Normal and Malignant Germ Cell Development

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Normale en Maligne Ontwikkeling van Kiemcellen

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“There is no science without fancy,
and no art without facts.”

Vladimir Nabokov

Meiner Familie

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Chapter 1

GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

The group of human germ cell tumours (GCTs) is heterogeneous, and comprises neoplasms found at different anatomical localizations (1 for review). They are of interest not only for aspects of their tumour biology, but also from the point of view of developmental biology. The clinical course of GCTs depends on factors such as sex of the patient, age at clinical diagnosis, histology, and anatomical site of the tumor. A classification of GCTs including 5 groups, based on these parameters, as well as their chromosomal constitution, has been proposed (2). Within the testis, only three of these different entities can be distinguished: I) the teratomas and yolk sac tumours of newborn and infants (referred to as type I tumours), II) the seminomas and nonseminomas of adolescents and young adults (referred to as type II tumours), and III) the spermatocytic seminoma of the elderly (referred to as type III tumours). Type IV and V are the dermoid cysts and the hydatidform moles, respectively (3, 4 for reviews). This distinction has recently been adopted by the 2003 World Heath Organisation (WHO) classification system for testicular GCTs (5). This thesis focuses on the type II tumours, the largest group of malignant GCTs.

Most likely related to their cell of origin, which resembles a primordial germ cell (PGC)/gonocyte to prespermatogonia, GCTs show significant similarities with germ cells at various developmental stages (this thesis, 6). Comparison of tumours and normal counterparts can help to define normal and disturbed development of germ cells. This thesis focuses on the developmental potential of germ cells, both normal and malignant.

1.1. Aspects of pathology and molecular biology of human germ cell tumours

Besides in the gonads, both ovary and testis, GCTs can be found at different sites along the midline of the body, i.e. the retroperitoneal, mediastinal, and hypothalamus/pineal gland regions. Possibly this is due to the migration route of PGCs during embryogenesis (7). In some of these tumours the germ cell origin is supported by the presence of VASA (8), a germ line specific protein (9), or their specific pattern of genomic imprinting (10, 11, 12). As they can mimic early embryonic development, GCTs are at the crossroads of developmental and tumour biology, and of interest from both these points of view. It is of relevance to keep this in mind when interpreting data in the context of the pathogenesis of GCTs. This will become most eminent in the context of treatment sensitivity and resistance, as discussed below.

1.2. Normal and disturbed male and female germ cell development

In humans and rodents, amongst other species, the embryonic precursors of the gametes are known as PGCs (13 for review). These cells are set aside to an extra-embryonic localization early during embryonic development, and migrate to the area where the genital ridge will be formed around the 5th

and 6th week of human development (14). Subsequently, the gonads and sexual differentiation occur in the 6th and 7th week (15).

Various risk factors for the maldevelopment of the fetal reproductive system like cryptorchidism (16) and gonadal dysgenesis are associated with an increased risk of testicular cancer, possibly due to disturbed migration and maturation of germ cells (17, 18, 19). The precursor of testicular seminomas and nonseminomas is well established, known as carcinoma *in situ* (CIS) (20), also referred to as intratubular germ cell neoplasia unclassified (IGCNU).

1.2.1. Female germ cell development

Between the 7th and 9th week during ovarian development (referred to as prefollicular stage) the germ cells (oogonia) and pre-granulosa cells characteristically arrange in cords and sheets without specific organization (21). The population of oogonia increases by undergoing multiple divisions, after which they become oocytes, stop proliferating, and enter meiosis. The first distinctive change in prenatal germ cell development in the ovary therefore is the onset of meiosis, which starts at 11 to 12 weeks of gestation and extends into the second trimester (22). Contradictory data exist with regard to the onset of folliculogenesis, i.e. the occurrence of primordial follicles, which has been described to take place between 16 and 21 weeks of gestation (23, 24). Considerable changes in the total number of germ cells are seen, first due to the proliferation of premeiotic oogonia and later the apoptotic elimination of both oogonia and germ cells at different stages of folliculogenesis during intrauterine maturation (25). Maturation is a gradual process, characterized by the initiation of certain stages of oocyte development at different time points. In humans, this process is not synchronized, as germ cells at different developmental stages can be detected at the same time in the same prenatal ovary. This suggests that unknown intracellular mechanisms may govern the individual pace of maturation.

1.2.2. Male germ cell development

In case of testicular development, early germ cells are referred to as gonocytes, which are predominantly found in the central areas of the newly formed tubules. Starting around the 14th week of foetal development, these gonocytes gradually migrate towards the tubular periphery. Once the cells have reached close contact with the basal lamina of the tubule, they are referred to as prespermatogonia. As in the female gonad, germ cell maturation in the foetal testis is a gradual process. At the 20th gestational week the foetal testis predominantly contains prespermatogonial germ cells (26). During the first years after birth and until puberty, morphological and functional changes occur, including a change from large, immature germ cells (prespermatogonia) to adult type A spermatogonia (27, 28, 29). In contrast to the situation in the female gonad, germ cells in the male gonad do not enter meiosis before the onset of puberty. With the onset of puberty, spermatogonia undergo further spermatogenic maturation, and after meiotic divisions finally produce spermatozoa.

1.2.3. Disturbed germ cell development in dysgenetic gonads

The term 'dysgenetic gonads' is used to describe the phenomenon of a disturbed gonadal sex determination or differentiation. Prolonged expression of immunohistochemical markers in various intersex conditions has been interpreted as a result of a maturation delay of germ cells, and has been linked to the high incidence of germ cell neoplasia seen in these individuals (30, 31, 32). The precursor lesion of the tumours of the dysgenetic gonad is called gonadoblastoma (16). Gonadoblastoma, like CIS/IGCNU, shows the potential to progress to an invasive GCT, mainly dysgerminoma, and less frequently to other tumour components, i.e. embryonal carcinoma, teratoma, yolk sac tumour, and choriocarcinoma.

1.3. Embryonic origin and developmental potential of germ cell tumours

CIS/IGCNU is located at the inner side of the basal layer of the seminiferous tubules, where normally the spermatogonia reside. Various phenotypical characteristics support the model that the PGC/gonocyte is the benign counterpart of the CIS cell (33, 34, 6). Accordingly, if the PGC/gonocyte is the cell of origin for testicular GCT, the initiation of tumourigenesis has to occur during intra-uterine development. It has been suggested that the CIS cells are formed before the 9th gestational week. Various epidemiological and biological arguments support the concept of early initiation. Despite an early initiation, testicular GCTs become clinically manifest only after puberty. This lag period is most likely explained by hormonal influences on the growth and development of transformed (initiated) PGCs/gonocytes. The exact nature of these factors remains unknown.

So far, it is not resolved whether CIS/IGCNU are programmed to form only seminoma, nonseminoma, or a combination of the two, or whether these cells are multipotent or even totipotent. Differences between CIS/IGCNU surrounding nonseminomas and seminomas have been described regarding immunohistochemical markers and chromosomal constitution (35, 36). Therefore, a pre-determination of the later tumour histology already at the preinvasive stage has been suggested. However, the differences could also be an effect of the adjacent tumour on these cells, referred to as parallel evolution.

Seminomas are uniform tumours, composed of cells resembling PGCs/gonocytes (37, 38). In contrast, nonseminomas are heterogenous and can contain one or more histologic subtypes representing various differentiation lineages and stages of normal intra-uterine development. The embryonal carcinoma cell is the pluripotent stem cell component, which may differentiate towards extra-embryonal tissues (yolk sac tumour and choriocarinoma) and embryonic tissues with mesenchymal, epithelial and neuronal appearance (immature and mature teratoma) (39). As they have never been shown to give rise to the germ line in humans, they seem to fall one step short of totipotency.

1.3.1. Role of the transcriptional regulator POU5F1 (OCT3/4) on germ cell development

POU5F1 (also known as OCT3/4, which will be used in the following) is expressed in undifferentiated, pluripotent cells including embryonic stem cells (ES) and germ cells, both in mouse (40, 41, 42) and human (43, 44). It is involved in regulation of pluripotency, as levels of expression determine whether ES cells remain undifferentiated, or differentiate into either trophectoderm or extraembryonic endoderm and mesoderm (45). In fact, OCT3/4 is the determining factor for the first embryonic lineage specification. Inhibition of expression of this gene in mouse or human ES cells results in differentiation to trophoectoderm (46, 47). In contrast, PGCs without OCT3/4 do not differentiate, but undergo apoptosis (48). This indicates that the role of OCT3/4 in embryonic stem cells and PGCs is different.

It has been shown that expression of OCT3/4 in GCTs is highly similar to the pattern found during normal development (49). OCT3/4 is found specifically in CIS/IGCNU, seminoma and embryonal carcinoma cells, while yolk sac tumour, teratoma and choriocarcinoma are consistently negative. Immunohistochemical detection of OCT3/4 is of high diagnostic value for GCTs (50). In addition, its expression was also found in gonadoblastoma, dysgerminoma, and germinoma (49). This has been confirmed in multiple studies (50 for review).

OCT3/4 has been termed a dose-dependent oncogenic fate determinant in the mouse model (51). However, this conclusion was drawn from results obtained in mouse ES cells, which are not a proper animal model for type II GCTs. Furthermore, no correlation between treatment response and OCT3/4 staining in human germ cell tumours was observed (49). The fact that OCT3/4 expression is regulated during differentiation of pluripotent cells suggests that the gene is under control of a physiological expression machinery, rather than being an oncogene in the classical sense. However, it remains to be shown whether the presence of OCT3/4 is indeed a driving force in the pathogenesis of this disease in humans, or merely reflects the origin of these tumours and their developmental potential.

1.3.2. Role of TSPY (testis-specific protein,Y-encoded) in germ cell development

The human *TSPY* (testis-specific protein, Y-encoded) gene is organized as a repetitive gene family. It maps to the critical region of GBY (GonadoBlastoma on the Y chromosome) (52, 53, 54) locus on the short arm of the Y chromosome (55, 56). Expression of *TSPY* sequences has been found by RNA analysis in prenatal and adult human testis (57, 58, 56). It is mainly present in humans during early spermatogenesis. It has been suggested to play a normal role in spermatogonial proliferation and an oncogenic role in early germ cell tumorigenesis (59, 60). In addition to CIS/IGCNU, gonadoblastoma cells were also found to express TSPY in a limited number of cases (61, 60). Even though a role in the proliferation of human germ cells has been suggested, the function of TSPY remains speculative.

1.3.3. Role of the microenvironment in germ cell development

Mammalian germ cells originate from the proximal region of the epiblast. In mouse experiments, embryonic stem cells can be stimulated to differentiate into germ cells and extra-embryonic mesoderm

when grafted to the proximal epiblast (62). This indicates that the potential to become a germ cell may not be restricted to predetermined progenitor cells, but are under control of extracellular factors and/or cell-cell interactions (63). Even though a number of factors involved in germ cell survival have been suggested (64 for review), their exact nature is largely unknown. Recent findings indicate that in mouse embryonic stem cells, BMP4 might be crucial for germ cell development (65). It is noteworthy that differentiation occurred only when the embryonic stem cells were coaggregated with BMP4-producing cells; no effect was seen when BMP4 was added to a suspension culture of embryonic stem cells, underlining the importance of a unique three-dimensional configuration of these cells (65). Besides BNP4/BNP8b, also stem cell factor (SCF)/KIT, and ß-catenin/E-cadherin, have been found to have an effect on germ cell development. The SCF/KIT system is involved in survival and proliferation of migrating germ cells in mice (66). In humans, recent studies suggest an important role of this system in female germ cells (67). Whereas some data is available on the cadherin-catenin complex in mouse PGC development and oocyte maturation (68, 69), little is known about the role of these factors in human germ cell development.

