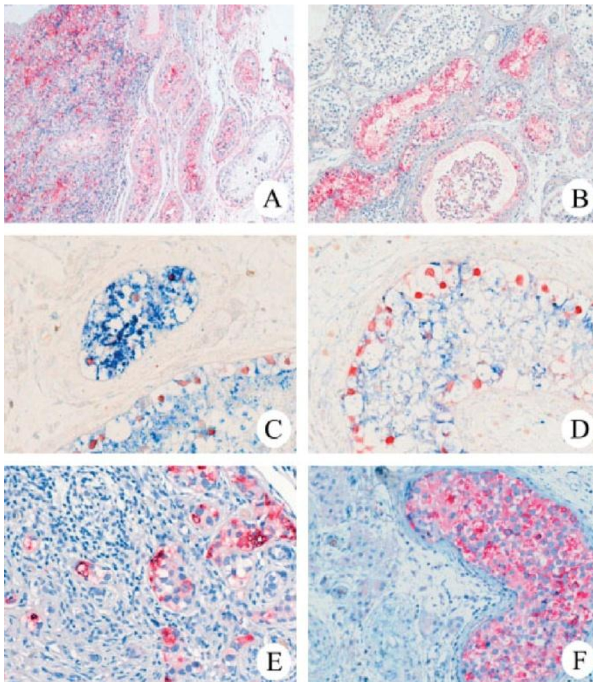


Figure 2. Immunohistochemical staining for SCF (red). **(A)** CIS adjacent to an invasive seminoma as well as **(B)** CIS adjacent to normal testicular tissue. **(C, D)** double staining for OCT3/4 (red) and SCF (blue) on CIS showing copresence; **(E)** gonadoblastoma and **(F)** intratubular seminoma are both positive for SCF as well.

Hypovirilization and infantile patients

A series of patients with various forms of hypovirilization was investigated, all known to have a certain delay in germ cell maturation, previously characterized using PLAP, c-KIT, and OCT3/4 [27] (Figures 3A–3C). No CIS was diagnosed based on the WHO criteria and no SCF was detected using immunohistochemistry (Figure 3D). In contrast, we reported three cases of hypovirilization with CIS, based on patient age, staining, and distribution of OCT3/4-positive cells [27]. These cases were indeed positive for SCF (Figures 3E and 3F), restricted to the CIS-containing seminiferous tubules.

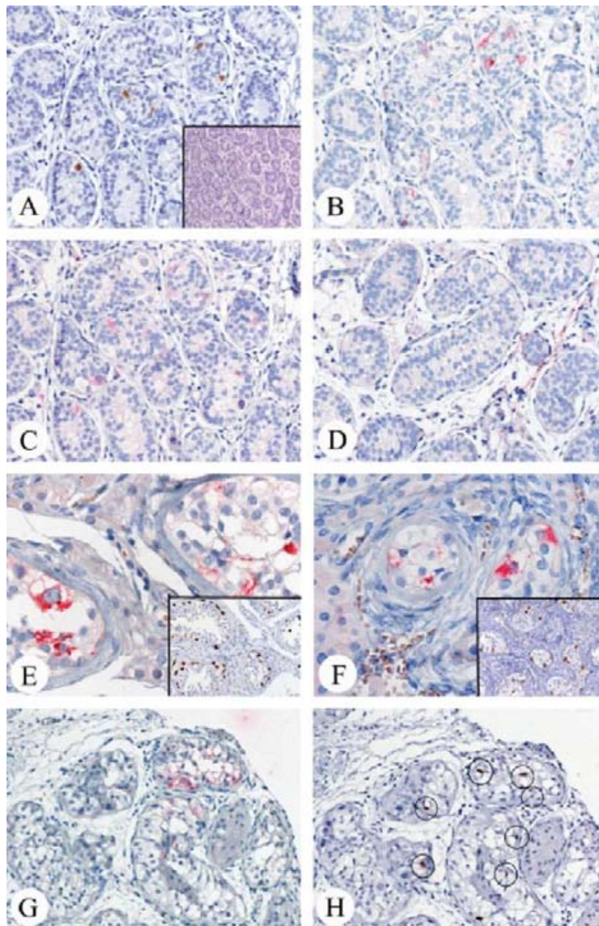


Figure 3. Immunohistochemistry on parallel sections of gonadal tissue of a DSD patient with hypovirilization without CIS. (A) OCT3/4. Inset H&E staining **(B)** PLAP, **(C)** c-KIT, and **(D)** SCF.
There is colocalization of all markers except SCF, which is negative **(D)**.
In contrast, SCF is detected in the gonads of two DSD patients with CIS **(E, F)**, identified by OCT3/4 staining (inset). **(G)**Staining of the gonadal biopsies of an infantile DSD patient demonstrating that SCF-positive cells and OCT3/4-positive cells (indicated by circles in **H**) are seen in the same areas. See text for details.

Two additional DSD patients were included: the first case was a girl (46,XY) with ambiguous genitalia (biopsy revealed a streak gonad and a dysgenetic testis). OCT3/4-positive cells at the basal lamina of the seminiferous tubules were identified in the testis, although not judged as classical CIS. This case also showed SCF staining, although not of the same cells (Figures 3G and 3H). The second case was a 1-year-old DSD patient (46,XY) with ambiguous genitalia (severe hypospadias, bilateral intra-abdominal gonads, and a vagina). OCT3/4-positive cells were found in the lumen of some seminiferous tubules, although in addition there were some on the basal lamina (data not shown). Indeed, again, a limited number of SCF-positive germ cells were identified. Both patients are under active and close clinical surveillance. A series of testicular biopsies (n = 7) and testes obtained by orchidectomy (n = 20), taken in routine paediatric urological practice, was investigated. All samples were negative for SCF staining (data not shown).

Relationship between SCF staining and c-KIT

Based on parallel staining for SCF and c-KIT, no relationship was observed between these markers (data not shown), with the exception of CIS/GB and seminomas. Two additional seminomas with a genomic c-KIT amplification were investigated [65], showing the same finding. Of the samples investigated (see above), three cases have a c-KIT exon 17 mutation (two codon 816 and one 822) and 15 are wild type. Again, no relationship with SCF was observed.

mRNA analysis of SCF and c-KIT

Seminomas showed variable levels of *c-KIT* (Figure 4A), which was overall lower in non-seminomas (Figure 4B). Furthermore, expression of *c-KIT* was low in normal testicular parenchyma, and in parenchyma containing various amounts of CIS, with-out a relationship with the percentage of CIS. No *c-KIT* expression was found in peripheral blood, whereas *SCF* mRNA was present (Figure 4B). *SCF* expression was heterogeneous in seminomas, with high expression in four cases, which showed *c-KIT* expression that was significantly lower (indicated by asterisks in Figure 4A, Mann–Whitney test, p = 0.027). No difference in SCF immunohistochemistry, which is non-quantitative, was found in these four cases compared with the others. Non-seminomas showed heterogeneous *SCF* mRNA expression, in accordance with immunohistochemistry. Normal testicular parenchyma expressed *SCF* mRNA, as did the samples with various amounts of CIS, without a relationship between these two parameters (Figure 4B). Overall, there was no significant correlation between *SCF* and *c-KIT* mRNA levels (Spearman's rank correlation, p = 0.35). When the different subgroups were analysed, only the non-seminomas showed a significant correlation between *c-KIT* and *SCF* mRNA expression (Spearman rank correlation coefficient: + 0.557, p < 0.001). Although of interest, this was not investigated further in this study.

To discriminate between the mRNA expression of membrane-bound and soluble SCF, RT-PCR (Figure 5A) was performed on normal testis, CIS, seminomas, and non-seminomas. In general, both forms of SCF were found (Figure 5B), although one seminoma and one non-seminoma were negative for both forms, possibly due to heterogeneity of *SCF* expression by immunohistochemistry or differences between FFPE and frozen material.

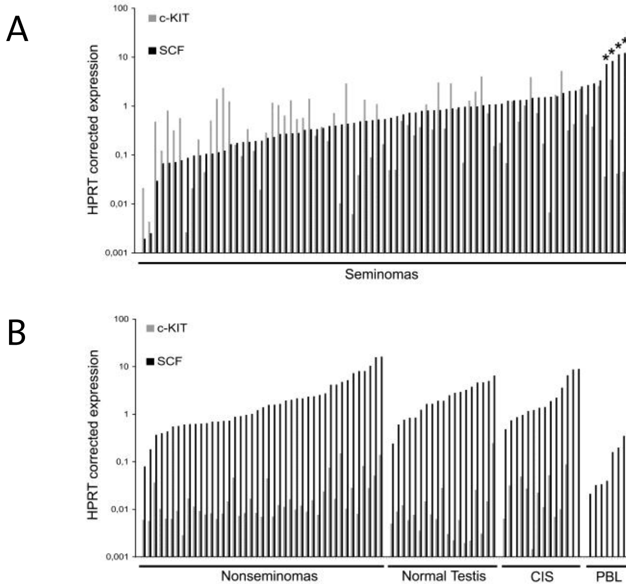


Figure 4. Quantitative RT-PCR for *c-KIT* and *SCF* expression (A) seminomas (*n* = 79) and **(B)** non-seminomas (*n* = 43), normal testis (*n* = 19), testes with different amounts of CIS (*n* = 14), and peripheral blood leukocytes (PBL) (*n* = 7). All samples are displayed sorted by their *SCF* expression level. Seminoma samples with the highest expression of *SCF* and low expression of *c-KIT* are indicated (asterisks in A). Expression of *c-KIT* and *SCF* mRNA is normalized based on *HPRT* mRNA expression that was determined in parallel as a control. Note the logarithmic scales on the *y*-axis

TGCT cell lines, c-KIT, and SCF

Whereas there are no cell lines for CIS or GB, cell lines representative of embryonal carcinoma and, most recently, also seminoma are available. With the exception of NCCIT, being negative for both SCF and c-KIT protein, all the other cell lines showed at least some positive staining for SCF and c-KIT (Figure 6A). Only a minority of positive cells were found in NT2, 2102EP, and Tera-1, while the majority of TCam-2 cells were positive. Because of technical limitations, we could not prove that c-KIT and SCF are present on the same cells, although this is most likely. Expression of *c-KIT* and *SCF* was confirmed on the mRNA level by quantitative PCR, which showed that all the cell lines, including NCCIT, express *c-KIT* and *SCF* at varying levels (Figure 6B), again without a relation-ship between the two. Like the invasive tumours, the cell lines also expressed overall both

membrane-bound and soluble SCF (Tera-1 and TCam-2 the lowest levels of membrane-bound form) (Figure 6C). The absence of a relationship between mRNA and protein could be due to specific microRNAs. However, this was not observed for three miRNAs known to interact with c-KIT mRNA, ie hsa-miR-146, -221, and -222 [67] (Figure 6D).

Discussion

Clinical diagnosis of CIS in adolescents and adults is based on the detection of markers such as PLAP, c-KIT, and OCT3/4 [11]. These markers are specifically found in PGCs/gonocytes, but not at later developmental stages of germ cells. This hampers CIS diagnosis at an early age and in cases of germ cell maturation delay [9]. This may result in overdiagnosis, possibly preventable in cases of undervirilization based on additional criteria for the diagnosis of CIS. These include the presence of OCT3/4 and TSPY-positive cells on the basal lamina of seminiferous tubules, showing clonal expansion, especially at ages above 3 months [27]. However, the identification of a marker that discriminates malignant germ cells from germ cells showing delayed maturation would be of clinical diagnostic value.

SCF–c-KIT signalling is important for PGC migration, survival, proliferation, and maturation [41,68–71]. Activating mutations of c-KIT have been reported in type II TGCTs, predominantly in bilateral cases [72–74], and specific amplification of the c-KIT locus has been described [65]. We therefore studied the presence of SCF during normal and pathological gonadal development, including type II TGCTs. SCF is possibly involved in the survival of the precursors of type II TGCTs, CIS and GB, in their specific micro-environment.

CIS and GB were investigated using immunohistochemistry for SCF, alongside the established markers OCT3/4, PLAP, and c-KIT. Interestingly, all precursor lesions stained positive for SCF, independently of the presence of an invasive component. In contrast, a heterogeneous pattern was found in invasive tumours. Testes of fetuses and newborns showed no SCF staining, with the exception of two cases, one of which belonged to a risk group for type II GCT. Moreover, gonads of DSD patients were investigated; those with no (or low) tumour risk lacked SCF staining, while cases previously identified with a high risk stained positively for SCF.

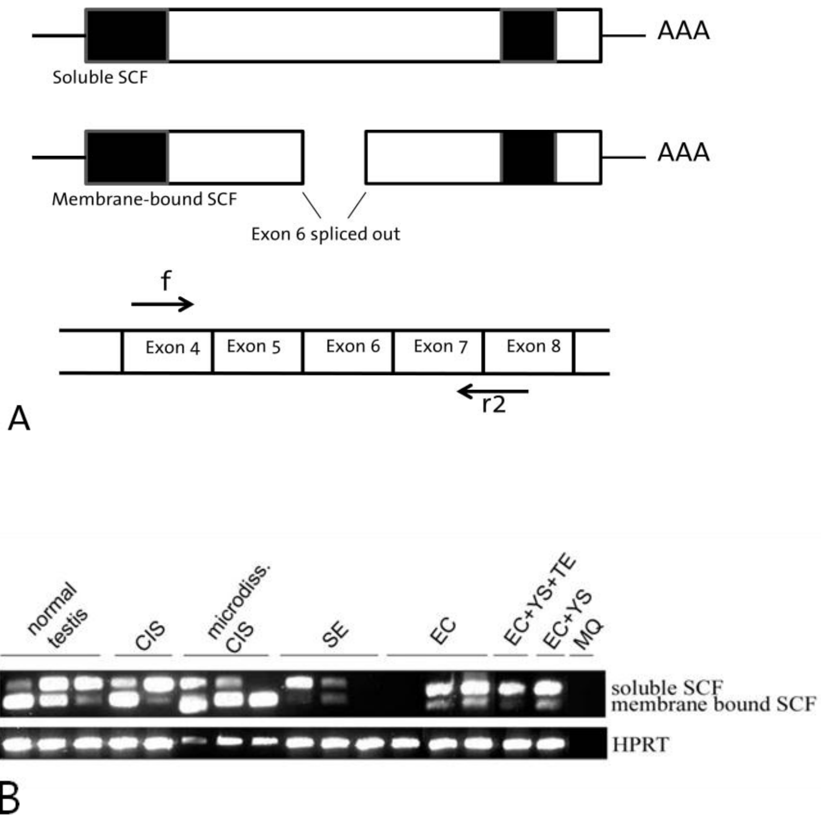


Figure 5. (A) Analysis of *SCF* variant RNA expression in type II TGCTs. Location of primers f and r2 (arrows) used to detect membrane-bound and soluble *SCF* by RT-PCR are indicated. (B) *SCF* RT-PCR demonstrating the expression of soluble and membrane-bound *SCF* transcripts in normal testis, testicular tissue containing CIS, seminoma (SE), and non-seminoma (EC = embryonal carcinoma; YS = yolk sac tumour; TE = teratoma; MQ = MilliQ negative control). *HPRT* expression was detected to check the quality of the cDNA

How SCF is involved in the pathogenesis of type II (T)GCTs remains to be elucidated. No relationship was found between SCF and c-KIT expression or mutations, and both the membrane-bound and the soluble form of SCF were present in most tumours and cell lines. The level of mRNA expression did not parallel the protein findings, which could not be explained by the limited number of miRNAs investigated. An alternative explanation might be accumulation of SCF in the CIS containing seminiferous tubules, possibly due to the presence of c-KIT. This might be in line with the absence of SCF on some individual CIS cells, despite its presence in (possibly interconnected) adjacent seminiferous tubules. However, the observations in cell lines support the model of an autocrine loop, a deviation from the physiological paracrine interaction during normal germ cell development and spermatogenesis. This may explain the cytoplasmic SCF

staining of CIS and seminoma cells, as reported before [60], although internalization might occur. If an autocrine loop is involved, it is probably predominantly of relevance in the early stages of pathogenesis.