1.4. Treatment sensitivity and resistance in germ cell tumours

Modern treatment of GCTs is based on all available treatment options in the field of oncology: surgery, radiotherapy and chemotherapy. Even in metastatic stages, cure rates of 80% can be achieved by multiagent, cisplatin-based chemotherapy. These treatment results are unique among solid tumours of adults. GCTs are therefore considered a model for a curative malignant disease (70). The exquisite chemosensitivity of GCTs has been regarded as a reflection of the characteristics of their cells of origin, PGC/gonocyte and early embryonal cells derived from them. From a teleologic point of view, it is assumed that it is of paramount importance to maintain the genetic information of stem cells intact. In order to achieve this goal, cells with damaged DNA are thought to go into apoptosis rather than try to repair the damage. Thereby, they avoid errors occurring during DNA-repair, which could be transmitted to their progeny with detrimental effects (71).

1.4.1. Biologic basis of treatment sensitivity

Seminomas are highly sensitive to both radiation and chemotherapy. Nonseminomas are less susceptible to radiation but show an overall high sensitivity to combination chemotherapy. Treatment resistance, which occurs in about 10-30% of patients diagnosed with a metastatic nonseminomatous germ cell tumour, might in fact be related to the loss of efficient defense mechanisms for DNA damage recognition or acquisition of effective DNA repair mechanisms. A number of reviews on this issue have been published recently (72, 73, 74). The majority of findings so far indicate that the crucial determinators of drug sensitivity in type II GCTs are downstream of DNA binding, e.g. in the intrinsic or extrinsic pathways of apoptosis or DNA repair. Most tumors lack export pumps with cisplatin affinity and show low levels of detoxification. It has been postulated that low levels of the DNA repair

proteins XPA and HRAD9/HRAD9B is related to the overall sensitivity of these tumours (75, 76). The presence of testis-specific high mobility group (HMG)-box proteins might also be relevant (77), as well as the FAS-ligand system (74 for review). Although suggested multiple times, it is now accepted that the TP53 status is not the sole determining factor whether type II GCTs will be responsive or resistant to treatment (78, 72 for review). More likely, the presence of TP53 in type II GCTs could again be related to their origin from PGCs (79).

1.4.2. Role of differentiation and a functional cell cycle control in treatment resistance

While most type II GCTs show an intact DNA mismatch repair pathway (MMR), a defect leading to microsatellite instability has been observed in tumours refractory to cisplatin (80). In addition, a defective caspase 9 function has been reported in treatment resistance (81). Although all these factors might be important, the overall notion is that most likely not one single factor alone will determine treatment resistance. This is nicely illustrated by the findings in mature teratoma of nonseminomas, which are characteristically resistant to chemotherapy (80), possibly due to epigenetic changes occurring during somatic differentiation.

Cell cycle control is crucial in determining whether a cell will remain proliferative, or will differentiate. Various studies on regulatory elements of the cell cycle have been performed in GCTs, with a number of interesting findings. For example, overexpression of cyclin D has been suggested to be involved in the development of GCTs. Interestingly, progression from CIS/IGCNU to invasive growth is associated with loss of the cell cycle inhibitor CDKN2C (p18), resulting in disturbed cell cycle regulation (82).

A number of studies have linked the development of GCTs to a deregulated G_1/S checkpoint. The absence of such a checkpoint could be related to the lack of the cell cycle regulator RB (83), and is reflected in the absence of CDKN1A (p21) upregulation after induction of TP53 following DNA damage. Interestingly, cells without p21 show reduced cisplatin-induced DNA damage repair, and increased sensitivity to cisplatin (84). These findings may again be related to the origin of the tumours (34), as neither RB nor CDKN2D (p19) are found in human foetal gonocytes or CIS/IGCNU (82, 85). Within the histological heterogeneity of type II GCTs, the mature teratomas are consistently resistant to DNA damaging agent (see above). In contrast to other invasive components, the mature teratomas are positive for MDR, MRP2, BCRP, LRP, and GSTπ. Moreover, mature teratomas show presence of RB, p21, and p27, suggesting the ability of these cells to activate G_1/S cycle arrest (82, 86), in contrast to less differentiated elements. Taken together, there is sufficient evidence to hypothesize that treatment sensitivity of GCTs most likely reflects the intrinsic characteristics of the cell of origin, and their derivatives. Furthermore, the potential loss of characteristics of embryonic cells, and in particular the acquisition of somatic differentiation could be a crucial phenomenon in the development of resistance.

1.5. Aims of the thesis

The objective of this thesis is to identify factors involved in the development of normal and malignant human germ cells. The particular marker profile of male and female germ cells during normal foetal development is established, and compared to germ cells in dysgenetic gonads. Special attention is given to the expression profile of OCT3/4 in germ cells during normal and disturbed gonadal maturation, and the role of pluripotency in germ cell tumour development is discussed. The intrinsic developmental potential of nonseminomatous germ cell tumours (with regard to pluri-/totipotency) and the role of extracellular factors like the microenvironment are investigated. Finally, links between pluripotency, cellular differentiation, and treatment response, are described.

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Chapter 2

DIFFERENTIATION AND DEVELOPMENT OF HUMAN FEMALE GERM CELLS DURING PRENATAL GONADOGENESIS: AN IMMUNOHISTOCHEMICAL STUDY

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Differentiation and development of human female germ cells during prenatal gonadogenesis: an immunohistochemical study

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BACKGROUND: In the development of the human ovary, the second trimester includes the transition from oogonial replication to primordial follicle formation. The present study was carried out to assess differentiation and proliferation of germ cells in a series of female gonads from 19 fetuses from the second and third trimester, and two neonates. METHODS: Using immunohistochemistry, the following markers were studied: placental/germ-like cell alkaline phosphatases (PLAP), the marker of pluripotency OCT3/4, the proliferation marker Ki-67, β-catenin and E-cadherin, the stem cell factor receptor c-KIT, and VASA, a protein specific for the germ cell lineage. RESULTS: PLAP and OCT3/4 were seen during oogenesis, but not in germ cells engaged in folliculogenesis. A similar pattern was observed for Ki-67. Loss of pluripotency occurs once oocytes engage in follicle formation, suggesting a role of cell–cell interactions in the process of germ cell maturation. VASA, c-KIT, β-catenin and E-cadherin were found in germ cells at all developmental stages of oogenesis and folliculogenesis. CONCLUSIONS: Immunohistochemically, two groups of germ cells can be distinguished. Germ cells that are predominantly found in the cortical region of the ovary before weeks 22–24 of gestation, showing an immature phenotype, are mitotically active, and express OCT3/4, a marker of pluripotency. On the other hand, germ cells undergoing folliculogenesis have lost their pluripotent potential and no longer proliferate.

Key words: differentiation/fetal ovary/germ cells/immunohistochemistry/proliferation

Introduction

In humans and rodents, amongst other species, the embryonic precursors of the gametes are known as primordial germ cells (PGC) (see Donovan, 1998, for review). These cells are set aside to an extra-embryonic location early during embryonic development, and migrate to the area where the genital ridge will be formed around weeks 5 and 6 of human development (Witschi, 1948). Subsequently, the gonads and sexual differentiation occur in weeks 6 and 7 (Falin, 1969). Between weeks 7 and 9 during ovarian development (referred to as prefollicular stage) the germ cells (oogonia) and granulosa cells characteristically arrange in cords and sheets without specific organization (Gondos, 1985). The population of oogonia increases by undergoing multiple divisions, after which they become oocytes, stop proliferating, and enter the first steps of meiosis. The first distinctive change in prenatal germ cell development in the ovary is therefore the onset of meiosis, which starts at 11–12 weeks of gestation and extends into the second trimester (Rabinovici and Jaffe, 1990). Contradictory data exist with regard to the onset of folliculogenesis, i.e. the occurrence of primordial follicles, which has been reported to take place between 16 and 21 weeks of gestation (Kurilo, 1981; Konishi *et al.*, 1986). Due to the proliferation of premeiotic oogonia on the one hand and the apoptotic elimination of both oogonia and germ cells at different stages of folliculogenesis during intrauterine maturation on the other hand, considerable changes in the total number of germ cells are seen (De Pol *et al.*, 1997). Whereas at 5 weeks of gestational age an estimated 700–1300 germ cells are present, germ cell number peaks between weeks 16 and 20, reaching an estimated 6×10^6 cells per ovary (Baker, 1963). At birth, the total number of germ cells has been estimated to be $\sim 1 \times 10^6$ cells, and the number of follicles has been reported to be in the range of 1.3–3.8×10^5, with 95% being represented by primordial follicles (Baker, 1963; Forabosco *et al.*, 1991). Maturation is a gradual process, as is demonstrated by the initiation of

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

Antibody	Company	Code	Pre-treatment	Dilutions	Secondary antibody (1:200) (biotinylated)	Visualization
PLAP	Cell Marque, Hot Springs, AR, USA	CMC203	HIAR[a]	1:200	Rabbit anti-mouse (Dako-Cytomation, E0413)	ABCplx-ap[b]
c-KIT	Dako-Cytomation, Glostrup, Denmark	A4502	HIAR	1:500	Swine anti-rabbit (Dako-Cytomation, E0413)	ABCplx-ap
OCT3/4	Santa Cruz Bio-technology, Santa Cruz, CA, USA	sc-8629	HIAR	1:1000	Horse anti-goat (Vector Laboratories, Burlingame, CA, USA, BA9500)	ABCplx-hrp[c]
VASA	Provided by D.Castrillon[d]		HIAR	1:2000	Swine anti-rabbit	ABCplx-ap
β-Catenin	Zymed Laboratories, South San Francisco, USA	CAT-5H10	HIAR	1:2000	Rabbit anti-mouse	ABCplx-hrp[c]
E-Cadherin	Zymed Laboratories, South San Francisco, CA, USA	HECD-1	HIAR	1:300	Rabbit anti-mouse	ABCplx-hrp[c]
Ki-67	Dako-Cytomation, Glostrup, Denmark	A047	HIAR	1:50	Swine anti-rabbit	ABCplx-hrp

[a]Heat-induced antigen retrieval (Shi *et al.*, 1991).
[b]ABC complex, alkaline phosphatase, DakoCytomation, Code: K0391.
[c]ABC complex, horse radish peroxidase, DakoCytomation, Code: K0377.
[d]Castrillon *et al.* (2000).
PLAP = placental/germ-like cell alkaline phosphatases.

certain stages at certain times, i.e. the temporal regulation of oocyte development. In humans, this process is not synchronized, as germ cells of different developmental stages can be detected at the same time in the same prenatal ovary. This indicates that unknown intracellular mechanisms govern the individual pace of maturation (Rabinovici and Jaffe, 1990).

In order to further investigate the distribution and timing of proliferation and differentiation, we undertook an extensive study on the presence of a number of markers involved in either proliferation or maturation of normal female germ cells spanning the intrauterine period between week 15.5 gestational age and the neonatal period. In the human ovary, this crucial period includes the transition from oogonial replication by mitosis to primordial follicle formation. In this study we investigated the maturation process seen in normal development of the ovary and assess its potential as a reference for future identification of pathological processes of maturation.

Materials and methods

Tissue samples

Use of tissues for scientific reasons has been approved by an institutional review board (MEC 02.981). The samples are 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 gonads of 19 females from the second and third trimester after spontaneous or induced abortions (gestational ages 15.5–40 weeks), or premature and term neonates that had died shortly after birth were obtained from post-mortem examinations in our department. Ovaries were dissected and fixed in 10% formalin and processed into paraffin. To assure satisfactory quality, poor preservation of tissue samples assessed by haematoxylin–eosin staining led to exclusion from this analysis. Cases showing conditions that can possibly affect gonadal development such as trisomy 18 and 21, hydrocephalus, maldeveloped kidneys, or gross intrauterine growth retardation, were excluded from the study. Gestational ages were calculated in relation to the mothers' last menstrual cycle and were in accordance with the foot length and the crown–heel length measurements at autopsy.