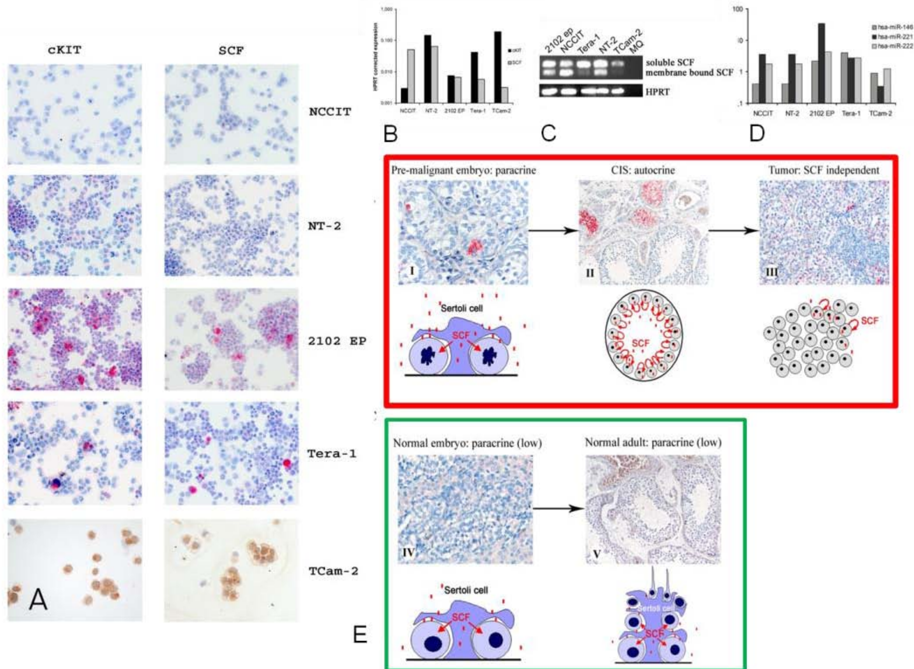


Figure 6. Expression of SCF and c-KIT studied in a panel of embryonal carcinoma (NCCIT, NT-2, 2102 EP, and Tera-1) and seminoma (TCam-2) cell lines. **(A)** Immunohistochemistry; **(B)** quantitative RT-PCR; (C) RT-PCR discriminating soluble and membrane-bound SCF; **(D)** expression analysis of three miRNAs (miR 146, 221 and 222) reported to target *c-KIT*. With the exception of NCCIT, all the embryonal carcinoma cell lines showed a certain percentage of c-KIT and SCF-positive cells, while almost all cells of the seminoma cell line TCam-2 were positive. All the cell lines express *SCF* and *c-KIT* mRNA. There is no relationship between SCF and c-KIT protein expression on one side versus the mRNA levels, or the presence of both the membrane-bound and the soluble *SCF* form. Moreover, no relationship between the expression level of three (*c-KIT* targeting) miRNAs and the level of *c-KIT* mRNA or protein was identified. **(E)** Proposed model for the role of SCF–c-KIT signalling during the development and progression of type II TGCTs. In the initial, embryonal pre-malignant stage, SCF signalling is paracrine, produced by the Sertoli cells (I), possibly with higher SCF levels than in the normal embryonal situation (IV). Upon progression to CIS, the cells are dependent on SCF (illustrated by our observation that all CIS are positive for SCF) and an autocrine stimulation loop has developed (II). Upon progression to invasiveness, the cells become independent of SCF, resulting in heterogeneous expression patterns of SCF, eg a seminoma with diminished expression of SCF (III). During normal embryonal to adult development, the germ cells are dependent on a low level of paracrine SCF stimulation, only detectable with sensitive methods (IV, V, and Figure 1C)

The cell lines can be informative in this context, especially TCam-2, which is representative of seminoma and has significant similarities to CIS [58]. Reduced viability of TCam-2 cells after siRNA-induced down-regulation of c-KIT has indeed been demonstrated recently, suggestive of an autocrine stimulation loop [59]. It is not clear whether bovine SCF, capable of activating human c-KIT, present in the fetal calf serum (FCS) of the cell culture medium, may also play a role [75,76].

Growth stimulation by SCF of non-seminoma cell lines was seen when grown in the absence, but not when grown in the presence, of FCS [35], which might indeed indicate interference of bovine SCF and that the autocrine loop may not be present in non-seminomas. However, this needs further investigation. The exact mechanism by which SCF is involved in the pathogenesis of type II TGCTs remains to be elucidated. The most interesting model is a paracrine to an autocrine switch during early development and the subsequent independence of this pathway upon further progression (Figure 6E). This altered bioavailability of SCF (it appears to accumulate on CIS, so less SCF may be available for activating Leydig cells) may lead to reduced testosterone production, resulting in the activation of a feedback loop to produce LH [77]. This could explain the occurrence of Leydig cell hyperplasia, as is seen in about half of the cases of testicular parenchyma containing CIS [78,79].

Our study demonstrates that SCF, in addition to OCT3/4, can be used as a marker allowing the distinction of malignant germ cells from those with delayed maturation, particularly in gonads at risk for TGCT and showing germ cell maturation delay. The diagnostic possibilities of detecting CIS cells in young individuals and patients with various forms of DSD will benefit significantly from this observation. Moreover, the findings support the model that a switch from paracrine to autocrine SCF–c-KIT signalling could be relevant in the early pathogenesis of type II GCTs.

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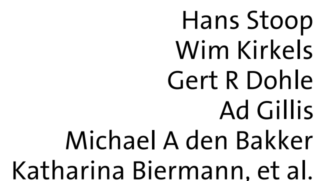
Chapter 11

Diagnosis of testicular carcinoma *in situ*, seminoma and embryonal carcinoma using direct enzymatic alkaline phosphatase reactivity on frozen histological sections

Histopathology 58: 440-446, 2011



Hans Stoop
Wim Kirkels
Gert R Dohle
Ad Gillis
Michael A den Bakker
Katharina Biermann, et al.

Abstract

Aims: Testis-sparing surgery might benefit quality of life, but can only be applied with histological examination for the presence of invasive germ cell tumour components, and the precursor carcinoma *in situ* (CIS). Currently, diagnosis is based on paraffin-embedded tissue, therefore a delay in further surgery is mainly unavoidable. The aim was to develop an intraoperative assessment technique using alkaline phosphatase as a marker.

Methods and results: A total of 4093 snap-frozen samples and matched paraffin-embedded tissue of 1500 patients were included. Besides standard haematoxylin and eosin (H&E) staining, the direct enzymatic alkaline phosphatase reactivity (dAP) test (duration 15 min) was applied on frozen sections, while H&E and immunohistochemistry for detection of OCT3 / 4, α-fetoprotein, human chorionic gonadotrophin (hCG) and cytokeratin was performed on the paraffin-embedded slides. Endothelial cells served as control for the dAP test. Positive staining was found in all CIS (*n* = 965), seminoma (*n* = 1035) and embryonal carcinoma (*n* = 584), either intratubular, microinvasive or invasive. Differentiated non-seminomas (*n* = 1238) showed variable staining. No staining was identified in spermatocytic seminomas (*n* = 5), testicular lymphomas (*n* = 42), testicular rhabdomyosarcomas (*n* = 7), Leydig cell tumours (*n* = 31), Sertoli-cell-only nodules (*n* = 4), (epi) dermoid cyst (*n* = 16), normal testicular parenchyma (*n* = 116), testicular torsion (*n* = 32) and inflammation of the epididymis (*n* = 19). The dAP test results matched H&E-stained parallel sections, as well paraffin-embedded tissue analysis, including immunohistochemistry.

Conclusions: The dAP test is an informative, reproducible and easy tool to diagnose CIS, (intratubular and microinvasive) seminoma and embryonal carcinoma on frozen tissue sections, being of great value in the context of sparing surgery.

Introduction

Testicular tumours are a heterogeneous group of lesions, with different origin and pathogenesis. These include Leydig cell tumours, Sertoli cell tumours, lymphomas, sarcomas and various types of tumours originating from the germ cell lineage.[1] Based on clinical and pathobiological criteria, testicular germ cell tumours can be subdivided into three categories, i.e. teratomas and yolk sac tumours of neonates and infants (type I), seminomas and non-seminomas, referred to as TGCTs (type II), and spermatocytic seminomas (type III). In addition, there are benign teratomatous lesions with the histology of somatic tissue, including (epi)dermoid cysts. Although TGCTs are rare in the general population, with an incidence of between six and 11 per 100 000 in Caucasian males, they represent the most common cancer in men aged between 15 and 45 years and the incidence is still rising.[2] High success rates in clinical management of TGCTs have been achieved using a combination of surgery, irradiation and chemotherapy.[3] In fact, standard care for therapy for TGCTs is radical orchiectomy, which may be combined with irradiation and / or cisplatin-based chemotherapy, depending on the histology of the tumour as well as the disease stage. In the diagnostic work-up, the presence of elevated serum markers a-fetoprotein (AFP), ß-human chorionic gonadotrophin (ß-HCG) and / or lactate dehydrogenase (LDH) is informative. However, in present clinical practice a tumour in a testis is, in itself, enough reason to perform a radical orchiectomy, without a histological diagnosis of the testicular lesion. Recently, more attention has been given to the possibility of testis-sparing surgery in specific patients. This alternative procedure may be of interest for patients with a bilateral TGCT or an affected monotestis, in whom standard care would result in complete castration and lifelong dependence upon hormone substitution (testosterone replacement).[4] To avoid these sequelae organ-sparing surgery might be an option, in which the tumour is enucleated and the normal appearing testis parenchyma is preserved.[5,6] Other patients who may benefit from such an approach are those without elevated serum markers and a small, possibly benign tumour.[7] However, a limitation is that no distinction can be made between a benign and a malignant lesion using the available imaging techniques, such as ultrasonography or magnetic resonance imaging. Therefore, by definition, exploratory surgery and frozen section analysis of tumour tissue is required. In fact, histological investigation should be performed of the lesion and its adjacent parenchyma.

Carcinoma *in situ* (CIS), also known as intratubular germ cell neoplasia unclassified (IGCNU)[8] or testicular intratubular neoplasia (TIN), is the precursor of all TGCTs.[9] Identification of CIS is sufficient for the diagnosis of a TGCT, which is by definition malignant. CIS is present in the majority of patients with an invasive

TGCT, more frequently in patients with non-seminomas than with seminomas, which is probably related to the immune response.[10] It is reported that 70% of patients with CIS will develop an invasive tumour within 7 years, which is extrapolated to 100% at 10 years.[11] Of importance is the fact that CIS can be treated effectively by local low-dose irradiation with preservation of hormonal function of the testis in most cases.[11] In daily practice, CIS is diagnosed using (formalin-, Stieve's- or Bouin-) fixed, paraffin-embedded tissue, preferably supported by immunohistochemistry, for example (OCT)3 / [4,12–15] or c-KIT (CD117) and placental alkaline phosphatase (PLAP).[8] The required time to diagnose CIS, based on paraffin-embedded tissue, possibly supported by immunohistochemistry, precludes its application as an intraoperative procedure. Therefore, if malignant cells are found in the adjacent parenchyma a second intervention, usually surgical, is required. Alternatively, frozen tissue diagnosis might be applied to determine the presence of CIS, as well as invasive tumour components, such as (intratubular and microinvasive) seminoma or embryonal carcinoma. The problem is that the morphology of frozen haematoxylin and eosin (H&E)-stained sections is often not sufficient to diagnose CIS reliably. This shortcoming of frozen section diagnosis could be compensated for with a specific marker that can be applied intra-operatively on frozen section. Alkaline phosphatase is such a marker.

Alkaline phosphatases are membrane-bound glycoproteins occurring in various forms and different tissues, which are encoded by different genes, i.e. tissue non-specific alkaline phosphatase (TNAP), tissue-specific alkaline phosphatase (TSAP), being placental-, intestine- and germ-cell alkaline phosphatase (PLAP and GCAP).[16] No specific antibodies against all these various isozymes are currently available. Alkaline phosphatase is present in migrating primordial germ-cells (PGCs), and in gonocytes as well as in pre-spermatogonia in embryonal gonads.[17] In fact, this enzymatic activity was used initially to identify mouse PGCs during normal embryogenesis.[18,19] In normal testis parenchyma of adolescents and adults, no endogeneous activity of alkaline phosphatase is present, except in the endothelial cells of small blood vessels.[20] It is known that PLAP and GCAP are expressed abundantly in seminoma and embryonal carcinoma.[21,22]

Because the enzyme is also present in CIS, it may be applied as a marker for the early diagnosis of TGCTs.[17] The aim of this study was to develop the direct histochemical detection of alkaline phosphatase enzyme reactivity, which detects the activity of all enzymes, to diagnose CIS, (intratubular and microinvasive) seminoma and embryonal carcinoma on frozen tissue sections.

Materials and methods

The use of tissues for scientific purposes as included in this study was approved by an institutional review board (MEC 02.981 and CCR2041), and the samples were used according to the '*Code for Proper Secondary Use*' (FMWV) (version 2002).

Primary tissue handling

The material used was handled by routine procedures. Upon incision of the specimen a digital image was generated, and possible heterogeneity of the specimen identified. Representative parts of the tumour area(s) (possibly including different elements) and adjacent parenchyma were cut and fixed in 10% buffered formalin for maximum 24 h, and subsequently embedded in paraffin. Parallel areas of both the tumour and parenchyma were cut and snap-frozen in liquid nitrogen, according to special requirements. Small fresh biopsies need to be prepared as quickly as possible after surgical intervention. The biopsies were placed on a small piece of thin cork and covered with a small piece of wet filter paper, after which they were directly frozen in liquid nitrogen. Larger pieces of testis tissue were snap-frozen, without the cork support, again in liquid nitrogen. In addition, special care was taken in cutting the sections: under no circumstances could the frozen tissue be thawed, which would have compromised morphology and enzyme-histochemical staining and thus the interpretation of the slides.

The snap-frozen samples were investigated for histology using both the H&E staining and the dAP test (see below) on parallel slides, allowing optimal comparison. The diagnoses made based on the snap-frozen sections stained with either H&E or the dAP test were compared directly. The pathologist (W.O.) studied the H&E-stained sections(s) first and decided whether CIS and/or invasive tumour components were present. This was confirmed for CIS, (intratubular and micro-invasive) seminoma and embryonal carcinoma based on the dAP-stained slides. In addition, the findings on the frozen samples were compared to the diagnosis made on the formalin-fixed, paraffin-embedded tissues. Therefore, H&E staining was performed again, as well as immunohistochemistry using antibodies specifically detecting OCT3 / 4, AFP, hCG and cytokeratin (CAM5.2). All tumours were diagnosed according to the WHO classification.

Direct alkaline phosphatase reactivity detection

Frozen tissue sections of 5-lm thickness were air-dried (at room temperature for at least 30 s) and fixed for 10 s in 100% acetone. Subsequently, the slides were air-dried for 30 s and incubated for 5–10 min in 60 ml Tris HCl pH8.4 (0.2 m) with 20 mg Fast Red (Sigma Steinheim, Germany; F8764) and 20 mg Naphtol AS-MX phosphate salt (N5000; Sigma). After washing in running tapwater the slides were fixed for 30 s in 10% buffered formalin and counterstained with haematoxylin. Subsequently they were covered with an aqueous mounting medium (Imsolmount; Klinipath Duiven, The Netherlands; 4059) and inspected visually, after which they could be stored in the archive.