Histochemical and immunohistochemical stainings

Stainings were performed as described before (Stoop *et al.*, 2001). For immunohistochemistry, sections were incubated with the primary antibody overnight at 4 °C [placental/germ-like cell alkaline phosphatases (PLAP), c-KIT, VASA, β-catenin, E-cadherin] or 2 h at room temperature (OCT3/4, Ki-67). The primary and secondary antibodies used are indicated in Table I. All slides were counterstained with haematoxylin. For PLAP, β-catenin and E-cadherin, positive staining of the Fallopian tube, and for c-KIT, the presence of mast cells were used as an internal positive control. Negative controls were performed by omitting the primary antibody, resulting in complete absence of signal.

Double-stainings were performed by using a combination of the same detection methods but with different substrates: Fast Blue/-Naphtol ASMX phosphate (F3378 and N500; Sigma, Germany) for blue staining and 3-amino-9-ethyl-carbazole (A.5754 and D4254; Sigma)/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, USA) to prevent background staining.

To semiquantitatively assess expression of the markers investigated, cells showing a positive signal were counted in representative visual fields (magnification × 200) containing surface areas of both the medullary and the cortical region of the ovary in comparable proportions in all cases. In addition, cells positive for PLAP, OCT3/4 and Ki-67 were counted in three representative high power fields (magnification × 400) of both the medullary and cortical regions of seven cases (gestational ages 15.5, 20, 23, 28, 33, 35 and 36 weeks) to assess regional differences. For quantitative analysis of OCT3/4, the percentage of positive cells among the total number of germ cells (as identified by histology) was assessed in three cases (gestational ages 15.5, 28 and 36 weeks) counting three representative visual fields (magnification × 200). The counting was performed independently by three different observers (H.S., F.H. and M.C.) who were unaware of the gestational age at the time-point of the investigation.

Results

The presence of a number of markers during normal fetal and neonatal ovarian development (week 15.5 gestational age until 6 weeks postnatally) was studied in 19 ovaries. Figure 1 summarizes the results of a semiquantitative assessment of the different markers. Each black dot represents one case.

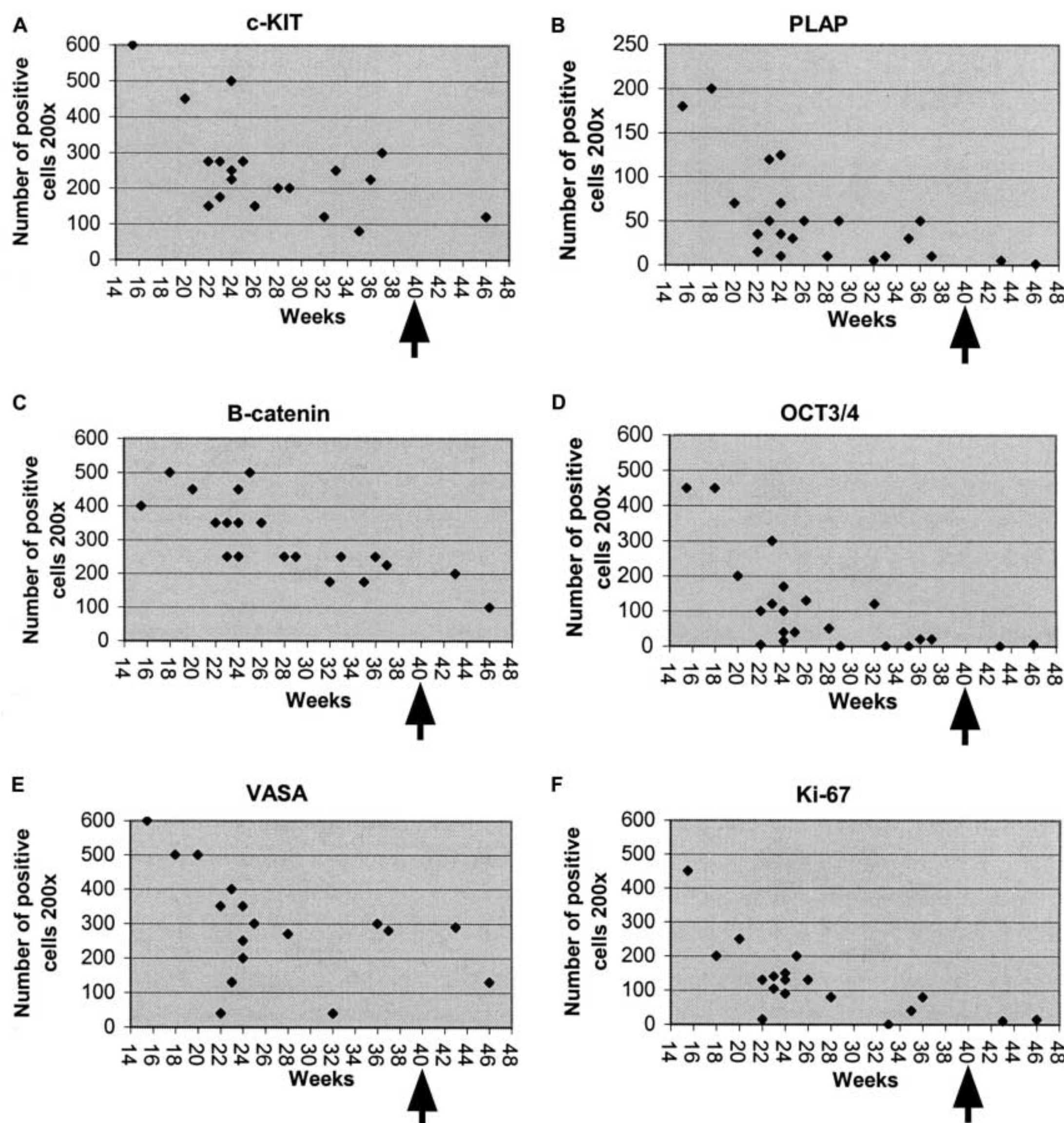

Figure 1. Absolute numbers of cells per representative visual field (magnification factor × 200) showing a positive staining with immunohistochemistry for the markers at different gestational ages (weeks). The earliest case studied in this series was 15.5 weeks, the oldest was the ovary of a neonate of 6 weeks. The arrow indicates partus (median 40 ± 2 weeks). The left panel (**A**, **C**, **E**) shows markers that remain positive throughout pre- and perinatal oogenesis and formation of primordial follicles. The right panel (**B**, **D**, **F**) illustrates factors associated with germ cell differentiation and proliferation. Each black dot represents one case. Note smaller scale of the *y*-axis (lower overall numbers) in **B** (placental/germ cell alkaline phosphatases, PLAP). As β-catenin and E-cadherin were expressed in the same cells in adjacent slides with similar localization, cell numbers for β-catenin (**C**) are also representative for E-cadherin.

Table II. Differences in the expression of placental/germ-like cell alkaline phosphatases (PLAP), OCT3/4 and Ki-67 between the cortical and the medullary region of fetal ovaries aged 15.5–36 weeks

	Gestational age (weeks)						
	15.5	20	23	28	33	35	36
PLAP							
Cortex	94 (72–115)	35 (23–44)	73 (54–85)	9 (3–12)	11 (9–13)	0	14 (12–17)
Medulla	34 (12–52)	4 (2–6)	4 (1–6)	5 (1–9)	0	2 (1–3)	3 (2–4)
OCT3/4							
Cortex	131 (106–157)	57 (56–57)	122 (99–162)	32 (29–33)	2 (0–3)	0	9 (1–20)
Medulla	49 (36–58)	12 (1–21)	26 (14–43)	8 (2–14)	0	2 (0–5)	4 (1–6)
Ki-67							
Cortex	234 (221–252)	136 (112–148	110 (95–118)	77 (62–98)	4 (2–7)	15 (9–18)	60 (46–75)
Medulla	163 (152–172)	86 (70–110)	61 (55–65)	53 (36–75)	0	26 (16–32)	45 (24–69)

The average and the range of cell numbers found positive for the indicated markers in three independent high power magnification fields (× 400) is given. Note that expression of the early markers PLAP and OCT3/4 and the proliferation marker Ki-67 is predominantly found in the cortical region. Overall, the number of positive cells decreases between 15.5 and 28 weeks, but shows some inter-individual variation during the third trimester.

Table II shows the results of a differential analysis, comparing expression of PLAP, OCT3/4 and Ki-67 in the medullary and cortical regions of the ovaries of seven cases, aged 15.5, 20, 23, 28, 33, 35 and 36 weeks. Table III gives both the absolute numbers and the percentage of cells positive for OCT3/4 in three of these cases, aged 15.5, 28 and 36 weeks. Illustrations of representative stainings are shown in Figures 2 and 3. Results of single- and double-stainings are described below. The data in Figure 4 (grey bars) were compared to previously published findings (black bars). Figure 5 shows a model of the progression of markers from oogonia to primordial follicles.

Immunohistochemical detection of Ki-67, PLAP and OCT3/4

Ki-67, PLAP and OCT3/4 were all found much more frequently at earlier fetal ages, and expression decreased with advancing gestational age (Figure 1). The various regions of the ovary showed different stages of germ cell maturation: cells positive for Ki-67, PLAP and OCT3/4 were mainly observed in the cortical region of the developing ovaries, i.e. the region where most immature germ cells are located during the process of oogenesis (Tables II and III). In the medulla, where the majority of maturing germ cells are found during ovarian development, positive cells were much less frequent.

The proliferation index was assessed using Ki-67, which showed a nuclear staining in both early germ cells and interstitial cells throughout the whole period of ovarian development investigated in this series. A marked decrease in the number of positive cells was observed after weeks 22–24 of gestational age, mainly due to the cessation of mitosis of germ cells. This indicates that proliferating germ cells contribute significantly to the overall number of Ki-67 positive cells in fetal ovaries. In germ cells, Ki-67 was observed mainly in oogonia (see Figure 2A). Yet, whereas the majority of oocytes engaged in folliculogenesis did not show Ki-67 expression, it was occasionally observed in oocytes of primordial follicles, where it was strictly confined to the nucleolus. Perinatally, only a few cells, mainly interstitial cells or granulosa cells, were positive.

The highest number of germ cells positive for PLAP was seen in the earliest stages of fetal development examined, and decreased sharply after 25 weeks gestational age (Figure 1). PLAP was detected in early germ cells predominantly located in the cortical region (Figure 2B and Table II). After birth, PLAP positive germ cells were hardly ever seen, with a maximum of one to three positive cells per visual field. PLAP expression was not restricted to germ cells, but was also seen in the Fallopian tube and occasionally in the epithelial lining of the ovary.

Nuclear staining for OCT3/4 was restricted to germ cells and the overall staining pattern was comparable to PLAP, yet total numbers of germ cells positive for OCT3/4 were somewhat higher (Figure 1 and Table II). In addition to oogonia, OCT3/4 was occasionally seen in early oocytes, but was never detectable in cells involved in folliculogenesis (Figure 2C). Similar to the expression pattern of Ki-67 and PLAP, a decline in the expression of OCT3/4 in fetal ovaries was seen at ~24 weeks of gestation. At term and in ovaries of neo-

Table III. Absolute numbers compared to total present (and percentage) of cells positive for OCT3/4 in three ovaries of 15.5, 28 and 36 weeks gestational age

OCT3/4	Gestational age (weeks)		
	15.5	28	36
Cortex	231/681 (34%; range 32–38)	5/324 (1.5%; range 0–4)	13/242 (5.4%; range 0–7)
Medulla	35/271 (13%; range 6–18)	2/201 (1%; range 0–2)	0/188 (0)

The total number of germ cells, identified by histology, and the number of germ cells expressing OCT3/4 immunohistochemically was assessed in three independent low power fields (magnification × 200) in both the cortical and the medullary region of three fetal ovaries. In parentheses, the percentage of germ cells expressing OCT3/4 is given, together with the range (as percentage) of three different counts of three different fields per case. Whereas a significant number of germ cells express OCT3/4 at 15.5 weeks, predominantly in the cortex, the percentage of positive cells decreases markedly during the third trimester. Note the decrease in overall numbers of germ cells.