Results

During a period of more than 15 years of collecting primary testicular tumours, in close collaboration with pathologists and urologists in the south-western part of the Netherlands, a large archive has been established. This tissue bank contains more than 1500 tumours from individual patients, from which frozen tissue as well as (matched) formalin-fixed, paraffin-embedded tissue is available. Representative slides of all frozen tissue samples were stained routinely using H&E and investigated for dAP; the findings showed no discrepancies. However, identification of CIS was more unequivocal using the dAP test. This was especially the case when only a relatively limited number of seminiferous tubules contained CIS cells, and when the affected tubules also retained spermatogenesis (see below). In addition, the findings were compared to the observations made on formalin-fixed paraffin-embedded tissues, which were also stained using H&E and investigated using immunohistochemistry (see Materials and methods section).

All cases were examined by an experienced pathologist with a special interest in TGCTs (J.W.O.). All tumours were described in a standardized, authorized pathology report in which various items were scored, including the presence of CIS, presence of the various invasive components and intratubular variants (seminoma and embryonal carcinoma). Again, no discrepancies were found between the various approaches. The results are summarized in Table 1, as well as the diagnosis made. Representative examples are shown in Figure 1.

Normal parenchyma and non-neoplastic conditions [normal parenchyma (Figure 1A), testicular torsion and chronic inflammation] showed no alkaline phosphatase reactivity, with the consistent exception of endothelial cells. The same pattern was found in all spermatocytic seminomas (Figure 1B) and non-TGCTs testicular lymphoma (Figure 1C), rhabdomyosarcoma, Leydig cell tumour (Figure 1D), Sertoli cell-only nodule, dermoid cysts and epidermoid cysts (Figure 1E). The endothelial cells, showing positive staining pre-dominantly of the

smaller vessels, served as a positive internal control for this enzymatic staining. In contrast to the consistent negative finding as indicated above, all CIS (Figure 1F,G) and (intratubular and microinvasive) seminoma (Figure 1H,I) and embryonal carcinoma (Figure 1J,K) samples were positive. Interestingly, the CIS cells were not restricted to seminiferous tubules without spermatogenesis. The preinvasive tumour cells were located on the basal membrane in the niche beneath the tight junctions between the Sertoli cells. Some cases did not contain CIS, which was due to the absence of testicular parenchyma available for analysis, because of the overgrowth of the tumour. The same staining pattern as in CIS was seen in intratubular seminoma, although in this case the seminiferous tubule was completely filled with tumour cells, and Sertoli cells may have been completely absent.

Table 1. Results of direct alkaline phosphatase staining on frozen tissue sections of more than 4000 samples from 1500 patients. The number of cases investigated (=tested) and positive results (%) are indicated

	Histology	Tested positive
Benign lesions		
Normal parenchyma	116	0
Torsion	32	0
Chronic inflammation	19	0
Sertoli cell-only nodule	4	0
Non-TGCTs		
Spermatocytic seminoma	5	0
Lymphoma	42	0
Rhabdomyosarcoma	7	0
Leydig cell tumour	31	0
(Epi)Dermoid cyst	16	0
TGCTs and precursor		
Carcinoma in situ	965	100
Intratubular seminoma	244	100
Seminoma	791	100
Intratubular embryonal carcinoma	71	100
Embryonal carcinoma	513	100
Yolk sac tumour	402	*
Choriocarcinoma	41	*
Teratoma	795	*

*A heterogeneous staining pattern was identified, with both positive and negative regions, even within a single tumour. This observation does not negatively influence the power of the direct alkaline phosphatase method to detect carcinoma *in situ*, seminoma and embryonal carcinoma, both intra-tubular, microinvasive and invasive.

Upon invasive growth the staining pattern stayed the same. A different pattern was observed in embryonal carcinoma, in which not every single cell was positive. Instead, groups of tumour cells were defined by a polarized membranous staining for alkaline phosphatase. This pattern was similar for intratubular, microinvasive and frankly invasive embryonal carcinoma. In

contrast, a more heterogeneously staining pattern of the enzymatic reactivity was identified in the various differentiated non-seminomatous components, such as yolk sac tumour (Figure 1L), choriocarcinoma (Figure 1M) and teratoma (Figure 1N). However, identification of the dAP test was not required in these cases, because diagnosis could be made based upon histological investigation (H&E-stained section) alone.

Gonadoblastoma, which is the CIS-counterpart of the dysgenetic gonad,[23,24] also showed positive staining for alkaline phosphatase (Figure 1O, n = 7 independent cases). Recent examples illustrate the power of the method to guide intraoperative surgical decision-making. In two cases the suspicious testis (based on physical examination and ultrasound analysis) was not removed because staining for alkaline phosphatase ruled out the presence of a TGCT or CIS. In another case CIS and a small seminoma were demonstrated in a patient whose other testis had been removed earlier for a TGCT. During the operation it was decided to excise the seminoma and to spare the testicle to preserve its hormonal function. The remaining CIS was treated with local irradiation. The patient is under surveillance. An opportunity for testis-sparing surgery was missed in a 17-year-old boy. Microscopic examination of the removed testis demonstrated a small epidermoid cyst (11 mm in diameter), lacking an invasive component or CIS, thus constituting a benign lesion. In this case, testis-sparing surgery could have been performed if snap-frozen analysis had been carried out on both the tumour and the adjacent parenchyma to rule out the presence of CIS, (intratubular and microinvasive) seminoma and embryonal carcinoma.

Discussion

Individualized treatment of cancer patients is becoming a major issue in current clinical practice. This is aimed at achieving optimal treatment success rates and at the same time reduction of treatment side effects to ensure quality of life, in both the short and long terms. Although intraoperative frozen section examination is applied commonly in surgical management for various malignancies, its role in clinical management of testicular masses is still in its infancy. However, in some cases organ-preserving surgery can be the preferred therapeutic intervention. These may include patients with a benign testicular lesion, as demonstrated by, for example, Heidenreich and coworkers in a series of 18 patients treated with enucleation of a benign tumour and analysis of frozen biopsies of the adjacent parenchyma.[25]

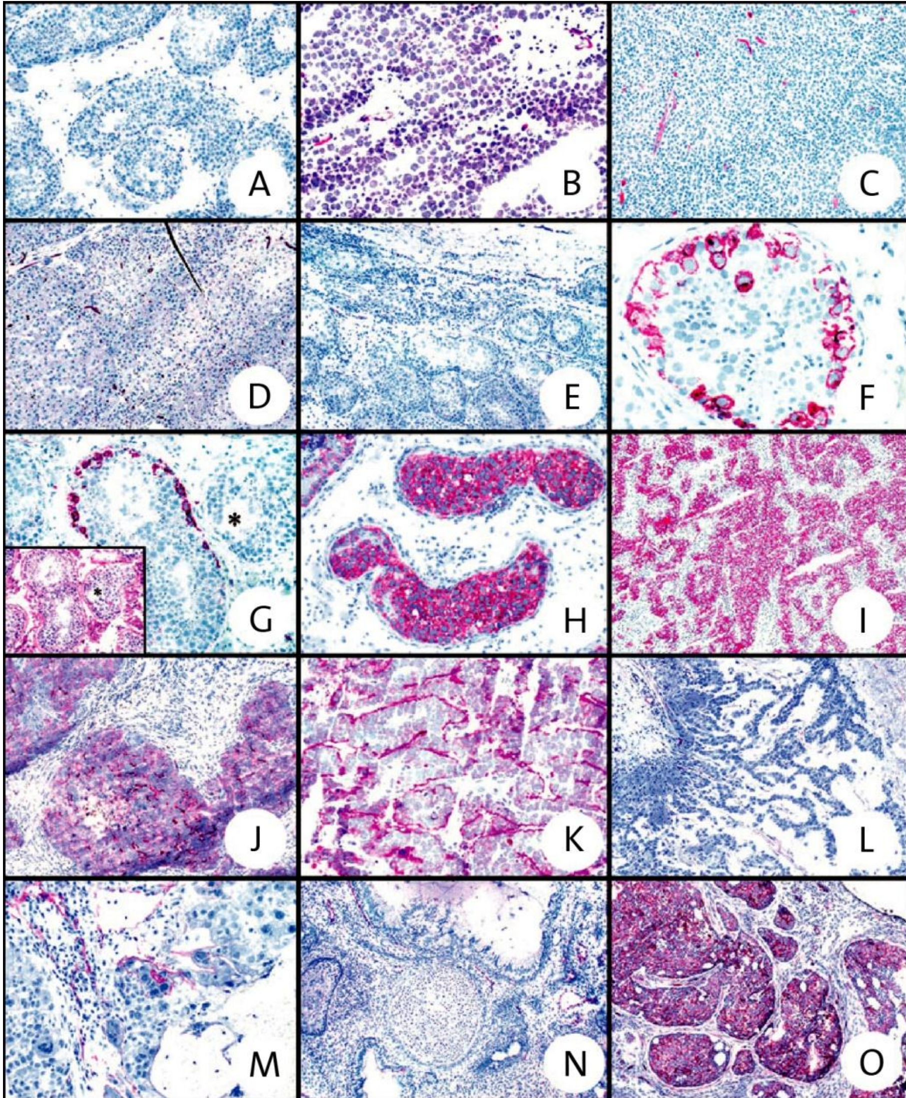


Figure 1. Examples of direct alkaline phosphatase staining on frozen tissue sections: **(A)** normal adult testis parenchyma; **(B)** spermatocytic seminoma; **(C)** lymphoma; **(D)** Leydig cell tumour; **(E)** epidermoid cyst, all showing only positive staining in endothelial cells; **(F, G)** carcinoma *in situ* (CIS) (parallel haematoxylin and eosin staining as insert, *position normal tubule); **(H)** intratubular seminoma; **(I)** invasive seminoma; **(J)** intratubular embryonal carcinoma; **(K)** invasive embryonal carcinoma, all showing a positive staining with a pattern as described in the text; **(L)** yolk sac tumour; **(M)** choriocarcinoma; **(N)** teratoma; **(O)** gonadoblastoma (showing a similar staining pattern to CIS).

In addition, an adenomatoid tumour of the testis was treated by testis-sparing surgery after it was diagnosed as benign by intraoperative frozen section examination.[26] Moreover, a segmental testicular infarction, simulating a TGCT, was treated by partial orchiectomy after a frozen section diagnosis.[27]

Currently, the standard treatment of a primary TGCT is radical orchiectomy, followed by additional treatment depending upon the stage of the disease and characteristics of the tumour, being either irradiation or chemotherapy.[3] However, some patients might benefit from a more conservative surgical approach. This might prevent complete castration in the case of patients with a tumour in a solitary testis, a bilateral testicular tumour or a second testicular tumour after an initial unilateral orchiectomy. In fact, organ-preserving surgery is not yet standard care in these patients.[28,29] Furthermore, sufficient endocrine function of the remaining testicular parenchyma should remain after the operation. If organ-preserving surgery is to be performed, detailed investigation of the adjacent testicular parenchyma is required to determine the presence of CIS, as well as (intratubular and microinvasive) seminoma and embryonal carcinoma. The other histological elements are easier to diagnose based on standard histological analysis. If CIS is documented histologically, adjuvant radiotherapy of the remaining testicular tissue is recommended strongly according to the management strategy for CIS in unilateral tumours.[7] If invasive tumour components are identified, orchiectomy must be considered. Histological examination is performed predominantly on fixed tissue, which benefits from optimally preserved morphology. This is significantly reduced using snap-frozen tissue.

The procedure described in this paper has been investigated in a large prospective series of 1500 patients, in whom more than 4000 frozen and matched formalin-fixed paraffin-embedded samples were investigated. The dAP test allows fast and reliable identification of CIS (intratubular, microinvasive and invasive) seminoma and embryonal carcinoma on frozen tissue sections within 15 min of surgical removal. The absolute sensitivity and specificity of enzymatic alkaline phosphatase detection on frozen tissue makes identification of CIS, seminoma or embryonal carcinoma highly reproducible compared to standard snap-frozen tissue analysis based on H&E staining. The dAP test is able to detect even a single CIS cell in a seminiferous tubule with spermatogenesis, as well as a limited number of CIS containing seminiferous tubules in a background of mostly unaffected tubules. This method, which includes an internal positive control, i.e. endothelial cells, which were present in all investigated samples, was as informative as the immunohistochemical detection of OCT3/4 on paraffin-embedded tissue, based upon comparison of matched frozen and paraffin section analysis of all cases (data not shown). In fact, no discrepancies were

found between the diagnoses made using the two methods. In addition, the test can be implemented easily in routine clinical histopathology.

The dAP test is a valuable diagnostic tool in testisconserving surgery, allowing direct evaluation of the presence of CIS, seminoma or embryonal carcinoma. With the availability of this intraoperative test the development and implementation of testis-sparing surgery is more feasible than hitherto. Even so, testissparing surgery should be performed at a centre with sufficient experience in the management of this rare clinical situation.[30] However, some limitations must be borne in mind. Although CIS is found in most affected samples of testicular parenchyma, in the case of a type II GCT, it can show a heterogeneous pattern. This patchy distribution can result in a false negative finding upon biopsy analysis, which is not influenced by which detection technique is applied.16 In this context it is of relevance that two independent surgical biopsies are recommended for detection of CIS in a normal-sized testis, each with a diameter of at least 3 mm.[31] However, although the risk will be small, the presence can never be absolutely excluded with a negative finding on biopsy material. Therefore, the proper size of the biopsy and subsequent handling and analysis are of great importance for optimal diagnosis.

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Chapter 12

Global DNA methylation in fetal human germ cells and germ cell tumours: association with differentiation and cisplatin resistance

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Abstract

Differences in the global methylation pattern, ie hyper- as well as hypo-methylation, are observed in cancers including germ cell tumours (GCTs). Related to their precursor cells, GCT methylation status differs according to histology. We investigated the methylation pattern of normal fetal, infantile, and adult germ cells (*n* = 103) and GCTs (*n* = 251) by immunohistochemical staining for 5-^{m}cytidine. The global methylation pattern of male germ cells changes from hypomethylation to hypermethylation, whereas female germ cells remain unmethylated at all stages. Undifferentiated GCTs (seminomas, intratubular germ cell neoplasia unclassified, and gonadoblastomas) are hypomethylated, whereas more differentiated GCTs (teratomas, yolk sac tumours, and choriocarcinomas) show a higher degree of methylation. Embryonal carcinomas show an intermediate pattern. Resistance to cisplatin was assessed in the seminomatous cell line TCam-2 before and after demethylation using 5-azacytidine. Exposure to 5-azacytidine resulted in decreased resistance to cisplatin. Furthermore, after demethylation, the stem cell markers *NANOG* and *POU5F1* (*OCT3/4*), as well as the germ cell-specific marker *VASA*, showed increased expression. Following treatment with 5-azacytidine, TCam-2 cells were analysed using a high-throughput methylation screen for changes in the methylation sites of 14000 genes. Among the genes revealing changes, interesting targets were identified: ie demethylation of *KLF11*, a putative tumour suppressor gene, and hypermethylation of *CFLAR*, a gene previously described in treatment resistance in GCTs.

Introduction

DNA methylation is one of the major epigenetic modifications of the genome, occurring in both temporally and spatially defined patterns. In somatically differentiated cells, genomic methylation is in general stable and heritable [1]. It is involved in the regulation of gene expression, both of protein encoding and of non-encoding genes [2]. Both global demethylation and regional hypermethylation have been described in malignant cells [3]. Investigation of the global DNA methylation status on tissue sections using an antibody recognizing the presence of a methyl group on the 5' carbon of cytidine (5-^{m}C) has been reported [4]. This approach allows the examination of single cells within the histological context and at different stages of development and maturation. Here we report for the first time the global DNA methylation status of male and female fetal, infantile, and adult germ cells within their histological context in the gonads.

Human germ cell tumours (GCTs) originate from transformed primordial germ cells (PGCs)/gonocytes [5]. Although most cases show a unique sensitivity to cisplatin-based chemotherapy [6], GCTs are still a deadly disease in a minority of cases. The mechanisms underlying treatment failure are not well defined [7]. Methylation of CpG islands has been associated with drug sensitivity in several cell lines [8] and promoter methylation has been described in cisplatin resistance of testicular GCTs (TGCTs) [9]. In addition, we have demonstrated hypermethylation of the promoter of *hMLH1* in cisplatin-resistant GCTs [10].

Using immunocytology, we analysed the global methylation pattern of cell lines and correlated it with levels of cisplatin resistance. In addition, the effects of chemically induced demethylation by 5-azacytidine on cisplatin sensitivity and the mRNA expression levels of a set of selected genes involved in differentiation were investigated. Furthermore, a set of 14 000 genes was analysed for changes in CpG island promoter methylation after 5-azacytidine exposure.

Materials and methods

Patients and samples

Formalin-fixed, paraffin-embedded tissues of human normal fetal, infantile, and adult gonads including those from autopsies collected between 2000 and 2006 were analysed. Formalin-fixed, paraffin-embedded tissues of (T)GCT cases were collected between 1991 and 2001 in collaboration with urologists and pathologists in the south-western part of The Netherlands. In total, 251 cases of different histologies were available. All cases were reviewed by a pathologist with special experience in GCT pathology (JWO) and classified according to the WHO classification. Research on human tissue samples was performed according

to the *Code for Proper Secondary Use of Human Tissue in The Netherlands*, as developed by the Dutch Federation of Medical Scientific Societies (FMWV), version 2002, and was approved by an institutional review board (MEC 02.981).

Immunohistochemistry

Immunohistochemical detection of 5-^{m}C was performed using a mouse monoclonal antibody (Eurogentec (Liège, Belgium), #BI-MECY-0100; diluted 1 : 500). Sections of the formalin-fixed, paraffin-embedded tissue were cut at 4 µm and treated, after deparaffinization and blocking of endogenous peroxidase, by pressure cooking (120^{0}C, 1.2 bar) in Na citrate buffer (0.01 M, pH 6). Cytospin slides were formalin-fixed and subjected to the same antigen retrieval procedure as tissue sections. The slides were blocked for endogenous biotin and subsequently incubated at room temperature for 2 h with the primary antibody, for 30 min with a biotin-labelled secondary rabbit anti-mouse immunoglobulin, and again for 30 min with a biotinylated horseradish peroxidise– streptavidin complex (DAKO, Glostrup, Denmark and Vector, Burlingame, USA, respectively). 3,3'-Diaminobenzidine (DAB) was used as a chromogen. For immunohisto-chemistry of VASA, a rabbit polyclonal antibody (Abcam Cambridge, MA, USA, #ab13840; diluted 1 : 300) was used. No antigen retrieval was necessary for cytospins. Apart from the use of a biotinylated goat anti-rabbit secondary immunoglobulin, the same procedure as described above was applied.

Table 1. Global methylation pattern of different GCT subtypes and corresponding cell lines

Entity	Global methylation pattern*	*n*
Carcinoma *in situ*	-	68
Gonadoblastoma	-	11
Dysgerminoma	-	8
Germinoma	-	2
Seminomas		
Seminoma (control series)	-	62
Resistant seminoma	+++	1
Seminoma cell line (TCam-2)	+++	1
Non-seminomas		
Embryonal carcinoma	++	57
Yolk sac tumour	+++	51
Choriocarcinoma	+++	20
Mature teratoma	+++	50
Non-seminoma cell lines (NT2, 2102EP, NCCIT)	+++	3

* Results from 5-^{m}C immunohistochemistry.
-= no staining; ++= positive staining; +++= intense positive staining.

The results of 5-^{m}C immunohistochemistry (only nuclear staining) on testicular samples were scored by defining the ratio of positive to negative cells per seminiferous tubule. Representative visual fields (200-fold magnification) were counted and the average number of negative cells was reported. On GCT

samples, three staining intensities were distinguished: no staining (-) when there was no positive nuclear signal in tumour cells (in the presence of a positive internal control); weak staining (++) in the case of an irregular pattern of staining in 20–80% of the tumour cell nuclei; and intense positive staining (+++) in the case of homogeneous positive tumour cell nuclei staining. All samples were scored independently by two investigators (HW and HS).

Western blot analysis

Protein isolation

Pellets from the cell line TCam-2 were used to extract protein. Samples were washed in cold PBS and centrifuged at 0°C for 5 min at 1500 rpm. Lysis buffer (1× PBS, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS) including protease inhibitors (complete mini-cocktail; Catalogue No 1 836 153, Roche Diagnostics, Mannheim, Germany) was added. Protein lysates were stored at -80°C.

Western blot

Samples of 10 µg protein were run on a 10% SDSpolyacrylamide gel, blotted on a PVDF membrane for 1.5 h, and incubated with the primary antibody for 2 h (anti-VASA rabbit polyclonal antibody, same as that used for immunohistochemistry; diluted 1 : 500) at room temperature. Binding of the primary antibody was visualized by using IRDye conjugated donkey anti-rabbit secondary antibody and the blots were scanned on the Odyssey infrared imaging system (LI COR Biosciences, NE, USA). As loading control, anti-ß-actin was used (Abcam, #ab8226; diluted 1 : 50 000).

MTT vitality assay on cisplatin exposure with and without 5-azacytidine treatment

The effect of cisplatin on TCam-2 cell number was assessed using the MTT (3-[4,5-dimethylthiazol-2-yl]- 2,5-diphenyltetrazolium bromide) colorimetric assay as described previously [11]. TCam-2 cells were plated in 96-well plates at a density of 2 × 10^3 cells/100 µl medium per well in eight-fold replicates and allowed to adhere. After 24 h, TCam-2 cells were exposed to medium either with or without addition of 5-azacytidine (10 µM final concentration). After another 24 h pre-incubation, cisplatin was added in different concentrations and after 72 h, MTT (Sigma Chemical Co, St Louis, MO, USA) was added at a final concentration of 0.5 mg/ml. Cells were further incubated for 2 h. Plates were centrifuged for 5 min at 3000 rpm and the medium was carefully aspirated. Solubilization of formazan crystals was achieved by adding SDS (Sigma) and gentle shaking of the plates for 10 min. Finally, absorbance was read at 570 nm and results were expressed as the percentage of treated (cisplatin) versus untreated cells.

mRNA expression profiling

Quantitative RT-PCR
Total RNA was extracted from lysates of TCam-2 and TCam-2 treated with 5-azacytidine (24 h, 10 µM) using an RNAqueous-4PCR kit (Ambion/Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) according to the manufacturer's instructions. Samples were treated with DNase-I and checked for residual DNA contamination by PCR using a primer set for SOX2, which is a single exon gene. cDNA was generated as previously described [12]. Only samples found to be DNA-negative were included for further investigation. Quantitative PCR was performed using the 7900HT Fast Real-Time PCR System (Applied Biosystems). A specific set of primers was used to detect the expression of a set of genes known to be related to various differentiation lineages (see Supporting infor-mation, Supplementary Table 1). Quantitative values were obtained from the Cts as follows: target mRNA was quantified relative to HPRT (target mRNA value = $2^{(\text{mean Ct HPRT-Ct target})}$) [12].

High-throughput methylation profiling
The seminomatous cell line TCam-2 was analysed by high-throughput methylation profiling before and 22 passages after chemical demethylation with 5-azacytidine (one time exposure for 24 h; 10 µM final concentration). DNA bisulphite conversion was per-formed using the Zymo EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA) according to the manufacturer's protocol with the modifications described in the Infinium Assay Methylation Protocol Guide (Illumina Inc, San Diego, CA, USA). Sub-sequent analysis steps were performed according to the Infinium II Assay Lab Setup and Procedures and the Infinium Assay Methylation (http://www.illumina.com/technology/infinium_ methylation assay.ilmn; accessed August 2009) Protocol Guide (http://www.Illumine.com/ products/infinium_humanmethylation27_beadchip_kits.ilmn#documentation; accessed August 2009) (Illumina Inc). The processed DNA samples were hybridized to the HumanMethylation27 DNA Analysis BeadChip (http://www.illumina.com/products/infinium_humanmethylation27_beadchip_ leits.ilmn; accessed August 2009) (Illumina Inc), which allows assay at 27578 CpG sites selected from more than 14000 genes. Differential methylation analysis (DMA) was performed using the BeadStudio software (default settings; version 3.1.3.0, Illumina Inc). The global DNA methylation data showed a bimodal distribution in which beta values below 0.25 define the unmethylated CpGs and beta values above 0.75 the methylated CpGs. Thus, beta values below 0.25 and above 0.75 were selected as threshold values to define unmethylated and methylated CpG loci for further analysis. The thresholds for hyper- and hypomethylation have been established previously [13–15]. CpG loci were classified as differentially methylated if they showed a significant difference (< 0.001,

corresponding to a DiffScore of less than -30 or more than 30) in their DNA methylation values of at least 0.3 (delta-beta >0.3) between the samples analysed. The gene detection rate ($p < 0.01$) was high: 27552 of 27568 (99.94%; TCam-2 untreated) and 27548 of 27568 (99.93%; TCam-2 5-azacytidine treated), respectively, demonstrating a high quality of the sample DNA and the experimental performance. The reproducibility and accuracy of the HumanMethylation27 Bead Array technology has been demonstrated in several analyses published before using different techniques: eg bisulphite sequencing, bisulphite pyrosequencing, methylation-specific PCR or GoldenGate analysis [13,16–18].

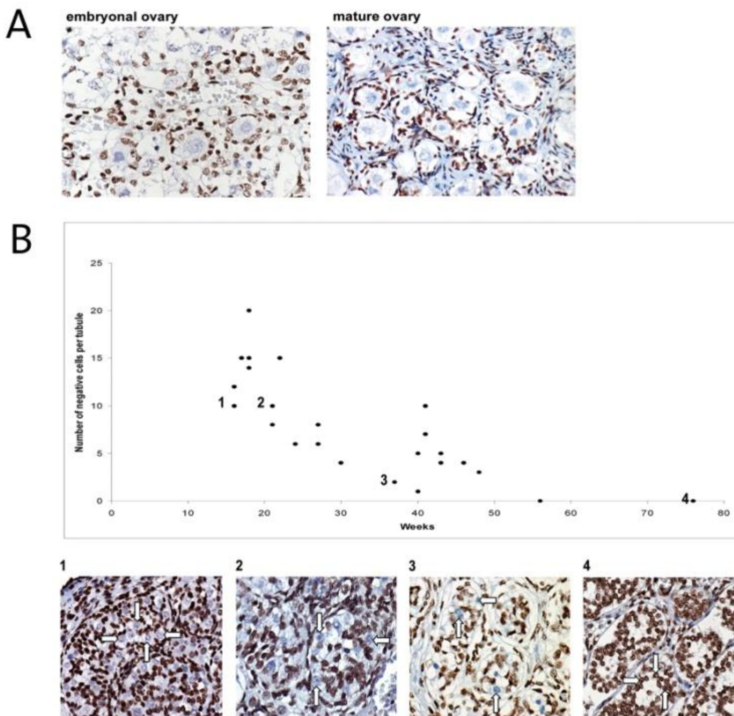

Figure 1. **Methylation status of male and female gonads during pre- and post-natal development**.
(A) Representative examples of 5-^{m}C immunostaining of fetal and mature female gonadal tissue (23 weeks gestational age: left image; 3 years: right image). **(B)** Methylation pattern in testicular tissue. Upper panel: schematic representation of absolute numbers of cells per seminiferous tubule showing no immunohistochemical staining for 5-^{m}C at different weeks of gestational age (median time of birth: 40 ± 2); lower panel: representative examples of 5-^{m}C immunostaining at the indicated time points (arrows: germ cells).

Results

5-^{m}C detection by immunostaining in fetal and adult gonadal tissue

The global methylation pattern of normal fetal germ cells during maturation from fetal to adult stages was assessed by 5-^{m}C immunostaining. Both fetal, infantile testes (*n* = 45) and ovaries (*n* = 19) starting from week 15 gestational age to post-pubertal age were analysed. In female gonads, germ cells, ie oogonia, oocytes, and follicles at different stages of maturation, are always unmethylated, both prenatally and after birth (see Figure 1A for representative illustrations). A more heterogeneous pattern was found in testes. Germ cells in fetal testes at early stages (starting from 15 weeks gestational age onwards) are predominantly hypomethylated (see Figure 1B for the scoring results and representative illustrations). With time, the number of demethylated germ cells declines and after birth, almost all germ cells are methylated. Interestingly, around the time of birth (week 40 ± 2), a short rise in the number of germ cells showing global demethylation is observed.

5-^{m}C detection by immunostaining in germ cell tumours

A distinct methylation pattern is found in undifferentiated and more differentiated TGCTs. Immunohistochemical findings are presented in Table 1 and Figure 2. The known precursor lesions intratubular germ cell neoplasia unclassified (IGCNU) of the testis and gonadoblastoma of the dysgenetic gonad, which morphologically resembles seminomatous cells, are both demethylated (Figures 2A and 2B). In addition, all seminomas are demethylated (Figure 2C). This pattern can also be seen in histologically similar tumours originating in the brain (germinomas) and the ovary (dysgerminomas), all of which are demethylated (data not shown). While embryonal carcinomas show an intermediate pattern (Figure 2E), the more differentiated non-seminomas, ie yolk sac tumours, choriocarcinomas and teratomas, are consistently hypermethylated (Figure 2F). Overall, a clear correlation between differentiation and methylation can be described: more differentiated tumour histologies show a higher degree of methylation. In contrast to a large series of unselected seminomas all showing demethylation, a single case of a relapsed seminoma after cisplatin treatment from our archive was not demethylated, but showed an atypical pattern of hyper-methylation (see Figure 2D). The only available cell line with seminomatous characteristics (TCam-2) as well as three embryonal carcinoma cell lines showed a high level of methylation (Figures 2G and 2H).

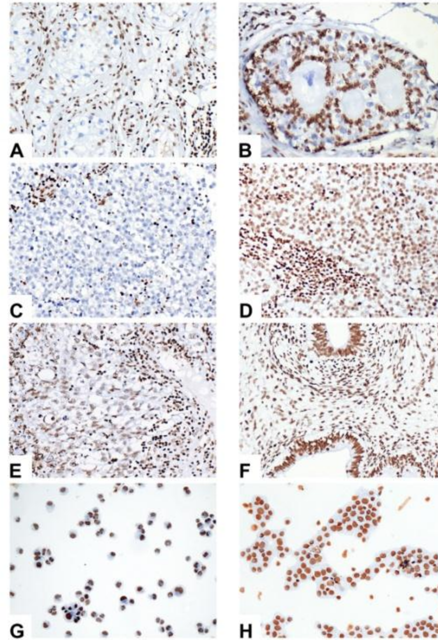

Figure 2. Representative examples of 5-^{m}C immunostaining of the different GCT entities and corresponding cell lines: **(A)** intratubular germ cell neoplasia unclassified (IGCNU); **(B)** gonadoblastoma. Note that the positive cells are supportive cells;**(C)** seminoma;**(D)** seminoma showing relapse after cisplatin-based therapy. Note that in both cases, the most intensive staining is observed in infiltrating lymphocytes, which can be used as an internal staining control;**(E)** embryonal carcinoma;**(F)** mature teratoma;**(G)** seminoma cell line TCam-2;**(H)** embryonal carcinoma cell line NT2.