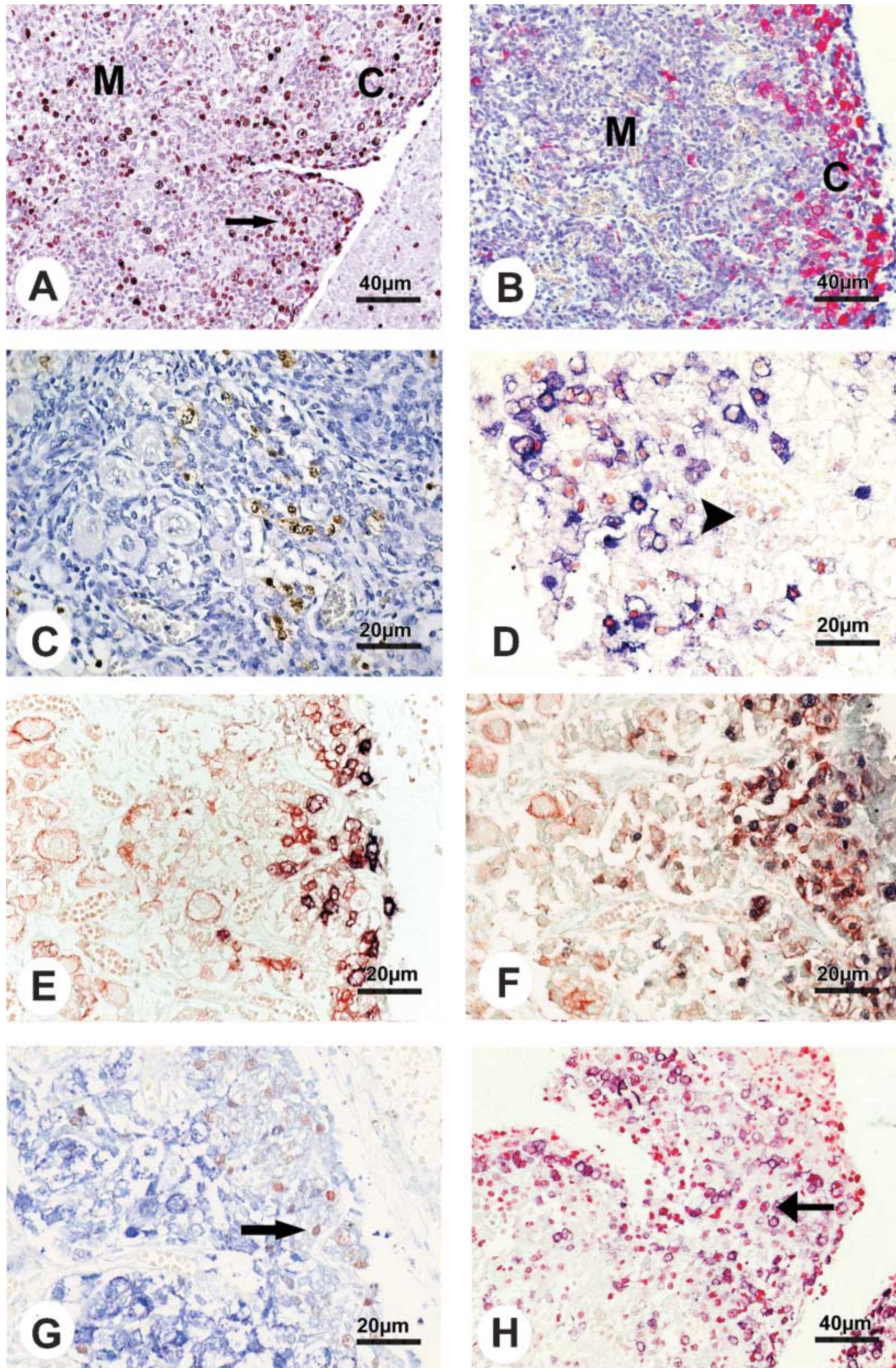

Figure 2. Results of immunohistochemical analysis of different markers in fetal germ cells. The cortex (C) and medulla (M) are indicated. (**A**) Fetal ovary (15.5 weeks of development) (magnification ×200); Ki-67 (brown nuclear signal) seen both in germ cells and cells of non-germ cell origin (e.g. stromal cells, endothelial cells, granulosa cells). Positive cells are mainly seen in a cortical localization (indicated by an arrow). (**B**) Fetal ovary (same case) (magnification ×200); placental/germ cell alkaline phosphatase (PLAP); red cytoplasmic signal. Note high number of positive cells (oogonia) at a cortical localization. (**C**) Fetal ovary (22 weeks of development) (magnification ×400); OCT3/4 (brown nuclear signal). Oogonia and immature oocytes, mainly cortically localized, are positive, whereas primordial follicles are negative. (**D**) Fetal ovary (same case) (magnification ×400); 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 occasionally cells are positive for OCT3/4 but negative for

nates, hardly any positive germ cells were detectable.

Immunohistochemical detection of c-KIT, β-catenin/ E-cadherin and VASA

A comparable staining pattern was seen for c-KIT, β-catenin and E-cadherin (Figure 3A–F). Both in early, immature germ cells (oogonia) and at all later stages of folliculogenesis, these factors were predominantly localized at the membrane, but sometimes also showed a cytoplasmic localization (see also Figure 1). In primordial follicles, all three factors showed strong signal intensity at sites of cell–cell interaction (Figure 3A–F). As β-catenin and E-cadherin were expressed in the same cells in adjacent slides in similar localization, these factors could be linked, forming an E-cadherin–catenin complex in germ cells. In addition to their presence in germ cells, these factors were also seen in epithelial cells of the Fallopian tube, serving as an internal positive control.

VASA was observed in the cytoplasm of germ cells, both oogonia and oocytes, at all gestational ages and after birth (Figure 1G and H). In addition to oogonia and early oocytes, all stages of follicle maturation showed presence of VASA. Signal intensity varied with maturation of germ cells: early germ cells showed only weak staining for VASA, and signal intensity increased with maturation and was strongest in primordial follicles (Figure 3G). The total number of germ cells, determined by VASA, decreased with gestational age: whereas at 15.5 weeks ~600 germ cells, mostly oogonia and early oocytes, were seen per visual field, the average number of germ cells at term was ~100–200 per visual field.

Results of double-stainings

Double-stainings were performed to assess correlations or differences of markers at different stages of maturation. A total of nine cases of 15.5, 18, 24 (three different cases), 28, 33, 36 weeks gestational age, and a neonate of 2 weeks, born at term, were stained for six different combinations: OCT3/4 and PLAP, PLAP and c-KIT, OCT3/4 and c-KIT, OCT3/4 and VASA, OCT3/4 and β-catenin, and PLAP and Ki-67 (Figure 2D–H respectively). The results of all double-staining experiments were in accordance with the results of the respective single-staining experiments. Staining for OCT3/4 and PLAP revealed that a higher number of germ cells were positive for OCT3/4 than for PLAP, with a constant ratio of 1.4:1 observed for all ages investigated (Figure 2D). PLAP was never detected in germ cells negative for OCT3/4, and expression of OCT3/4 was still observed at later stages of development, when PLAP was already undetectable. 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 oogonia without expression of c-KIT, but in contrast to PLAP, c-KIT remained positive throughout maturation, including different stages of folliculogenesis (Figure 2E). In accordance with these findings, OCT3/4 and c-KIT were found in comparable numbers only in early germ cells, whereas during folliculogenesis, c-KIT remained positive in germ cells that had already lost OCT3/4 expression (Figure 2F). Similar staining patterns were observed for the combinations OCT3/4 and VASA, and OCT3/4 and β-catenin (not shown), where co-expression was restricted to early germ cells, whereas expression of OCT3/4 was no longer seen at later stages of maturation. Interestingly, an inverse correlation was found between the presence of OCT3/4 and the staining intensity of VASA. Oogonia were positive for OCT3/4, but only weakly positive for VASA, whereas primordial follicles staining strongly for VASA no longer showed OCT3/4 (Figure 2G). Double-staining for PLAP and Ki-67 revealed that significantly more cells were positive for Ki-67 than for PLAP at all ages. Furthermore, not all early germ cells (identified by PLAP expression) were proliferating at the same time (Figure 2H). A proportion of germ cells of between 50% at earlier and 37% at later gestational ages showed co-expression of both markers. This indicates that in addition to a decrease in the absolute number of early germ cells, these cells are also less likely to proliferate at later stages of ovarian development. During the third trimester, expansion of the pool of early germ cells (indicated by co-expression of Ki-67 and PLAP) has basically ceased.

Discussion

The aim of this study was to examine the differential expression of a number of factors involved in proliferation and differentiation of human fetal germ cells. The findings give an indication of the temporal scale as well as the regional differences during the maturation of normal female germ cells in the human ovary. The overall picture of the presence of the different factors in fetal and neonatal ovaries is shown graphically in Figure 4 (grey bars) and compared to findings in humans published so far in the literature (black bars). Figure 5 gives a scheme of marker progression during maturation from oogonia to primordial follicles.

PLAP (indicated by an arrowhead). (**E**) Fetal ovary (same case) (magnification ×400); double-staining for PLAP (blue cytoplasmic signal) and c-KIT (red membranous signal). Primordial follicles express only c-KIT, and only a minority of cortically located immature germ cells is positive for both markers. (**F**) Fetal ovary (same case) (magnification ×400); double-staining for OCT3/4 (blue nuclear signal) and c-KIT (red membranous signal). Similar staining pattern as seen in E, but note that more germ cells are positive for OCT3/4 than for PLAP. (**G**) Fetal ovary (same case) (magnification ×400); double-staining for OCT3/4 (red nuclear signal) and VASA (blue cytoplasmic signal). Primordial follicles, mostly located in the medullary region, show a strong intensity of VASA and are negative for OCT3/4, whereas immature germ cells, mostly found in a cortical localization, are positive for OCT3/4 but show only weak VASA staining. (**H**) Fetal ovary (15.5 weeks of development) (magnification ×200); double-staining for Ki-67 (red nuclear signal) and PLAP (blue cytoplasmic signal). Significantly more cells are positive for Ki-67 than for PLAP. The majority of proliferating cells are of non-germ cell origin, as demonstrated by positive staining for Ki-67 but absence of PLAP; however, a few oogonia (identified by PLAP expression) show co-expression of Ki-67, indicating that these immature germ cells are mitotically active (indicated by an arrow).