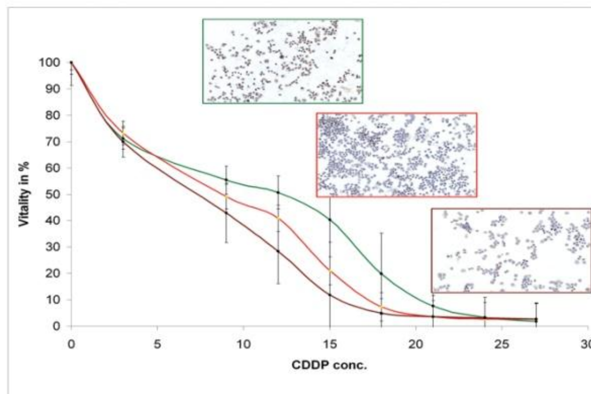

Figure 3. **Cisplatin resistance levels of the seminoma cell line TCam-2.** Vitality curves of untreated (green) and 5-azacytidine pretreated (red: 24 h; brown: 24 h + five passages) TCam-2 cells under cisplatin exposure for 72 h. Immunohistological images are shown as insets of matching cytospins stained for 5-^{m}C displaying the respective methylation state. All curves represent the results of eight-fold analyses (standard deviation indicated in bars). The assays were repeated in three independent experiments, yielding comparable results.

Cisplatin resistance level of the seminoma cell line TCam-2 before and after 5-azacytidine treatment

To investigate the influence of the methylation status on the resistance level of the seminoma cell line TCam-2, vitality assays were performed under cis-platin incubation with or without pretreatment with 5-azacytidine. TCam-2 showed an IC_{50} concentration of cisplatin of 12 μM after 72 h of treatment (see Figure 3). Upon pretreatment with 5-azacytidine for 24 h, a significant decrease in resistance to cisplatin (IC_{50} of 8 μM) was observed. This effect remained stable in TCam-2 cells after further passaging with-out further 5-azacytidine treatment: the IC_{50} cisplatin concentration decreased from 12 to 7 μM after five passages in culture. Cytospins were carried out to confirm the efficiency of the demethylation procedure; they demonstrated that the cells were predominantly demethylated and remained so up to the eighth passage after a single 5 azacytidine exposure (see also Figure 3, insets).

Gene expression changes in TCam-2 after 5-azacytidine treatment

The effect of 5-azacytidine treatment was investigated by quantitative RT-PCR analysing the transcript levels of a set of 29 genes related to differentiation. The absolute values (normalized to an internal control using the housekeeping gene *HPRT* of the untreated cell line) are shown in Figure 4A and are in line with data from previous genome-wide expression profiling [19]. The relative changes after 5-azacytidine treatment in comparison to the untreated cell line are shown in Figure 4B. The pluripotency markers *NANOG* and *POU5F1* (*OCT3/4*), already strongly expressed in untreated TCam-2 cells, further increased in expression. In addition, the expression of several markers associated with differentiation, such as *TEAD1* (late trophectoderm), *BMPR1A* (cardiac differentiation), and *SMAD4* (signalling), increased after 5-azacytidine treatment. The most distinguishing marker between untreated and treated TCam-2 cells is the germ cell-specific marker *VASA*, showing a 16-fold increase in expression in demethylated cells. No changes in histomorphology or cell growth characteristics were observed *in vitro* (data not shown).

VASA protein expression changes after 5-azacytidine treatment

To verify the observed changes in gene expression, western blot analyses were performed for VASA (see Figure 4C). In line with the mRNA expression levels, the amount of VASA protein increased in lysates from demethylated TCam-2 cells, albeit to a lesser extent than on the mRNA level. A 17% gain of signal in digital image quantification was detected compared with the original TCam-2 (Figure 4C, left panel). This was also confirmed by immunohistochemical staining of cytospins for VASA, which showed stronger expression in cells after exposure to 5-azacytidine (Figure 4C, right panel).

Changes in the methylation of 27 578 CpG sites in the seminoma cell line TCam-2 after 5-azacytidine treatment

Upon continuous passaging after 5-azacytidine treatment, initially demethylated TCam-2 cells showed, from the tenth passage onwards, regeneration of their level of global methylation (immunohistochemical detection; data not shown). Interestingly, however, they remained more sensitive to cisplatin than untreated cells. This phenomenon was further analysed using a high-throughput methylation screen of 27 578 CpG sites, representing a subset of 14 000 genes. TCam-2 cells at passage 22 after single exposure to 5-azacytidine treatment showed significant hypomethylation in 54 CpG sites (23.5%), whereas 176 loci (76.5%) showed hypermethylation. Figure 5 shows the change in methylation of all analysed CpG sites, high-lighting the 15 most hyper- and de-methylated sites. Remarkably, the tumour suppressor gene *KLF11* was the most highly demethylated CpG site, whereas the top three hypermethylated CpG sites include two proto-oncogenes, namely *CFLAR* and *ERBB2*.

Discussion

In this study, we demonstrate that the global methylation state of fetal human germ cells shows a specific pattern of temporal regulation and is different in female and male gonads. During testicular development, the pattern changes from a generally hypomethylated status in fetal gonads to a hypermethylated status in more mature germ cells. In contrast, female germ cells remain unmethylated during all developmental stages investigated. Previously, it has been reported that PGCs, the cell of origin of both female and male mature germ cells, are completely unmethylated after undergoing a methylation erasure [2,20]. As all type II TGCTs are believed to originate from a common precursor, a transformed PGC/gonocyte, which has not been re-programmed by *de novo* methylation, the global methylation status of particular TGCT subtypes is supposed to differ according to the time point of their developmental arrest [21–23]. It has been reported that the global methylation status of non-seminomas is different from both seminomas and IGCNU [24]. In line with these findings, our investigation shows that the precursor lesions IGCNU and gonadoblastomas and (T)GCTs that resemble PGCs/gonocytes, ie semi-nomas, germinomas, and dysgerminomas, are largely unmethylated. Non-seminomatous GCTs have a global methylation status according to their degree of differ-entiation. Embryonal carcinoma, the undifferentiated non-seminoma component, shows intermediate levels of methylation, while choriocarcinomas, yolk sac tumours, and teratomas are highly methylated.

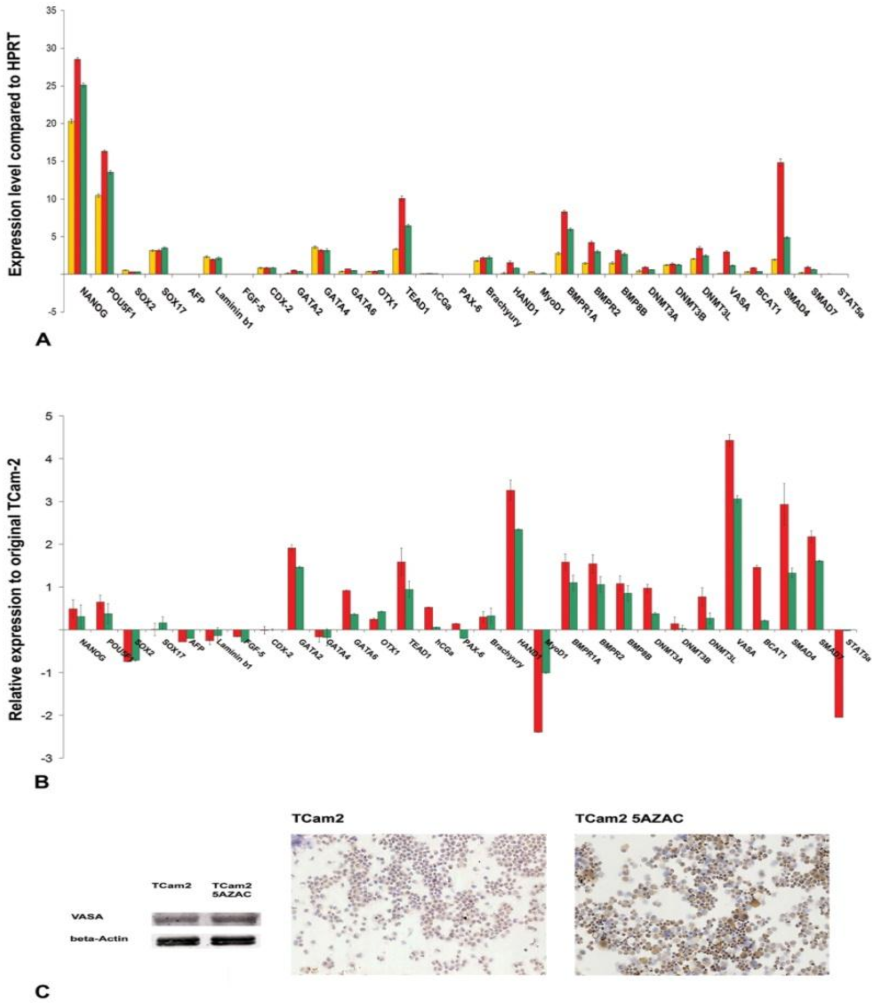

Figure 4. qRT-PCR results from a set of 29 genes investigated in untreated TCam-2 (yellow), TCam-2 after demethylation by single 5-azacytidine treatment (24 h) (red), and TCam-2 eight passages after demethylation by 24 h treatment with 5-azacytidine (green). **(A)** Absolute values are normalized to *HPRT*. **(B)** Results are depicted as changes in gene expression after demethylation, expressed as a ratio per gene compared with untreated TCam-2. **(C)** Immunoblots detecting VASA protein in TCam-2 cell lysates, untreated and after demethylation, passage 17 after 5-azacytidine exposure. Corresponding cytospins showing VASA staining are shown. VASA signal is detected by DAB (brown nuclear and cytoplasmic signal).

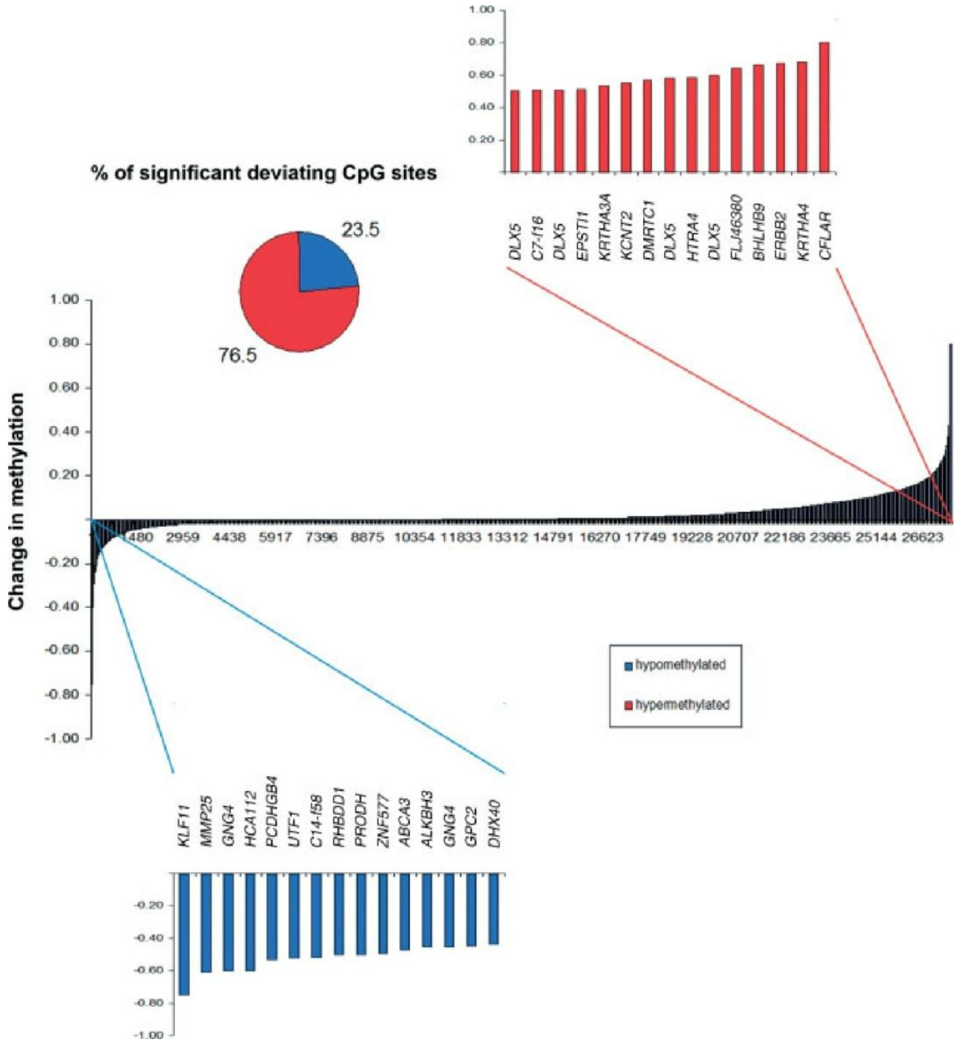

Figure 5. Results from methylation profiling of 27 578 CpG sites in TCam-2 cells. Changes in methylation after 22 passages after demethylation by 24 h treatment with 5-azacytidine are depicted. The pie diagram shows changes in the percentages of significant deviating CpG sites (see text for further explanation). The 15 CpG sites showing the highest degree of hyper- and demethylation are highlighted.

Another distinguishing factor between seminomas and non-seminomas with evident clinical implication is their responsiveness to chemotherapy, mainly cisplatin. Seminomatous GCTs usually show an excellent response to cisplatin, whereas the response of non-seminomatous GCTs to chemotherapy can differ according to histology: highly differentiated mature teratomas show no response to cisplatin [25,26]. Furthermore, in non-seminomatous GCTs, cisplatin resistance is a clinical problem, rendering the tumour a deadly disease in a minority of cases

[27–29]. Our observations suggest an association between differentiation, global DNA methylation status, and response to chemotherapy. Regarding global DNA methylation in seminomas, a hypermethylated genome was found in only one case showing relapse after cisplatin-based chemotherapy, whereas no other seminoma out of 62 cases from our tumour archive showed hypermethylated DNA. Clearly, this finding warrants further investigation of the methylation status of relapsed seminomas in a larger series. Interestingly, the only available cell line with seminoma characteristics, TCam-2, which compared to non-seminoma cell lines shows surprisingly high resistance to cisplatin (IC_{50}12 μM versus 0.2–5 μM, according to our own findings [30,31]), shows a high degree of DNA methylation. In our view, hypermethylation of TCam-2 could be a cause of the high cisplatin resistance in this cell line, which shares many similarities with seminomas [19] with the exception of the high level of methylation. Therefore TCam-2 can be regarded as an *in vitro* model for analysing mechanisms of resistance in seminomas. The impact on resistance of non-seminomatous components needs to be investigated.

To investigate the role of DNA methylation in cisplatin resistance, we analysed the effects of the demethylating agent 5-azacytidine on treatment resistance. Chemical global demethylation of TCam-2 cells led to a decrease in cisplatin resistance. Most recently, a study reported that treating embryonal carcinoma cell lines with 5-azacytidine induces cisplatin sensitivity [32]. Our findings are in line with this observation and describe the same effect in a seminoma cell line, making a general susceptibility of all GCTs to treatment with 5-azacytidine very likely.

To reveal differences in gene expression that could be associated with the observed change in cisplatin resistance, a panel of 29 markers of differentiation was analysed by quantitative RT-PCR in TCam-2 cells treated with or without 5-azacytidine. Upon demethylation, the expression of the pluripotency markers *NANOG* and *OCT3/4* increased, reflecting an association between hypomethylation and an undifferentiated state. However, no gross morphological changes in TCam-2 cells were observed *in vitro*. The most distinguishing marker between methylated and unmethylated TCam-2 cells is the germ cell-specific marker *VASA*. Whether expression of VASA has a direct impact on cisplatin resistance is unclear and warrants further investigations. In general, it seems likely that the observed change in cisplatin resistance levels after global demethylation involves the regulation of many genes and is therefore multi-factorial.

To further analyse potential target genes involved in cisplatin resistance, we performed an analysis of more than 14 000 genes by a high-throughput

methylation screen. In total, 27 578 CpG island sites of TCam-2 cells 22 passages after a single course of 5-azacytidine treatment were analysed and compared with CpG sites of untreated cells. Surprisingly, the vast majority of analysed CpG sites which show a significant change in the methylation status became hypermethylated with time, compared with untreated cells, whereas only a few remained demethylated (76.5% versus 23.5%, respectively). Among the genes showing the most significant changes in methylation, three genes seem particularly noteworthy: the most demethylated CpG site maps to *KLF11*, a putative tumour suppressor gene, whereas among the top three hypermethylated CpG sites, two proto-oncogenes were identified: *CFLAR (c-FLIP)* and *ERBB2*. Functionally, it remains to be elucidated in which way these genes are involved in cisplatin resistance in GCTs. Interestingly, *CFLAR* has been described among the top ten genes predicting overall survival in a recent analysis of tumour samples from GCT patients [33]. The c-FLIP protein is a direct inhibitor of active caspase 8 formation and therefore of executing apoptosis. A regulatory role of caspase activation has been reported for cisplatin resistance in TGCTs [34]. Furthermore, it has been shown in a cervical cancer cell line *in vitro* as well as in colorectal cancer *in vivo* that c-FLIP triggers response to cisplatin-based chemotherapy [35,36].

Conclusions

Germ cells at different stages of maturation as well as (T)GCTs of different histologies show marked differences in their global DNA methylation pattern. More differentiated cells show a higher degree of DNA methylation. The more undifferentiated GCTs such as seminomas and dysgerminomas are mainly demethylated, whereas non-seminomas show more methylated DNA. Interestingly, we found a single case of a seminoma after relapse from cisplatin-based chemotherapy that showed an unusual hypermethylation of the DNA in contrast to a large control series of seminomas. *In vitro*, chemical demethylation with 5-azacytidine decreased the intrinsically high resistance to cisplatin in TCam-2, a seminoma cell line. Further *in vitro* and *in vivo* experiments are warranted to explore the promising potential of demethylating agents such as 5-azacytidine in the treatment of cisplatin-resistant (T)GCTs.

Acknowledgment

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SUPPORTING INFORMATION ON THE INTERNET

The following supporting information may be found in the online version of this article.

Table S1. Details of quantitative RT-PCR analysis of 29 markers related to differentiation.

Chapter 13

General Discussion

13.1 Introduction

Histologically type II (T)GCTs mimick embryogenesis to a certain extent, although in an uncontrolled manner. The parallels between normal embryogenesis and tumorigenesis strengthen the need to understand mechanisms involved in early development, especially related to the different developmental stages of the gonads, including the germ cells. The studies described in this thesis deal with expression profiles of proteins during normal human testicular development and the translation of the observations into clinical practice, in particular for the diagnostic workup of (T)GCTs. The obtained results will be discussed in the context of optimal early diagnosis, as well as understanding the pathogenesis of this disease.

13.2 Normal testicular development and protein expression profile

Germ cells during normal development show a number of defined steps of maturation. They originate as PGCs, and after completing the process of spermatogenesis, induced after puberty, spermatozoa are formed, consisting of highly specialized cellular compositions and a haploid set of chromosomes, suitable for fertilization. However, already during embryonal and neonatal development, various maturation stages of male germ cells are defined. These are of specific relevance in the context of (T)GCTs, and were therefore the main topic of the investigations performed and described here. After migration of PGCs from the yolk sac to the genital ridge, the germ cells are referred to as gonocytes, which are associated with the supportive cells, being the Sertoli precursors cells. At this moment of development, the germ cells are positive for a number of markers, including PLAP, VASA, c-KIT, TSPY, and OCT3/4 [1]. Interestingly, under physiological conditions, TSPY is limited in expression to germ cells in a testicular micro-environment. Co-expression of these markers can identify various subpopulations of germ cells, supporting a stepwise maturation process [1-5]. However, the impact of the different subpopulations is unknown so far. Subtle differences in the identified subpopulations of germ cells are reported regarding their individual marker staining profiles [6-9]. Again, the value remains to be determined.

During subsequent development, the germ cells, which are initially located in the central position of the developing seminiferous tubule, move to a location at the basal lamina. These will eventually home to the niche under the tight junctions of the Sertoli cells, at which stage they are referred to as pre-spermatogonia. In association with this transition, the germ cells show a reduced protein expression of PLAP, c-KIT, and OCT3/4, while VASA and TSPY remain positive. In fact, PLAP

and OCT3/4 disappear completely, while c-KIT remains detectable depending on the method of fixation and detection, even in an adult (post-pubertal) testis (unpublished observations). A similar profile as the embryonic germ cell markers is found for the proliferative activity, using Ki-67 as marker. Interestingly, both VASA and TSPY are informative as markers for the presence of germ cells, independent of the developmental age of the testis. It is of importance to note that VASA is detected at multiple maturation stages, while TSPY is restricted to the pre-spermatogonial germ cells. . This is schematically Illustrated in Figure 1A.

In conclusion, after birth, the embryonic germ cell markers can be sporadically found, although they are absent after 6-12 month post partum, associated with a decline in proliferative activity.

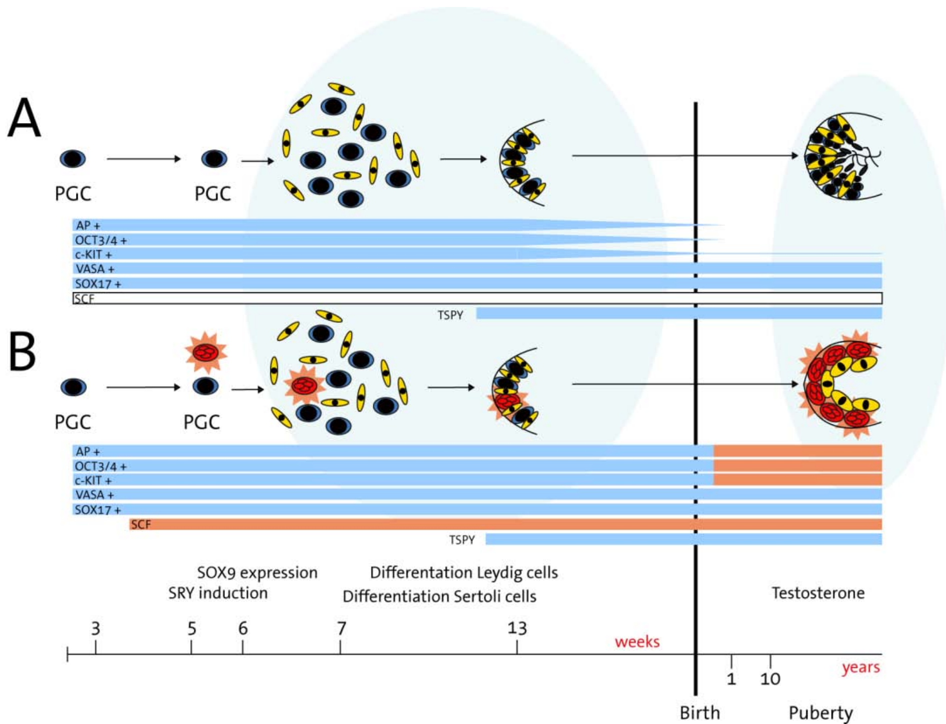

Figure 1. Protein expression in normal and aberrant germ cell development. (A) Expression of the different diagnostic markers alkaline phosphatase (AP), OCT3/4, c-KIT, VASA, SOX17 and TSPY during the normal development of the male gonad from fertilization till puberty. No detectable expression of SCF. **(B)** The expression of the different diagnostic markers during aberrant germ cell development, leading to CIS, is shown. There is an ongoing expression of the embryonal diagnostic markers after birth in CIS cells. This is in contrast with the normal expression in which about 6-12 months after birth all the proteins related to the embryonal stage are disappeared with an exception for VASA and TSPY. A significant difference is the expression of SCF only detectable around the niche of the (pre)CIS cells.

Besides based on protein expression profiling also regarding epigenetic (DNA) modification, different stages of germ cell development can be identified. Using immunohistochemical detection of $5^{-M}C$, the PGC/gonocyte stage is overall demethylated, while during transition towards pre-spermatogonia the level of methylation is increased, which is retained in later stages. The specific timing of down-regulation of the embryonic markers in germ cells in association to the methylation status is not investigated in detail. However, it can be stated that at the moment that no embryonic marker positive germ cells are identified anymore (after 6-12 months), all germ cells present are methylated [10].

13.3 From pluripotency to totipotency of (testicular) germ cell tumors

The histological composition of (T)GCTs indicates that these cancers are pluripotent, i.e., can generate derivatives of all three germ cell layers. However, the formation of the germ cell lineage itself was not identified in these tumors so far. In Figure 2 and 3 tumorigenesis and pathogenesis of type II (T)GCTs is illustrated. Using immunohistochemistry with a unique set of recent markers, including OCT3/4, TSPY, and VASA, the presence of germ cell formation was demonstrated in a number of independent nonseminomas [11]. Based on these findings, it is concluded that (T)GCTs can also generate the germ cell lineage, making them really toti- or omnipotent. This is in line with the observations that embryonic stem cells are also capable to form the germ cell lineage, both *in vitro* as well as *in vivo* [12-14]. This supports the use of (T)GCTs and derived cell lines for further investigation of regulation of pluripotency/totipotency, as recently demonstrated [15-16].

13.4 OCT3/4 as ultimate marker for diagnosis and non-invasive detection

The capacity of forming the germ cell lineage in individual tumors supports the similarities between pluripotent embryonic stem cells and the cells of origin of (T)GCTs. In this context, OCT3/4 is of particular interest. Originally, OCT3/4 has been identified as regulator of pluripotency in mouse and subsequently human embryonic stem cells [3,17]. In addition, it has been found to be expressed in mouse and human PGCs and gonocytes [18]. Absence of OCT3/4 in mouse PGCs results in induction of apoptosis [19-21]. This indicates that at least an additional, i.e., apoptosis-suppressing role of OCT3/4, exists in PGCs compared to pluripotency regulation in embryonic stem cells. In human germ cells, OCT3/4 is down-regulated upon maturation from gonocyte to pre-spermatogonia (see above), while it remains positive in mice [22-23]. This must be kept in mind when comparing different species, especially related to the development of GCTs [24].

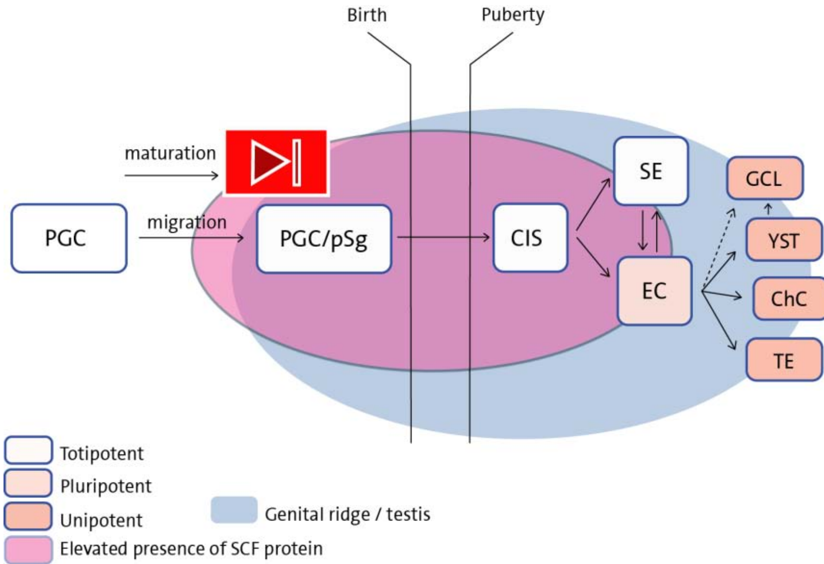

Figure 2. Tumorigenesis of the type II (T) GCT. The early PGC stops maturating and is now called a pre-CIS cell which migrates to the genital ridge. After puberty the CIS cell gives rise to an invasive germ cell tumor. SE and/or NSE. The embryonal carcinoma (EC) is the stem cell component of the nonseminomas and can give rise to the different differentiated components: Teratoma (TE), choriocarcinoma (ChC), yolk sac tumor (YST) and germ cell lineage (GCL). SCF is detectable as soon as the PGC becomes arrested and is still present until the CIS transforms to an invasive tumor cell.

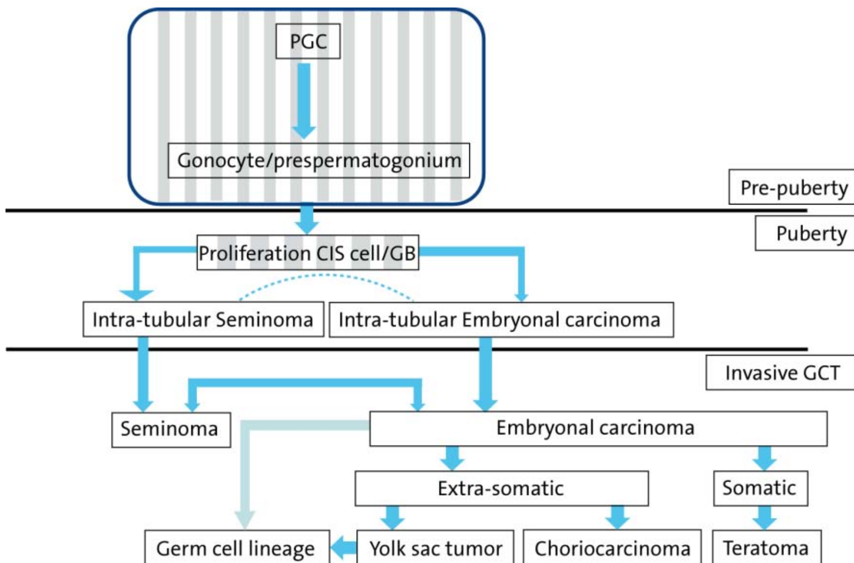

Figure 3. Pathogenesis of the type II (T)GCT. The arrested embryonal germ cell (pre-CIS cell) in the embryonal gonad, is supposed to start proliferating at puberty. Via the specific intratubular stage CIS/GB develops in an invasive germ cell tumor. Testicular cancer is the only totipotent cancer that can give rise to extra-somatic tissue, somatic tissues and also, which is for the first time described in chapter 5, to the germ cell lineage.