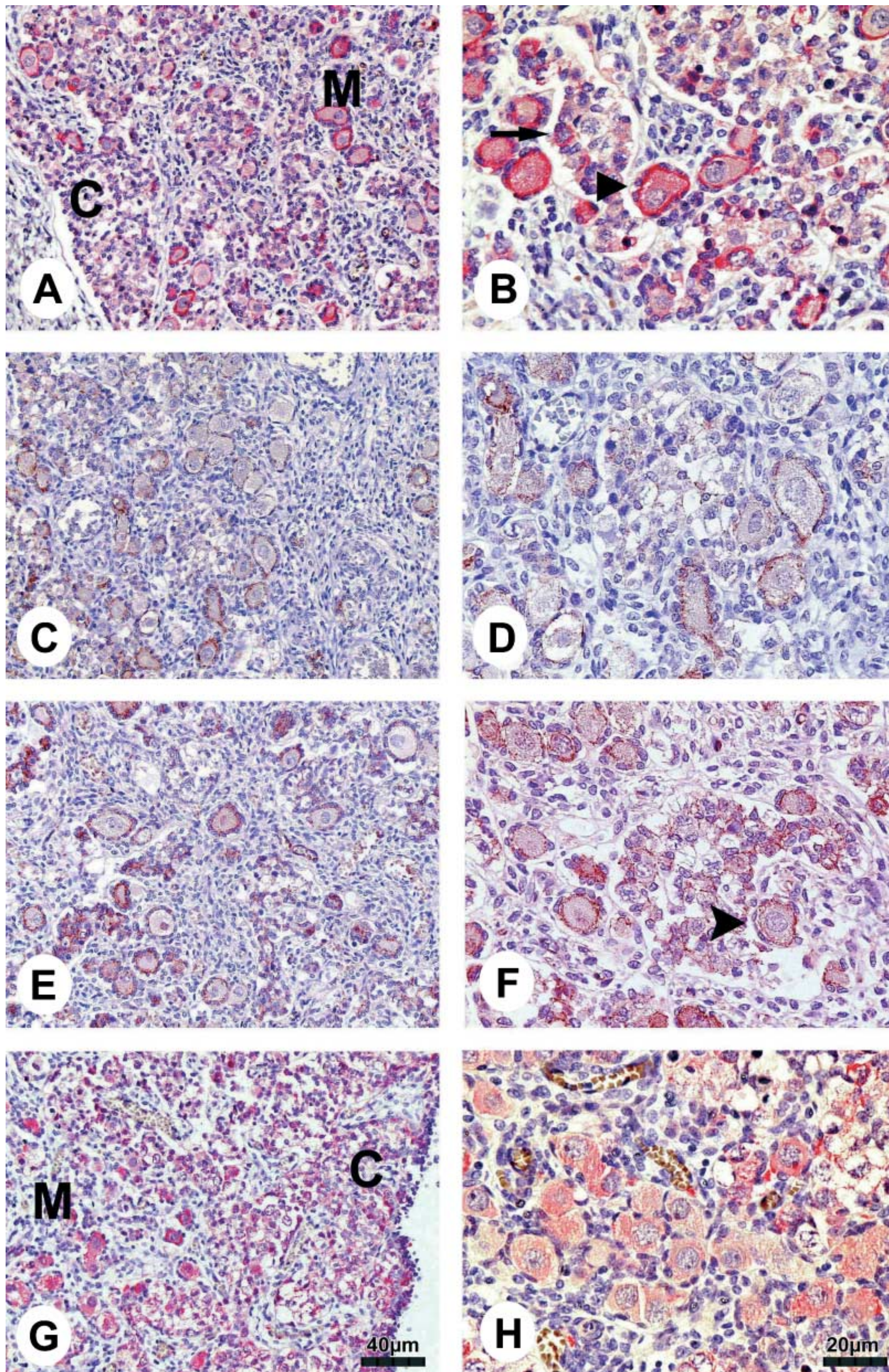

Figure 3. Results of immunohistochemical analysis of different markers in fetal germ cells. The cortex (C) and medulla (M) are indicated. (**A**) Fetal ovary (28 weeks of development); c-KIT (red cytoplasmic and membranous signal), positive in both immature oocytes and oocytes involved in folliculogenesis. (**B**) Fetal ovary (same case); c-KIT. Note staining of both immature oocytes (arrow) and primordial follicles (arrowhead). (**C**) Fetal ovary (same case); E-cadherin (brown-reddish membranous signal). (**D**) Fetal ovary (same case); E-cadherin. (**E**) Fetal ovary (same case); β-catenin (brown-reddish signal) is seen in oocytes at different stages of folliculogenesis. (**F**) Fetal ovary (same case); β-catenin. Note strong submembranous signal intensity in the primordial follicles (arrow), suggesting formation of an E-cadherin–catenin complex. (**G**) Fetal ovary (22 weeks of development); VASA (red cytoplasmic signal), seen both in germ cells of all stages of development. (**H**) Fetal ovary (same case); VASA. Magnifications: left panels ×200, right panels ×400.

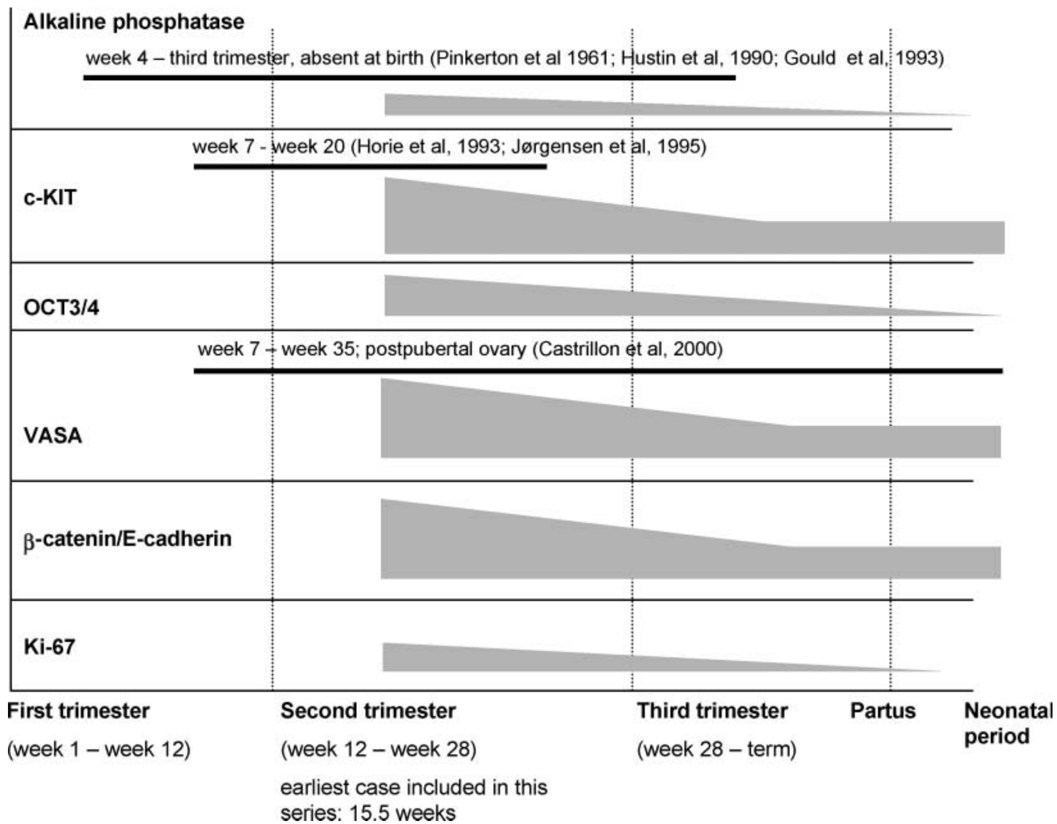

Figure 4. Overview of the data currently available from the literature on the expression of different antigens in germ cells during female intrauterine development (black bars), compared with findings of this study (grey bars). Heights of grey bars represent numbers of germ cells expressing the individual factor at different developmental ages.

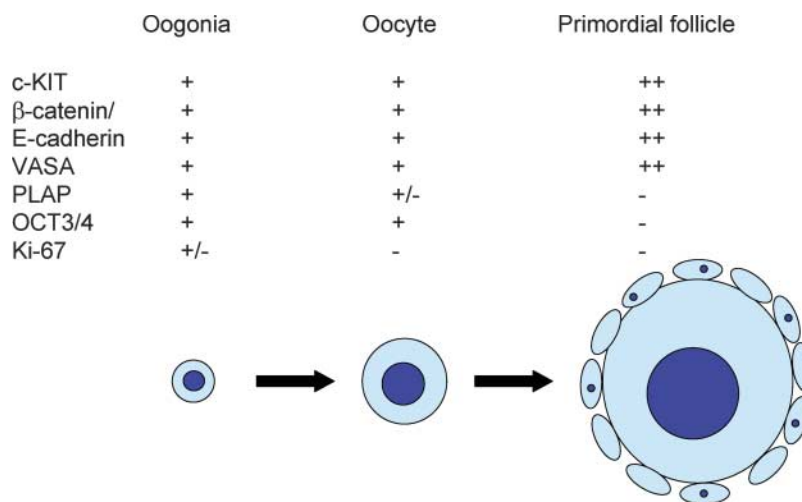

Figure 5. Progression of markers during germ cell maturation from oogonia to primordial follicles during the pre- and perinatal periods.

In the following paragraphs, the most interesting findings of the individual markers included in this study will be discussed in more detail.

To assess proliferation, we used Ki-67, a nuclear protein that is present in all phases of the cell cycle, but is absent in G_0-phase cells (Gerdes *et al.*, 1984). Overall expression of Ki-67 in both germ cells and cells of non-germ cell origin (mostly interstitial cells and granulosa cells) was highest in the earliest case investigated (15.5 weeks), and decreased steadily throughout the second and third trimester. Around term, almost all germ cells (mostly oocytes in primordial follicles) and the majority of cells of non-germ cell origin have entered a quiescent phase. Expression of Ki-67 was markedly higher in the cortex than in the medulla at earlier ages, whereas no differences between the two regions were found during the third trimester (Table II). To assess the proportion of germ cells of overall Ki-67 positive cells and examine the percentage of proliferating germ cells, we performed double-

stainings using a combination of PLAP and Ki-67. Of all cells positive for Ki-67, ~50% were early germ cells. The percentage of proliferating versus quiescent germ cells was 45 to 50% at 15.5 and 20 weeks, and between 37 and 40% of the few early germ cells found at week 28 gestation and 3 weeks after birth respectively. This indicates that immature germ cells at early stages show strong proliferation around weeks 15.5 to 20, whereas at later ages both the number and the fraction of proliferating germ cells decreases. At term, the expansion of germ cells has almost ceased. This finding is in line with earlier reports on germ cell numbers during the prenatal period (for review, see Rabinovici and Jaffe, 1990). Interestingly, a small number of oocytes in primordial follicles showed presence of Ki-67 in the nucleolus. Similar findings have been reported previously, suggesting a role of this factor not only during mitosis, but also during meiosis (Wrobel *et al.*, 1996; Traut *et al.*, 2002). The number of positive meiotic cells was too small to influence the overall conclusion, and did not allow conclusions regarding possible physiological meaning.

Alkaline phosphatases are regarded as archetypal oncofetal proteins. They are detectable at the mammalian blastocyst stage and have been described in germ cells of human embryos of <4 weeks gestational age (Pinkerton *et al.*, 1961). PLAP expression has been described in primordial germ cells at 8–13 weeks and at later ages in human oogonia not yet engaged in cell–cell interactions with somatic cells, whereas ovaries of neonates and infants were devoid of any immunopositivity (Hustin *et al.*, 1990; Gould *et al.*, 2000).

Table IV. Comparison of gene expression of female human and mouse germ cells during maturation

Marker	Early postmigratory germ cells (pre-meiotic)		Maturing germ cells		Late germ cells (post-meiotic prophase I)	
	Human	Mouse	Human	Mouse	Human	Mouse
OCT3/4	++	++*,[a]	+/− †	−[a,b]	?†	+[b]
c-KIT	+	+*,[a]	++	−*,[a]	++	+[c]
VASA	+	+*,[a]	++	++*,[a]	++[d]	++[e]

*Results are based on RT–PCR (expression of mRNA); all other results are based on immunohistochemistry.
†Note that the exact time-point of down-regulation of OCT3/4 with regards to onset of meiosis has not yet been established in human oocytes. We could not detect re-expression of OCT3/4 in primary and secondary follicles of 34 adult women (data not shown). However, the observation that OCT3/4 mRNA is not present in earlier stages of folliculogenesis but can be detected both in bovine and human mature oocytes and preimplantation embryos (Daniels *et al.*, 2000; Huntriss *et al.*, 2002) warrants investigation at what stage this protein is re-expressed in mature/ovulating oocytes or during fertilization.
[a]References: Hubner *et al.* (2003);
[b]Pesce *et al.* (1998);
[c]Horie *et al.* (1991);
[d]Castrillon *et al.* (2000);
[e]Toyooka *et al.* (2000).
The expression of a number of markers in human female germ cells (early postmigratory cells and cells at different stages of maturation), investigated in this analysis, is compared to data from the literature on the expression of these markers in mouse germ cells at comparable stages of maturation. The most striking difference between human and mouse germ cells is seen in expression of c-KIT, which is down-regulated in the mouse during oocyte maturation, but remains expressed in human oocytes throughout maturation.

Our finding that PLAP expression is restricted to early germ cells, most likely oogonia, is in line with these previous reports. The highest expression was seen in the cortex, where the number of positive cells decreased steadily with advancing age. In the medulla, differences at different ages were less pronounced after week 20. In our study, PLAP was still occasionally detected in germ cells of neonates shortly after birth, albeit at very low numbers.

OCT3/4 is a transcriptional regulator, expressed exclusively in pluripotent human embryonic stem cells and germ cells, including PGC (Goto *et al.*, 1999; Hansis *et al.*, 2000). In addition, presence of OCT3/4 has been described in germ cell tumour cells with pluripotent potential such as embryonal carcinoma and seminomas (Looijenga *et al.*, 2003). In fetal human testes, OCT3/4 has been found to be highly expressed in PGC between weeks 17 and 24 and to a lesser extent at later stages (Looijenga *et al.*, 2003). Expression of OCT3/4 in normal and dysgenetic human ovaries has been reported recently (Rajpert-De Meyts *et al.*, 2004). In our analysis, OCT3/4 was occasionally seen in early oocytes, but never in germ cells involved in folliculogenesis, indicating that human female germ cells irreversibly lose pluripotency once they progress to meiosis and engage in a close cell–cell interaction with granulosa cells. It is therefore tempting to speculate that both the processes of cell–cell interaction and down-regulation of OCT3/4 are important for germ cell maturation. It should be noted that this is different from the situation found in mouse follicles, where germ cells after completion of meiotic prophase I express OCT3/4 (see Table IV) (Pesce *et al.*, 1998). However, the exact mechanisms of interaction are not yet understood, and deserve further investigation. In this context it is interesting that ovarian teratomas originate from germ cells at different stages of meiosis (Surti *et al.*, 1990). The finding that these tumours show a restricted potential to differentiate and exclusively demonstrate somatic differentiation is in line with previous reports that the presence of OCT3/4 in embryonic stem cells is crucial to keep these cells in an undifferentiated state (Niwa *et al.*, 2000). We therefore speculate that ovarian teratomas arise from germ cells that have already lost expression of OCT3/4. Our findings demonstrate the value of both PLAP and OCT3/4 for the characterization of immature germ cells in the female gonad and indicate that these markers can be useful for the identification of developmentally arrested germ cells in pathological conditions, e.g. in dysgenetic gonads, as has been described recently (Rajpert-De Meyts *et al.*, 2004).

VASA is a member of the DEAD box family of RNA helicases and is specifically expressed in the germ cell lineage (Castrillon *et al.*, 2000). Furthermore, VASA expression has been detected by immunohistochemistry in migratory PGC at gestational week 7 and in germ cells both before and after birth (Castrillon *et al.*, 2000). It is most abundantly expressed in postmeiotic germ cells, i.e. in males in spermatocytes and in females in mature oocytes. VASA has also been described in testicular germ cell tumours retaining germ cell characteristics (Zeeman *et al.*, 2002). Interestingly, in murine embryonic stem cells, expression of Mvh, the mouse VASA homologue, has been described as an early event in the com-

mitment of stem cells to the germ cell lineage (Toyooka *et al.*, 2000, 2003). In our study, expression of VASA has been found both in germ cells at all gestational ages. In accordance with previous reports (Castrillon *et al.*, 2000), staining intensity was stronger in maturing oocytes than oogonia. Our results illustrate the usefulness of this protein as a marker of germ cells at all stages of development and allow an estimation of germ cell numbers at different ages.

c-KIT, β-catenin, and E-cadherin are all involved in cell signalling and cell–cell interaction. c-KIT is a type III receptor tyrosine kinase found on early germ cells, whereas the ligand stem cell factor (SCF or KIT-L) is present in granulosa cells (Matzuk *et al.*, 2002, for review). The c-KIT/SCF system has been found to be involved in survival and proliferation of migrating germ cells in mice (McLaren, 1992). In human intrauterine gonadogenesis, c-KIT expression has been described in female PGC at the period of arrival of PGC at the gonadal ridges at week 7 and later between 13 and 21 weeks of gestational age (Horie *et al.*, 1993; Jørgensen *et al.*, 1995; Robinson *et al.*, 2001). During folliculogenesis, c-KIT has been postulated to play a role in germ cell survival, possibly by up-regulating Mcl-1, an anti-apoptotic member of the Bcl-2 family (Hartley *et al.*, 2002).

Whereas some data are available on the cadherin–catenin complex in mouse PGC development and oocyte maturation (Ohsugi *et al.*, 1999; Di Carlo and De Felici, 2000), little is known about the role of these factors in human germ cell development. In an earlier analysis, the presence of E-cadherin on human oocytes has been demonstrated (Campbell *et al.*, 1995). Here we show that c-KIT, β-catenin and E-cadherin are all present in germ cells throughout all stages of female intrauterine development. The presence of these factors not only in maturing oocytes and germ cells during folliculogenesis, but also in oogonia could be demonstrated by double-stainings combining markers for early germ cells such as OCT3/4 and PLAP with c-KIT and β-catenin. As oogonia are often found in clusters, these factors seem to be involved in signalling between neighbouring immature germ cells. In primordial follicles, expression of these factors was predominantly seen in oocytes at the site of cell–cell interaction with granulosa cells. This suggests that cell–cell contacts play an important role, both for immature germ cells and later between oocytes and surrounding granulosa cells. In fact, E-cadherin has been reported to be required for germ cell determination in PGC (Okamura *et al.*, 2003), and in oocyte growth and acquisition of meiotic competence in mice (Carabatsos *et al.*, 2000). The functional importance of E-cadherin has further been demonstrated by dissociation/reassociation experiments in a mouse model: dissociated cells from gonads were unable to reform their initial contacts when cultured in the presence of an antibody to E-cadherin (Mackay *et al.*, 1999).

The total numbers of germ cells seen in one visual field, as assessed by either c-KIT, β-catenin or VASA at different gestational ages (see Figure 1), are in accordance with previous reports, where a steady decrease of germ cells from around weeks 18–20 gestational age resulted in numbers that were only one-sixth of peak values at term (Baker, 1963). We therefore conclude that assessment of cell numbers in one representative visual field at magnification ×200 and including comparable surface areas of cortical and medullary areas is a reliable method for semiquantitative analyses. Our results allow some interesting comparisons with data on mouse germ cell maturation (see Table IV).

Taken together, our data provide a number of interesting findings regarding the development and differentiation of human germ cells in the fetal ovary. Immature germ cells are characterized by expression of PLAP and show pluripotent potential, as is illustrated by the expression of OCT3/4. During the first part of the second trimester included in our study (gestational ages of weeks 15.5–20), the number of immature germ cells is high, and the pool of these cells decreases significantly after weeks 22–24. In accordance with previous reports, regional differences in the number of immature germ cells were found when comparing the cortex and medulla of the ovary. This finding supports the model of compartmentalization, in which the cortex is the area where expansion of immature germ cells take place, the medulla providing the environment needed for germ cell maturation. Accompanying the gradual shift from oogonia and early oocytes to germ cells engaged in formation of primordial follicles, loss of expression of first PLAP and consecutively OCT3/4 can be observed. Interestingly, in male fetal germ cells loss of pluripotency as judged by loss of OCT3/4 is observed at the time when gonocytes become attached to the basal membrane of the seminiferous tubules and come into close contact with nurturing Sertoli cells (Honecker *et al.*, 2004). As down-regulation of OCT3/4 in female germ cells occurs at a time when oocytes become surrounded by granulosa cells and start to form primordial follicles, a major role of cell–cell interactions in the maturation of germ cells in both sexes can be postulated. Expression of c-KIT, β-catenin, and E-cadherin in germ cells at all stages of germ cell development points towards a role of these factors for germ cell survival and maturation. Our findings help to define normal germ cell development and maturation in the human fetal ovary and will serve as a reference for further analyses investigating both normal and pathological processes in germ cell maturation, i.e. in dysgenetic gonads or in individuals showing chromosomal aberrations (Kersemaekers *et al.*, 2005, M.Cools *et al.*, unpublished data).

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Chapter 3

PATHOBIOLOGICAL IMPLICATIONS OF THE EXPRESSION OF MARKERS OF TESTICULAR CARCINOMA *IN SITU* BY FETAL GERM CELLS

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By Friedemann Honecker, Hans Stoop, Ronald de Krijger, Yun-Fai Chris Lau, Carsten Bokemeyer, and Leendert H.J. Looijenga

Original Paper

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 analysed. 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 prespermatogonial 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 prespermatogonia. 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.
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Keywords: germ cells; fetal gonads; carcinoma *in situ*/intratubular germ cell neoplasia unclassified (CIS/ITGCNU); immunohistochemistry; developmental arrest

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 prespermatogonia. Maturation is a gradual process; at the 20th gestational week, the fetal testis predominantly contains prespermatogonial 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 (prespermatogonia) 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

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

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

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

[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.

Histochemical and immunohistochemical staining

For immunohistochemistry, sections were incubated with the primary antibody overnight at 4 °C (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 and 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 throughout 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 prespermatogonia (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 prespermatogonia (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, prespermatogonia 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 prespermatogonia, 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.

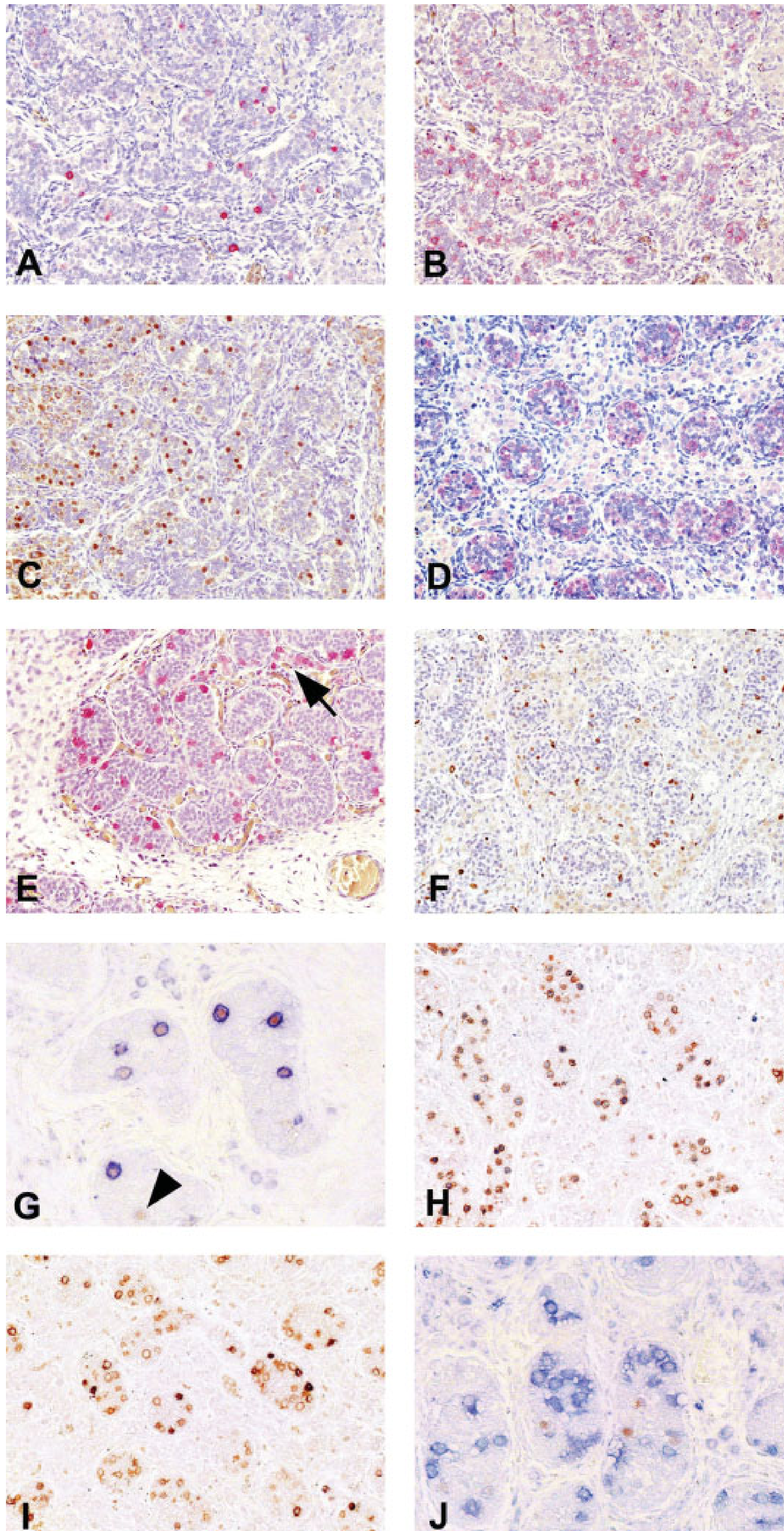
Ki-67 showed nuclear staining in a high number of cells, both within and outside the tubules, throughout the whole period of testicular development investigated. In developing tubules, until 24 weeks of gestational age, Ki-67 was predominantly seen in gonocytes (Figure 1F); after 24 weeks, both basally 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 prespermatogonia 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 prespermatogonia staining strongly for VASA mostly lacked OCT3/4 (Figure 1J).