All precursors of (T)GCTs, either CIS of the testis or GB of the dysgenetic gonad, are positive for OCT3/4, without a single exception [3,25-26]. The diagnostic utility of this marker can be considered as one of the recent significant contributions to the field of diagnosis of (T)GCTs. Besides the consistent staining of the precursor lesions, OCT3/4 is also positive in all seminomatous tumors, as well as all embryonal carcinomas. This is independent from the primary localization of the cancer, and whether it is metastatic or not. Based on this characteristic, an undiagnosed brain tumor in an adult female was classified as a germinoma (being a seminomatous tumor of the brain), after which an effective treatment approach could be initiated [27]. Detection of OCT3/4 can be done using suitable mono- and polyclonal antibodies, as well as after different methods of fixation. However, the use of acid fixatives, especially in case of over-fixation, can negatively effect detection of nuclear markers in general, including OCT3/4 [28]. The differentiated nonseminomatous elements of (T)GCTs are consistently negative for OCT3/4, both in case of *in vivo* tumors as well as *in vitro* cultured and manipulated cell lines. OCT3/4 is therefore the preferred initial diagnostic marker for (T)GCTs, including their precursor lesions. It is found to be more sensitive and specific than c-KIT (CD117) and PLAP [29-33]. Of notion is that these latter could not be detected in some case due to poor tissue preservation, while the cancer cells remained positive for OCT3/4. In specific well defined situations OCT3/4 might result in false positive conclusions, which will be discussed later.

The robustness of OCT3/4 as diagnostic marker on tissue sections, including biopsies and cytology specimens, stimulated testing of its applicability as marker in a non-invasive set up. CIS detection in semen of males at risk for development of a TGCT has been an attractive approach previously [34-36], although no real success has been reported. In fact, about 1% of Andrology clinic patients will develop a TGCT, and are therefore a putative population for screening. A pilot study of men at risk for CIS demonstrated that 85% showed OCT3/4 positive cells in semen, of which the presence of CIS was proven based on immunohistochemical methods on biopsy-obtained testicular tissue [37]. This positive finding indicates that a non-invasive test for CIS can in principle be developed, of which the characteristics (including positive- and negative predicting power) need to be investigated in ongoing multi-centered studies. A number of questions remain to be answered before this method can be applied in a clinical setting. These include amongst others which patients have to be selected for this procedure, how to deal with negative findings, and about the procedure to follow up in case of a positive finding (e.g. which testis is affected?). In spite of these possible limitations, the approach is of interest to be further developed for clinical implementation. It might be modified in various ways, including the recent possibility to detect protein (i.e., OCT3/4) by means of a

quantitative PCR-based approach [16]. This might result in an informative non-invasive test for screening of high risk individuals for a TGCT.

13.5 Differential diagnosis of seminomatous tumors and embryonal carcinoma

Both the seminomatous tumors as well as embryonal carcinomas are positive for OCT3/4 [3,38-40]. While the seminomatous cells have a characteristic nuclear staining pattern, embryonal carcinoma cells show both a nuclear and a cytoplasmic localization of the protein [38]. In addition, embryonal carcinomas are positive for a number of other markers, like CD30 and keratin, although these can also be sporadically positive in seminoma cells. A distinction between seminoma and embryonal carcinoma is however of patho-biological as well as clinical interest. In fact, seminomas can be treated using irradiation, while this is not used for embryonal carcinomas, which need chemotherapy [41]. Therefore availability of a robust diagnostic marker to distinguish seminoma from embryonal carcinoma is of interest. In addition, the presence of intravascular embryonal carcinoma is predictive for development of metastasis in stage I nonseminomas [42-43]. Based on a combined mRNA expression profile analysis and protein investigation, a consistent presence of SOX2 in embryonal carcinoma was identified [5]. This is in accordance with the knowledge on embryonic stem cells, as well as other studies on TGCTs [44-46]. Careful analysis showed that SOX2 is expressed in Sertoli cells within seminiferous tubules with a non-physiological constitution, either being deprived of or with reduced numbers of germ cells, or the presence of CIS. This can be falsely interpreted as intratubular embryonal carcinoma, leading to in fact over-diagnosis [5]. Using the same approach, SOX17 was found to be consistently expressed in seminomatous cells, including CIS and GB. However, normal spermatogonia were also positive, therefore SOX17 is not a marker suitable to distinguish normal adult- from malignant germ cells. Interestingly, SOX17 has been reported to be the regulatory transcription factor for the transition between embryonic and adult hematopoietic stem cells [47]. As SOX2 can be expressed in more differentiated elements of nonseminomas, SOX17 also shows a wider spectrum of expression. However, the combined positive staining of OCT3/4 with either SOX17 or SOX2, is absolutely informative to identify tumor cells of seminoma and embryonal carcinoma, respectively. It remains to be investigated whether SOX17 forms a similar complex with OCT3/4 as reported for SOX2 [48-51], with the same type of transcriptional outcome [5] (for review of transcriptional and signaling networks of pluripotency see [52]). Therefore, a limited set of (embryonic) transcription factors, showing a strong and consistent nuclear staining pattern, i.e., OCT3/4, SOX2 and SOX17, is informative for the diagnosis of TGCTs in a routine clinical setting.

13.6 SCF as diagnostic marker and early pathogenetic mechanism

Although OCT3/4 is an absolute marker for detection of malignant germ cells in the adult testis, it might result in false positive findings in case of delayed maturation of germ cells [53-57]. It is known that positive germ cells can be physiologically present during the first months after birth. In Figure 1B the protein expression in aberrant germ cell development is illustrated. However, this time window can be extended in various pathological conditions, including cryptorchidism, and cases of DSD [58-59]. These situations are generally characterized by a disturbed testis development, referred to as reduced "testicularization", associated with a slower or even absent maturation of gonocytes to pre-spermatogonia. Based on various descriptive parameters it has been tried to distinguish the identity of early malignant germ cells from delayed matured germ cells [60]. Although this approach is informative to a certain level, it is difficult to be used in a routine diagnostic setting. In this context, the application of immunohistochemistry for SCF (KITLG), is highly informative. No staining has been identified during normal development, while, in contrast, all CIS and GB cases are positive, as well as the high risk samples, showing embryonic germ cells at risk for malignant transformation. This finding is of relevance for a number of reasons. First it suggests a deviation from the normal regulation of SCF in the pathogenesis of (T)GCTs. Suggestive data is available that this is due to activation, or retention, of an autocrine loop, as found in early mouse gonocytes [61]. Indeed, a role in migration as well as survival has been reported for this ligand [62-64]. The possible impact of the presence of either the membrane bound or soluble form remains to be investigated [65-69]. Second, it links this diagnostic marker to genome wide association study results, in which an association between development of a TGCT and various single nucleotide polymorphisms (SNPs) has been found [70-72]. All identified SNPs seem to link to genes of which the encoded proteins have a role in generation of the germ cell niche, including SCF. The functional effect of the different SNPs, including those related to SCF, is unknown so far, but highly interesting and critical in elucidation of the pathogenesis of this type of cancer. Of specific value in a clinical setting would be the combined analysis of risk factors, including the SNPs, to develop a predictive model for TGCTs in selected groups of individuals.

13.7 Direct frozen tissue diagnosis of CIS and invasive testicular germ cell tumors

Frozen tissue diagnosis can be informative in a clinical context of testicular lesions in particular cases. These relate, amongst others, to the possible limited surgical approach of removing a putative benign lesion (enucleation), like in case of a mono-testis situation. However, detailed investigation of the adjacent parenchyma for the presence of CIS is required to exclude the presence of TGCT. PGCs and gonocytes are positive for PLAP detectable using immunohistochemistry, as well as found for CIS and GB. The enzymatic activity can also be visualized using a direct readout system on frozen tissue, an approach to be performed within a 5 minutes time frame. Accurate application of the method is feasible, due to the fact that endothelial cells of small vessels serve as positive controls. This approach is informative to detect CIS (and GB), as well as seminoma and embryonal carcinoma. It is much faster than standard fixation, paraffin-embedding and subsequent staining using immunohistochemistry, which requires at least 2 days. This so-called dAP test can be used for direct diagnosis during surgical intervention, allowing direct decision making regarding possible limited surgery, preventing possible need for a second operation [73].

13.8 Epigenetics

Epigenetic modification is proven to be a crucial regulator of gene expression, both during embryogenesis as well as pathogenesis of various diseases, including cancer [74]. One of the modifications is methylation of DNA, of which the overall status can be investigated using immunohistochemistry. Like PGC/gonocytes, CIS and GB have a demethylated pattern, in line with the close relationship between these types of cells. In addition, also the various seminomatous tumor cells are demethylated. In contrast, all differentiated nonseminomatous elements are hypermethylated, while the embryonal carcinoma cells show an intermediate pattern. In spite of a limited number of cases, it is of interest that chemo-resistant seminomas show higher methylation. This triggered a study using (T)GCT derived cell lines relating methylation pattern and (cisplatin) chemo-sensitivity. In spite of the observation that all cell lines show a high level of methylation, different levels of sensitivity to chemotherapy have been reported. Forced demethylation, using 5-azacytidine, in the cisplatin-resistant seminomatous cell line TCam-2 results in induced sensitivity [10]. High throughput methylation status investigation identified a number of regulatory sites of genes to be affected by this manipulation. One of the elements is associated with FLAR, also known as c-FLIP. This is a known oncogene, which is inhibiting formation of active caspase 8, which is required for execution of the apoptotic pathway [75]. Indeed, overexpression of FLAR is found to suppress apoptosis, also in TGCT-

derived cells lines [76], and it is found to be one of the predictive genes in an expression profile analysis of cisplatin-refractory TGCT [77], as well as in other cancers [78-79]. These results strongly suggest that apoptosis regulation is one of the determining factors in regulation of treatment sensitivity of TGCTs. Indeed, such a link has been reported before [76,80]. Further investigations are required to understand the impact of this finding in a clinical setting. For example, a combined approach, in which demethylating agents have a place, could be investigated in the treatment of cisplatin-refractory (T)GCTs.

13.9 Concluding remarks

Based on the studies performed, a number of conclusions can be drawn. Optimal diagnosis of (T)GCT, including their precursor lesions CIS and GB, is feasible using a limited set of markers in a routine clinical setting. This approach, including antibodies against OCT3/4, SOX17, SOX2 and SCF, is applicable independent of the age of the patient, as well as localization of the cancer. Moreover, an absolute test for the presence of CIS on frozen tissue is developed, suitable to be included into direct clinical decision making for limited surgical procedures. The overall and targeted methylation status is found to be involved in treatment sensitivity and resistance. FLAR is an interesting target for further investigation. Combining the observation of SCF as early diagnostic tool and the predictive power of SNPs related to this gene, opens a complete new field on investigation on the pathogenesis of (T)GCTs. In this context the interaction between Genetics and Environment, referred to as GENVIRONMENT, is an exciting development to be further explored in the context of the pathogenesis of (T)GCTs.

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Chapter 14

Summary/Samenvatting

CHAPTER 14

English

Introduction

The aim of the work described in this thesis is to improve the understanding of the pathobiology of testicular cancer (type II Germ Cell Tumors) to create possibilities for optimalization of diagnosis for this type of malignancy in routine pathology laboratories. The different studies presented here show valuable additional information on the microscopic diagnostics in daily practice. This enables proper and complete diagnosis of this relative rare variant of cancer ensuring the best possible treatment for the patient. A number of novel sensitive and specific immunohistochemical markers are presented, of value for (early) diagnostics. In addition an enzymhistochemical method is presented informative to detect the presence of (pre-)malignant cells during a surgical intervention. Based on these data, a decision can be made regarding a testis sparing procedure. The studies included in this thesis are part of a diagnostically decision tree in which immuno- and enzymhistochemistry plays an important role.

General

Testicular cancer is a relative rare variant of cancer with raising incidence in the Western population, in contrast to the rest of the world. Within the Netherlands, about 750 new patients are diagnosed each year. In particular this affects young males between the age of 15 – 45 years The current treatment possibilities is successful in approximately 95% of the patients. It consists of removing of the affected testicle (radical orchiectomy) and possibly additional treatment using irradiation and/or chemotherapy. In spite of the excellent prognosis, patients often will suffer from long-term side effects of the treatment like fatigue, disorders of the metabolism, heart diseases, neurotoxicity and infertility. A small group of patients (5%) will shows an insensitivity of the cancer treatment given, and will possibly die of the disease. An early and accurate diagnosis can prevent under- or overtreatment, ensure an optimal balance between the pro and conts of the treatment. This will have significant impact on their quality of life, for both short- and long term.

Specific

The cell of origin from which testicular cancer arises is already present during the first months of pregnancy (embryogenesis), as demonstrated in **Chapter 3**. It is a primitive germ cell formed after the first month after fertilization, which provide spermatozoa after puberty by spermatogenesis. In case this primitive germ cell does not maturate completely and keeps the properties of the embryonal cell. This cell can become carcinoma *in situ* (CIS), being the progenitor of testicular cancer. These CIS cell is located in the testis on the same place as the spermatogonium, below the Sertoli cells on the basal lamina. These cells start to

multiply in an uncontrolled manner after puberty and finally they can develop cancer. In **chapter 4** the totipotent character of this cancer is described for the first time in literature, shown by the presence of new formed germ cells. In other words testicular cancer is the only real totipotent cancer that can give rise to different germ lines the same as in embryogenesis. The application of a unique panel of immunological markers permitted this finding. In **chapter 5** the value of the presence of OCT3/4 in CIS, seminoma and embryonal carcinoma is for the first time described. This study was initiated by the presence of this marker in embryonal cells and germ cells of the mouse. The diagnostic significance of this extremely sensitive and specific marker is huge as described in **chapter 6**. Diagnostics of testicular germ cell tumors requires in most cases surgical removed tissue. In **chapter 7** a new method is described that offers the possibilities to detect OCT3/4 positive cells in semen. This method introduces the possibility to screen men with a higher risk for developing testicular germ cell tumor for CIS cells without surgical intervention. The robustness of this detection method for OCT3/4, even in sub optimal material, is illustrated in **chapter 8**. The malignant germ cells in the here described patient where only detectable by the presence of OCT3/4.

The two groups of testicular cancer: seminomatous and nonseminomatous tumors, differs in sensitivity for irradiation and chemotherapy. Therefore differentiation of these two groups is of great importance for an optimal treatment. The nonseminomas can be subdivided in different groups of which embryonal carcinoma is the stem cell population. In **chapter 9** detection of two SOX proteins (SOX2 and SOX17) is shown to be informative. Seminoma cells show expression of OCT3/4 and SOX17, embryonal carcinoma cells of OCT3/4 and SOX2. The other subgroups of the nonseminomas (yolk sac tumor, choriocarcinoma and teratoma) show a more heterogeneous expression.

A chance of overdiagnosis of CIS in the testicle based on the presence of OCT3/4 is risk in young children, especially in the first year after birth, a result of a possible delay in maturation of the normal developing germ cells. This is Important because maturation delay is common also in cases of an increased risk for the development of testicular cancer, for instance an undescended testicle. In **chapter 10** it is demonstrated how to distinguish a germ cell with maturation delay from a (pre-) malignant germ cell (CIS). Because SCF is only present in the direct neighbourhood of (pre-)malignant germ cells. This finding has an additional value in case of possible maturation delay when OCT3/4 positive cells are present. There is also a significant value for this finding concerning that specific DNA variants related to this gene, associated with an increased risk for development of testicular cancer in the entire population.

Organ sparing surgery may be preferred for different reasons, even so in cases of testicular cancer. Prevention of a second surgical intervention requires proper histopathological examination of frozen sections. In testis cancer this is mainly based on the detection of the presence of CIS. Unfortunately morphologic analysis of frozen material is not that easy, therefore formalin fixed tissue is needed which excludes often a single operation procedure. In **chapter 11** a direct enzymhistochemical method is described which makes it possible to detect the presence of these CIS cells in a few minutes on frozen tissue. By using this direct alkaline phosphatase (dAP) technique it is possible to have certainty about the presence of CIS cells in the available tissue. Based on these results a decision is possible about performing a testis sparing operation.