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 patho-biological 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 prespermatogonia.

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

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 prespermatogonia. (B) Same case; c-KIT staining (red signal) is seen in a large number of gonocytes and prespermatogonia. (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 prespermatogonia (on the basal membrane of tubules). (E) Testis of a neonate (6 weeks); TSPY (red nuclear and cytoplasmic signal), mainly in prespermatogonia. 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

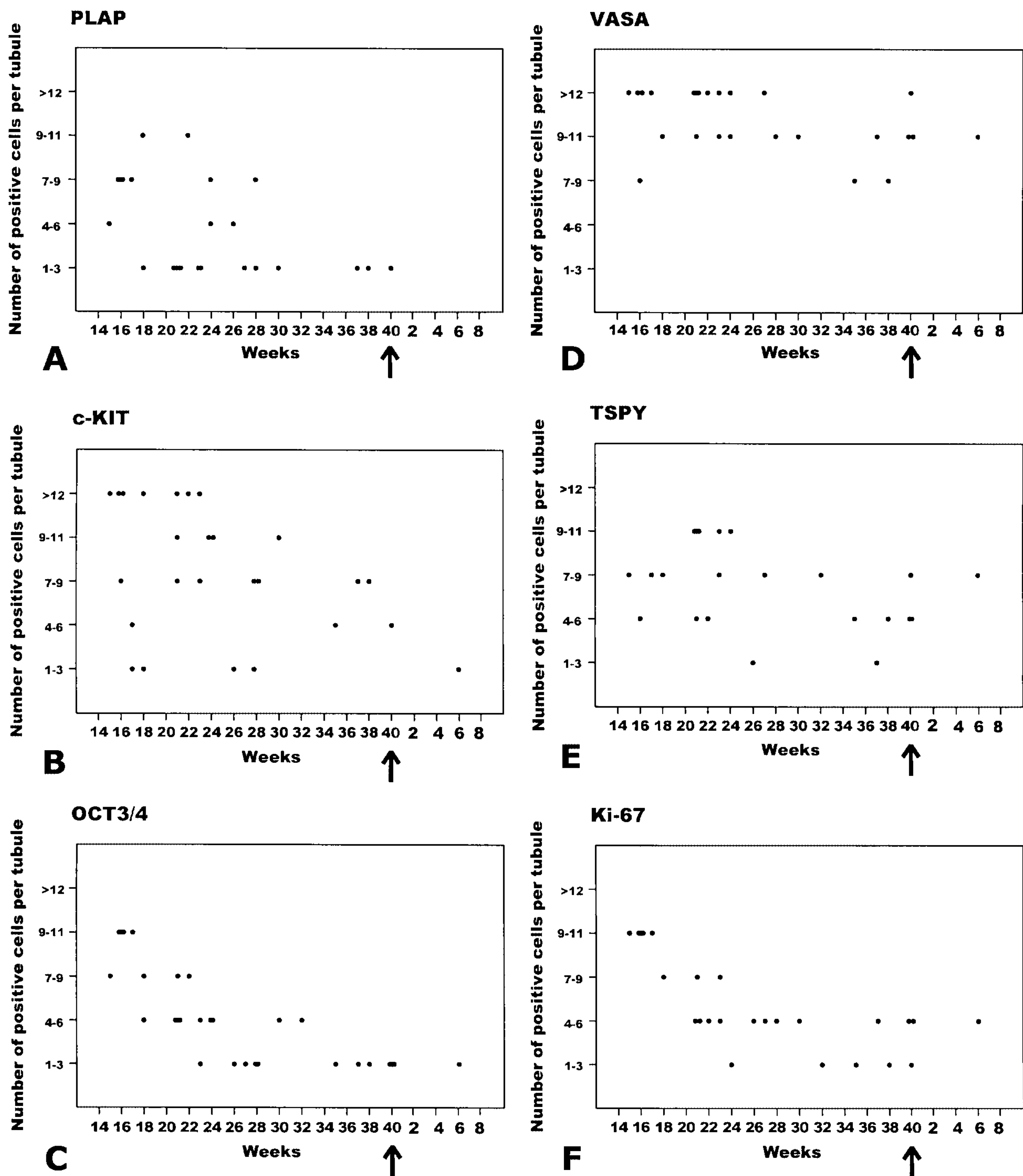


Figure 2. Absolute numbers of cells per tubule showing positive immunohistochemical staining for the markers at different gestational ages (weeks). The earliest case studied in this series was 15 weeks; the oldest was a neonate of 6 weeks. The arrow indicates the time of birth (median 40 ± 2 weeks). 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

in roughly equal numbers, suggesting that, in addition to gonocytes, the pool of more differentiated prespermatogonia 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

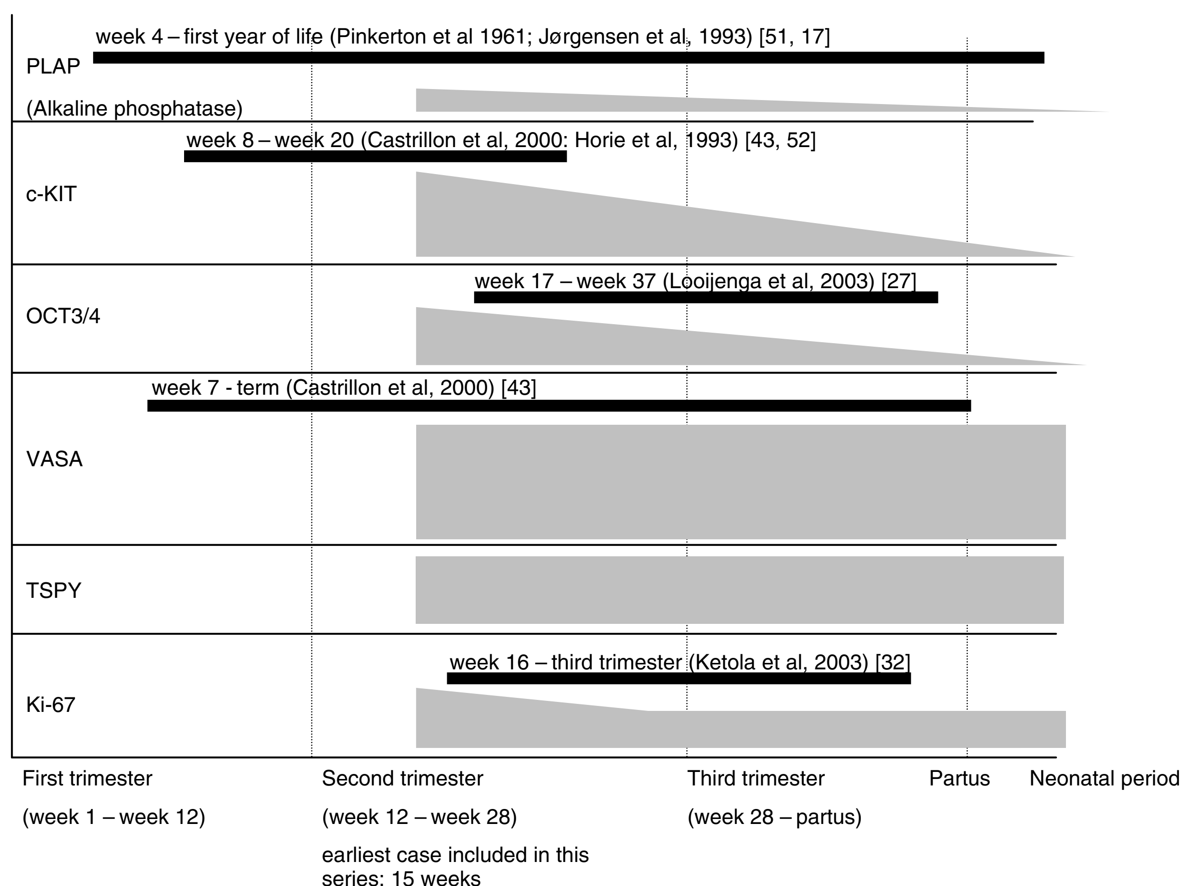


Figure 3. Overview of the data currently available from the literature on the expression of different antigens in germ cells during intrauterine development (black bars) compared with the findings of this study (grey bars). The sizes of the grey bars schematically represent the frequency of germ cells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

in groups of prespermatogonia 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].

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 prespermatogonia, these cells gradually lose these early markers. During further development, there is a relative decrease in the number of gonocytes compared with more mature prespermatogonia. 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 prespermatogonia 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 de-differentiation 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 prespermatogonia [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.

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Chapter 4

IDENTIFICATION OF GERM CELLS AT RISK FOR NEOPLASTIC TRANSFORMATION IN GONADOBLASTOMA. AN IMMUNOHISTOCHEMICAL STUDY FOR OCT3/4 AND TSPY

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Identification of germ cells at risk for neoplastic transformation in gonadoblastoma☆ An immunohistochemical study for OCT3/4 and TSPY

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Summary Carcinoma in situ (CIS) is the precursor of malignant testicular germ cell tumors (GCTs) of adolescents and young adults, being the neoplastic counterpart of primordial germ cells/gonocytes. Carcinoma in situ cells will develop into invasive seminoma/nonseminoma. Gonadoblastoma (GB) is the precursor of invasive GCTs in dysgenetic gonads, predominantly dysgerminoma (DG). In this process, part of the Y chromosome (GBY region) is involved, for which *TSPY* is a candidate gene. A detailed immunohistochemical survey was performed for the known diagnostic markers, germ cell/placental alkaline phosphatase (PLAP), c-KIT, and OCT3/4, as well as testis-specific protein on the Y chromosome (TSPY) on a series of GBs, and adjacent invasive DGs. All 5 patients were XY individuals (4 females and 1 male). In contrast to c-KIT, PLAP was positive in all cases. The immature germ cells of GBs were positive for OCT3/4, whereas the mature germ cells were negative for this

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Abbreviations: GB, Gonadoblastoma; CIS, Carcinoma in situ; CH, Choriocarcinoma; DG, Dysgerminoma; EC, Embryonal carcinoma; (T)GCT, (testicular) germ cell tumor; H&E, Hematoxylin and eosin; OCT3/4, Octamer binding transcription factor involved in regulation of pluripotency, also referred to as POU domain class 5 transcription factor 1 (POU5F1); PLAP, Germ cell/placental alkaline phosphatase; TE, Teratoma; TSPY, Testis-specific protein on the Y chromosome. This gene maps to the gonadoblastoma region on the Y chromosome (GBY); WHO, World Health Organization; YST, Yolk sac tumor.