Chapter 12 describes the low DNA methylation status of both CIS cells and embryonal germ cells in their natural environment. This is performed by the use of immunohistochemistry. Interesting is the possible connection between cancer cells which are sensitive to cisplatin and the status of methylation. DNA of the resistant tumors seems to be hypermethylated compared to sensitive tumors. Analysis of cultured cell lines indicates c-FLIP gene as a potential candidate. This gene regulates the sensitivity of cells for a programmed cell death (apoptosis).

Conclusions

The studies presented give possibilities to perform optimal diagnostics of testicular cancer, eventually in certain circumstances, even in a non-invasive set up. These findings demonstrate that understanding of the normal development of germ cells, especially during embryogenesis, is of importance to carry out these kind of studies. The results give a solid base for implementation in a diagnostic set up and for further translational research.

CHAPTER 14

Nederlands

Introductie

Het doel van het werk beschreven in dit proefschrift is het begrijpen van de pathobiologie van zaadbalkanker (teelbalkanker, kiemcelkanker), en daarmee mogelijkheden te creëren voor het optimaliseren van de diagnostiek van deze vorm van maligniteit, toepasbaar in elk pathologisch laboratorium. De hier beschreven studies geven een waardevolle aanvulling op de microscopische diagnostiek in de dagelijkse praktijk. Hierdoor is een betrouwbare en volledige diagnose te stellen van deze relatief zeldzame vorm van kanker die voor de behandeling van de patiënt van belang is.

Er worden een aantal nieuwe gevoelige en specifieke immunologische markers geïntroduceerd die voor de (vroeg)diagnostiek een belangrijke toegevoegde waarde bezitten. Tevens wordt een enzymhistochemische methode beschreven die de mogelijkheid biedt om op een eenvoudige toe te passen wijze gedurende een chirurgische ingreep aan de teelbal de aanwezigheid van (pre)maligne cellen te detecteren. Op grond van de bevindingen kan besloten worden tot eventueel een testis-sparende ingreep uit te voeren. De in dit proefschrift beschreven studies maken deel uit van een diagnostische beslisboom waarin immuno- en enzymhistochemie een belangrijke plaats innemen.

Algemeen

Zaadbalkanker is een relatief zeldzame vorm van kanker die steeds meer voorkomt in de Westerse populatie, in tegenstelling tot in de rest van de wereld. In Nederland wordt bij ongeveer 750 nieuwe patiënten per jaar deze diagnose gesteld. Vooral jonge mannen tussen de 15 en 45 jaar worden door deze vorm van kanker getroffen.

De huidige beschikbare behandelingsmethoden van zaadbalkanker leidt bij ongeveer 95% van de patiënten tot complete genezing. Dit bestaat uit verwijdering van de aangedane zaadbal (orchidectomie) en eventueel een nabehandeling met bestraling en/of chemotherapie. Ondanks de uitstekende prognose krijgen deze patiënten vaak te maken met de gevolgen van de behandeling die kunnen bestaan uit: vermoeidheid, stofwisselingsstoornissen, hart- en vaat ziekten, aandoeningen aan de zenuwen en onvruchtbaarheid. In ongeveer 5% van de patiënten blijkt tijdens de behandeling de kanker niet gevoelig te zijn, waardoor de patiënt de kans loopt aan de ziekte te overlijden. Een vroege en juiste diagnose kan onder- en overbehandeling voorkomen, waardoor de optimale balans tussen voor- en nadelen van de behandeling verkregen kan worden. Dit heeft significante effecten op de kwaliteit van leven, zowel op korte als op lange termijn.

Specifiek

De cel van oorsprong waaruit de zaadbalkanker ontstaat is al tijdens de eerste maanden van de zwangerschap (embryogenese) aanwezig, zoals beschreven in **Hoofdstuk 3**. Het is een primitieve kiemcel die na de eerste maand na de bevruchting gevormd wordt, welke na de pubertijd door middel van spermatogenese bij de man de uiteindelijke zaadcellen gaat vormen. In het geval dat deze primitieve kiemcel niet volledig uitrijpt en embryonale eigenschappen behoudt, kan deze cel carcinoma *in situ* (CIS) vormen, dat de voorloper is van zaadbalkanker. De CIS cel bevindt zich in de zaadbal op de plaats van normaal gesproken een zogenaamd spermatogonium zich bevindt (onder de Sertoli cellen, op de basale lamina). Het zijn deze cellen die zich na het starten van de pubertijd ongecontroleerd gaan vermeerderen, en uiteindelijk de kanker kunnen vormen. In **hoofdstuk 4** wordt voor de eerste maal in de literatuur de werkelijke totipotentie van zaadbalkanker beschreven, aangetoond door de aanwezigheid van de nieuw gevormde kiemcellen in de kanker. Met andere woorden, zaadbalkanker is de enige werkelijke totipotente kanker, in staat tot het vormen van alle verschillende differentiatielijnen die gevonden kunnen worden tijdens de embryogenese. Deze bevinding is mogelijk gemaakt door de toepassing van een unieke set van immunologische markers. In **Hoofdstuk 5** wordt voor de eerste keer de waarde van de aanwezigheid van OCT3/4 voor het aantonen van CIS, seminoom en embryonaal carcinoom beschreven. De reden voor het initiëren van deze studie was de beschrijving van deze marker voor embryonale stam cellen en kiemcellen in de muis. De diagnostische waarde van deze zeer gevoelige en specifieke marker is groot, zoals in **hoofdstuk 6** is weergegeven. Voor de diagnostiek van zaadbalkanker wordt in de meeste gevallen gebruik gemaakt van chirurgisch verwijderd weefsel. In **hoofdstuk 7** wordt een nieuwe methode beschreven die het mogelijkheid maakt om OCT3/4 positieve cellen in zaadvloeistof aan te tonen. Deze methode biedt de mogelijkheid om bij mannen met een verhoogd risico op het krijgen van zaadbalkanker, zonder een eerste invasieve ingreep, onderzoek te doen naar de aanwezigheid van CIS cellen. De robuustheid van de methode voor het aantonen van OCT3/4 wordt aangetoond, zelfs in sub-optimaal materiaal. Dit wordt geïllustreerd in **hoofdstuk 8**, waar een patiënt wordt beschreven waarbij de kwaadaardige kiemcellen enkel te detecteren waren door het aantonen van OCT3/4.

Seminomen en nonseminomen zijn twee varianten van zaadbalkanker, die verschillende gevoeligheden vertonen voor bestraling en chemotherapie. Voor het toepassen van de optimale behandeling is een differentiatie dus van belang. Binnen de nonseminomen worden verschillende subvarianten beschreven, waarvan embryonaalcarcinoom de stam cel populatie is. In **hoofdstuk 9** wordt beschreven dat het aantonen van twee verschillende SOX eiwitten en wel SOX2

en SOX17, hier informatief voor is. De seminoom cellen zijn positief voor OCT3/4 en SOX17 en de embryonaal carcinoomcellen voor OCT3/4 en SOX2. Een meer heterogeen beeld wordt gevonden in de andere nonseminoom componenten (dooierzaktumor, choriocarcinoom en teratoom).

De mogelijke overdiagnostiek van CIS van de zaadbal op grond van het aantonen van OCT3/4 is een risico bij jonge kinderen, met name in het eerste levensjaar. Dit is het gevolg van mogelijke rijpingsremming (vertraagde ontwikkeling) van de normale kiemcellen. Dit is van belang, omdat rijpingsremming voorkomt in die gevallen waarbij ook een verhoogd risico op zaadbalkanker gevonden wordt, zoals bijvoorbeeld een niet ingedaalde zaadbal. In **hoofdstuk 10** wordt aangetoond dat er onderscheid gemaakt kan worden tussen een kiemcel met rijpingsremming aan de ene kant en een (pre-)maligne kiemcel (CIS) aan de andere kant. Dit op grond van het aantonen van de aanwezigheid van SCF, dat alleen voorkomt in de directe omgeving van (pre-)maligne kiemcellen. Deze bevinding heeft additionele waarde in het geval dat OCT3/4 positieve kiemcellen aangetoond worden, waarbij de vraag is of er een vertraagde uitrijping aanwezig is. Deze bevinding heeft tevens significante waarde in het kader van het feit dat specifieke DNA varianten gerelateerd aan dit gen beschreven zijn, die een verhoogd risico geven op zaadbalkanker in de totale populatie.

Orgaansparende operaties kunnen op grond van verschillende redenen van belang zijn, zo ook in het geval van zaadbalkanker. Het voorkomen van het nodig zijn van een tweede ingreep vereist informatieve vriescoupe diagnostiek. In het geval van zaadbalkanker betreft dit voornamelijk het aantonen van de aanwezigheid van CIS. Echter blijkt dit door morfologische analyse van vriesmateriaal niet eenvoudig te zijn, waardoor formaline-gefixeerd weefsel nodig is. Hierdoor blijkt de ingreep in een eenmalige chirurgische uitvoering vaak niet mogelijk te zijn. In **hoofdstuk 11** wordt een directe enzymhistochemische bepaling beschreven, dat het mogelijk maakt om binnen enkele minuten te bepalen of CIS cellen aanwezig zijn. Met gebruikmaking van deze directe alkalische fosfatase (dAP) test is het mogelijk om met zekerheid vast te stellen of CIS cellen in het beschikbare weefsel aanwezig is. Op grond hiervan is een besluit omtrent het uitvoeren van een zaadbalsparende operatie mogelijk.

In **hoofdstuk 12** wordt aangetoond dat de CIS cellen, net als de embryonale kiemcellen een laag niveau van DNA methylatie bevatten. Dit is bepaald door middel van immunohistochemie, waardoor de informatie verkregen kan worden van cellen in hun natuurlijke omgeving. Interessant is de bevinding dat er een mogelijk verband bestaat tussen cisplatin gevoeligheid van de kankercellen en de methylatie status. Met name de ongevoelige tumoren blijken meer DNA methylatie te bevatten dan de gevoelige tumoren. Door cellijn analyse is hierbij

het c-FLIP gen als mogelijke kandidaat naar voren gekomen. Dit gen reguleert de gevoeligheid van cellen voor een geprogrammeerde celdood (apoptose).

Conclusies

De beschreven bevindingen geven de mogelijkheid optimale diagnostiek van zaadbalkanker uit te voeren, eventueel in een niet-invasieve opzet in bepaalde situaties. De bevindingen maken duidelijk dat het begrijpen van de normale ontwikkeling van kiemcellen, met name tijdens de embryogenese, van belang is voor het uit kunnen voeren van dergelijke studies. De resultaten geven een goede basis voor implementatie in een diagnostische setting als ook voor verder translationeel onderzoek.

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Curriculum Vitae

The author of this thesis was born in Bergen op Zoom on December 1st, 1955. In 1975 he finished his HBO-A pathology, histology, cytology and hematology at the *School voor Laboratoriumpersoneel* in Breda and started his career as a technician at the laboratory for histo-pathology (department of pathology) at the University Hospital Rotterdam. After 3 years he became a chief technician. In 1982 he started as a chief technician of the department of immunohistochemistry at the Pathological Anatomical Laboratory in Dordrecht. In June 1994 he graduated from the *Polytechnische Faculteit Hogeschool West Brabant* in Etten Leur: HLO-Biochemistry and started to study biology at the University Utrecht in the same year. During this period he finished his internship molecular genetics with the dissertation “The instability of chromosomes by an aberrant mitotic checkpoint in human testicular germ cell tumors” at the Laboratory for Experimental Pathology and Oncology (L.E.P.O.) of Prof.dr. Leendert Looijenga and Prof.dr. Wolter Oosterhuis at the Daniel den Hoed Cancer Center Rotterdam. In 1998 he graduated at the University Utrecht specialized in “Molecular Genetics” and “History and Philosophy of Biology”. Since January 1999 he has been working as a senior research technician with the group of Prof.dr. Leendert Looijenga at the Josephine Nefkens Institute, Erasmus Medical Center Rotterdam.

He is married to Els and they have two daughters and two grandsons.

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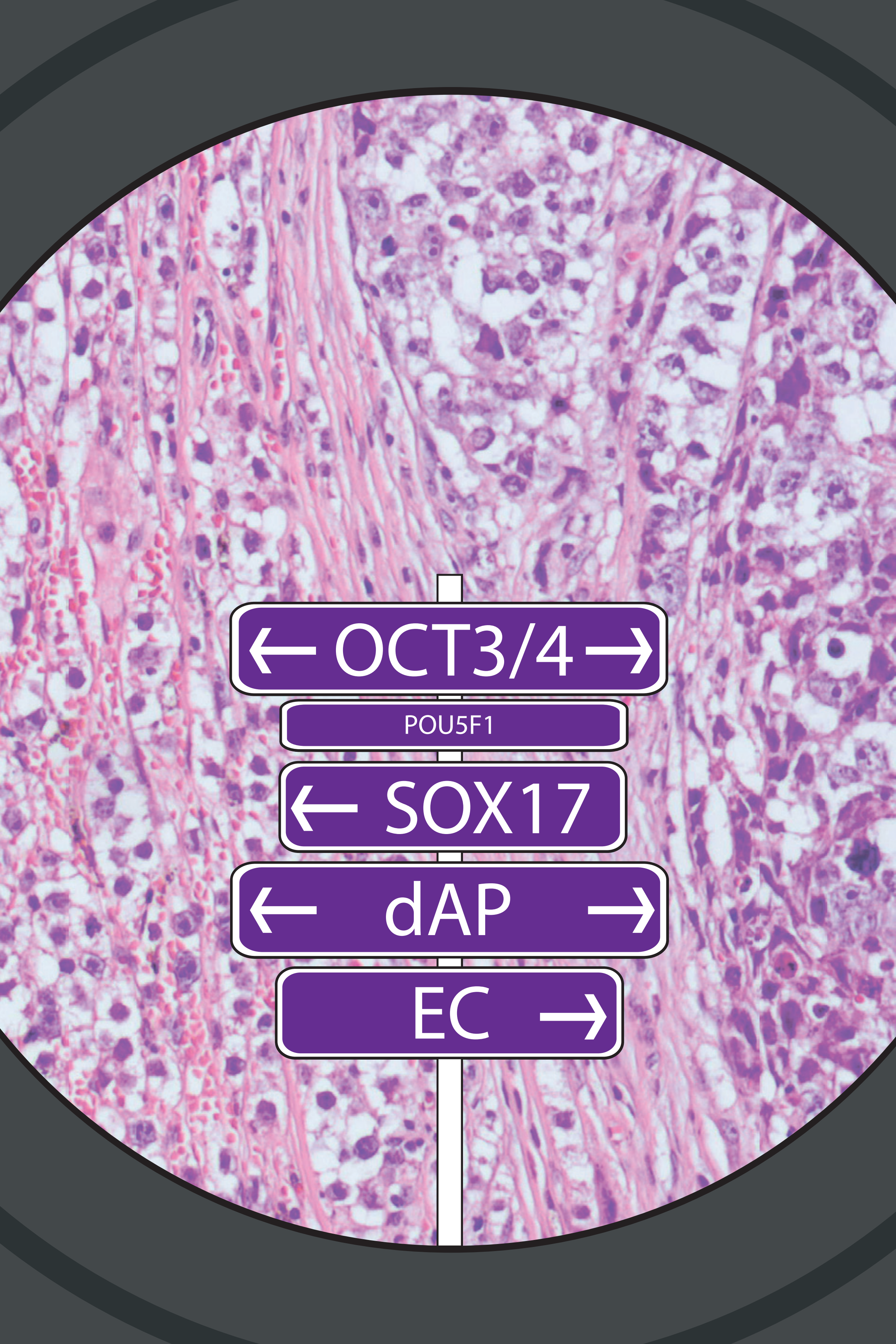
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