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marker, but positive for TSPY. In every GB, a minor population of germ cells positive for both markers could be identified, similar to most CIS cells and early invasive DG. On progression to an invasive tumor, TSPY can be lost, a process that is also detectable in invasive testicular GCTs compared to CIS. These results indicate that GB is a heterogeneous mix of germ cells, in which the OCT3/4-positive cells have the potential to undergo progression to an invasive tumor. These early invasive stages are initially also positive for TSPY (like CIS), supporting a positive selection mechanism. Therefore, OCT3/4 in combination with TSPY is valuable to identify malignant germ cells in dysgenetic gonads. This could allow better prediction of the risk of progression to a GCT. In addition, the data support the model that GB represents the earliest accessible developmental stage of malignant GCTs.
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1. Introduction

Human germ cell tumors (GCTs) represent a unique and complex group of neoplasms. Recently, we proposed a classification system on the basis of various parameters, including age at clinical presentation, anatomical site of the tumor, sex of the patient, histology, and chromosomal constitution (see Refs [1,2] for review). This classification has been acknowledged by the World Health Organization [3]. The group of tumors referred to as type II GCTs are the seminomas and the nonseminomas of the testis and mediastinum, known as (non)dysgerminomas of the ovary and dysgenetic gonad, and (non)germinomas of the brain. Various risk factors for these types of GCTs have been identified, in particular, related to those of the gonads, including cryptorchidism and gonadal dysgenesis [4-6]. The precursor of testicular seminomas and nonseminomas is well established and known as carcinoma in situ (CIS) [7], also referred to as intratubular germ cell neoplasia unclassified. The precursor lesion of the tumors of the dysgenetic gonad is called gonadoblastoma (GB) [4]. Like CIS, it shows the potential to progress to an invasive GCT, mainly dysgerminoma (DG), and less frequently to other tumor components, as embryonal carcinoma (EC), teratoma (TE), yolk sac tumor (YST), and choriocarcinoma (CH). Being a rare condition, GB has not been studied extensively, even though it might increase our understanding of the different pathogenetic steps involved in the development of malig-

Table 1 Clinical data and summary of immunohistochemical results of PLAP, c-KIT, OCT3/4, and TSPY staining experiments of germ cells in GB, DG, and TGCTs of adolescents and adults

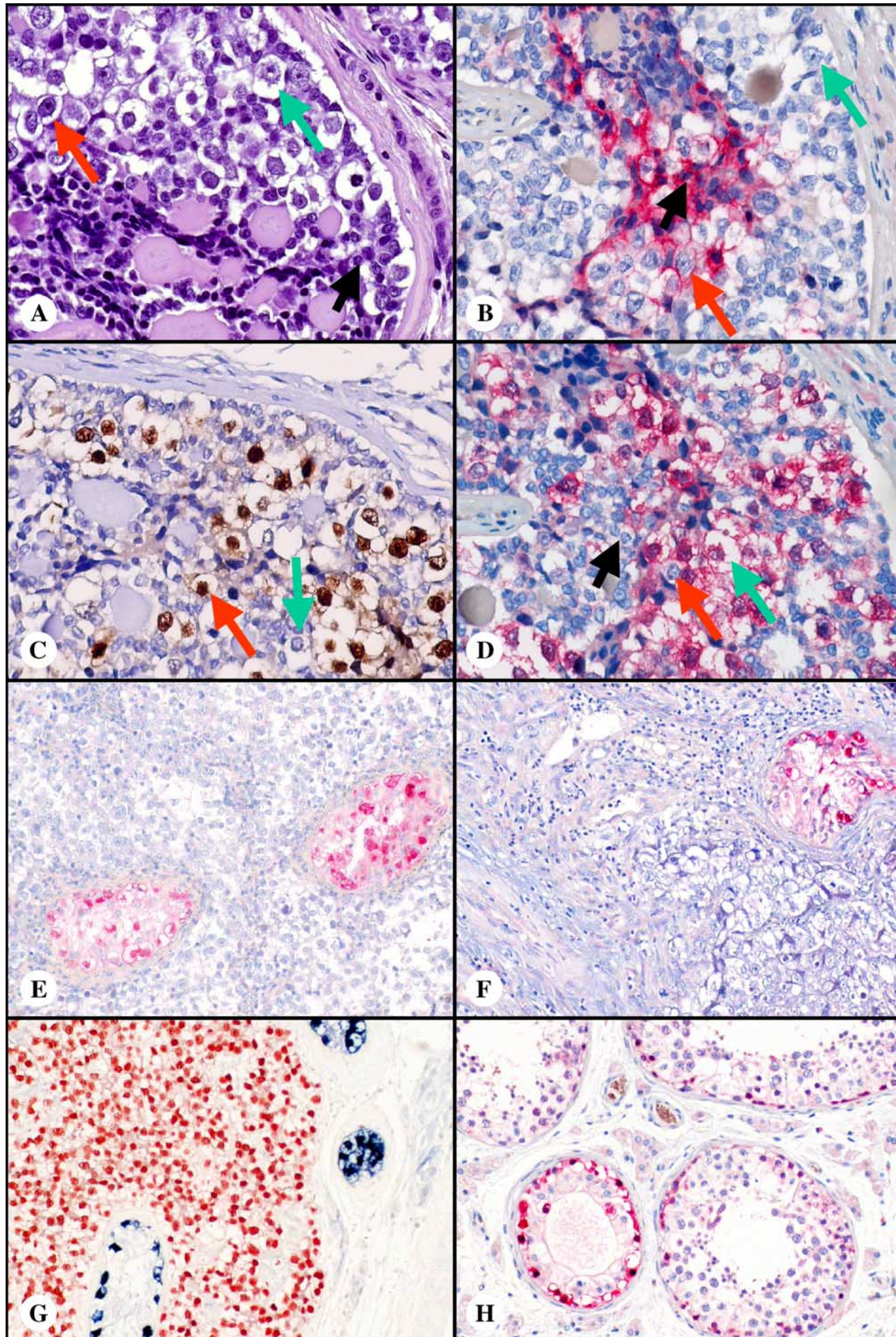
Organ	Sex	Case	Age (y)	Histology	PLAP	c-KIT	OCT3/4	TSPY
Dysgenetic gonad	F	1	18	GB	+–	–	++	+
				Early invasive DG	+	–	++	+
	F	2[a]	20	GB	+–	+	++	+
				Early invasive DG	+	+	++	+
	F	3	14	GB	+–	+	++	+
				Early invasive DG	+	+	++	+
	M	4	21	GB	+–	–	++	+
				Early invasive DG	+	–	++	+
				Progressed DG	+–/–	–	++	+/–
	F	5	16	GB	+–	–	++	+
Testis	M	n = 31	>16	CIS	++	+/–	++	++(–)
		n = 21	>16	Seminoma	++	+/–	++	+–
			>16	Nonseminoma:				
		n = 18		EC	–+	–+	+	–
		n = 9		TE	–	–	–	–
		n = 7		YST	–	–	–	–
		n = 4		CH	–	–	–	–

The organ, phenotype of the patient (all patients with a dysgenetic gonad had a 46,XY karyotype), age of the patient at time of tissue sampling (years), histology of the tumor, and staining results of immunohistochemistry at different stages of progression (GB, early invasive DG, progressed DG), EC, TE, YST, and CH are indicated. Immunohistochemistry for c-KIT was not always positive in GB; all cases consistently showed expression of TSPY, OCT3/4, and PLAP. Expression of the latter 2 was mainly found in immature germ cells (indicated by +/–). Staining results were the same on both sides (data not shown). – indicates negative; +/–, heterogeneity of staining; +, moderate; ++, strong intensity of a homogenous staining.

[a] Case 2 presented with bilateral gonadoblastoma.

nant GCTs. Both the comparatively young age at presentation [4] and the genomic constitution [8] suggest that GB could be the earliest accessible stage in the development of a malignant GCT.

There are strong indications that both GB and CIS are the result of a disturbance in germ cell maturation. This model is supported by epidemiological and morphological observations, as well as the presence of a number of immunohistochemical markers like germ cell/placental alkaline phosphatase (PLAP) and the protooncogene receptor c-KIT in CIS and GB [9-11]. Besides c-KIT and PLAP, GBs were recently found to express *TSPY* (testis-specific protein on the Y chromosome) in a limited number of cases [12,13]. This is of particular interest, because development of GBs has been linked to the presence of a specific part of the Y chromosome, namely the GBY (GB on the



Y chromosome) region [14-16]. A candidate gene in this region is *TSPY*. Although its function is still unclear, a role in the proliferation of germ cells has been suggested [13].

The model that CIS originates from an early germ cell, either a primordial germ cell or a gonocyte, is strongly supported by our recent finding that the octamer binding transcription factor POU5F1, also known as OCT3/4, is specifically found in CIS, seminoma, and EC [17]. OCT3/4 is a known marker for pluripotency. Its presence mimics the expression pattern during embryogenesis, where this factor is restricted to embryonic stem cells and early germ cells (as discussed in Ref [17]). We recently found that OCT3/4 is down-regulated during germ cell maturation both in male and female gonadogenesis [18,19]. In a large series investigating various malignancies, one case of GB was positive for OCT3/4, as were all cases with testicular CIS [17]. Recently, this was confirmed in larger series [20-25].

Presence of c-KIT, PLAP, OCT3/4, and TSPY proteins has not been investigated systematically in a single study in multiple GBs. To further investigate the pathogenesis of GB, and to shed light on its pathogenetic relationship with testicular CIS, we performed a detailed investigation of these markers in a series of 6 GBs and adjacent invasive tumor components. The findings were compared to those obtained in testicular CIS and adjacent invasive GCTs. Our results confirm that GB is a mixture of germ cells at different stages of maturation, which can be identified by morphology as well as the presence of various markers. A process of selection of immature germ cells leads to the precursor cells of invasive GCTs. This is demonstrated by the consistent and homogenous staining pattern for both OCT3/4 and TSPY in these cells, as well as in early invasive tumor cells and CIS. Upon further progression, eventually resulting in an invasive tumor, TSPY can be lost, whereas OCT3/4 remains positive. These observations are in line with the model that GB is an intermediate between normal immature germ cells and CIS of the testis.

2. Materials and methods

2.1. Material

Use of tissues for scientific reasons has been 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," developed by the Dutch Federation of Medical Scientific Societies (FMWV) (version 2002).

Six dysgenetic gonads (including one individual with bilateral dysgenetic gonads) containing GB with or without an invasive tumor component were collected in the south-western part of the Netherlands in collaboration with pediatric surgeons, urologists, and pathologists (Table 1). Furthermore, 21 seminomas and 38 nonseminomas of different, sometimes mixed, histologies (18 EC, 7 YSTs, 9 TEs, 4 CHs) of the adult testis were included, also, 31 cases of CIS adjacent to invasive testicular germ cell tumors (TGCTs) were retrieved from our archive. In addition, intratubular seminoma and nonseminoma could be identified in a number of cases. Two testicular CIS cases without the presence of an invasive tumor were also included. Two normal testes from fetuses of 16 and 21 weeks gestational age were obtained after spontaneous miscarriages. The postmortem examinations were carried out in our department. Tissues were routinely formalin fixed and paraffin embedded. Diagnosis of GB and invasive tumor parts was made according to the World Health Organization classification by a pathologist experienced in GCT pathology (JWO).

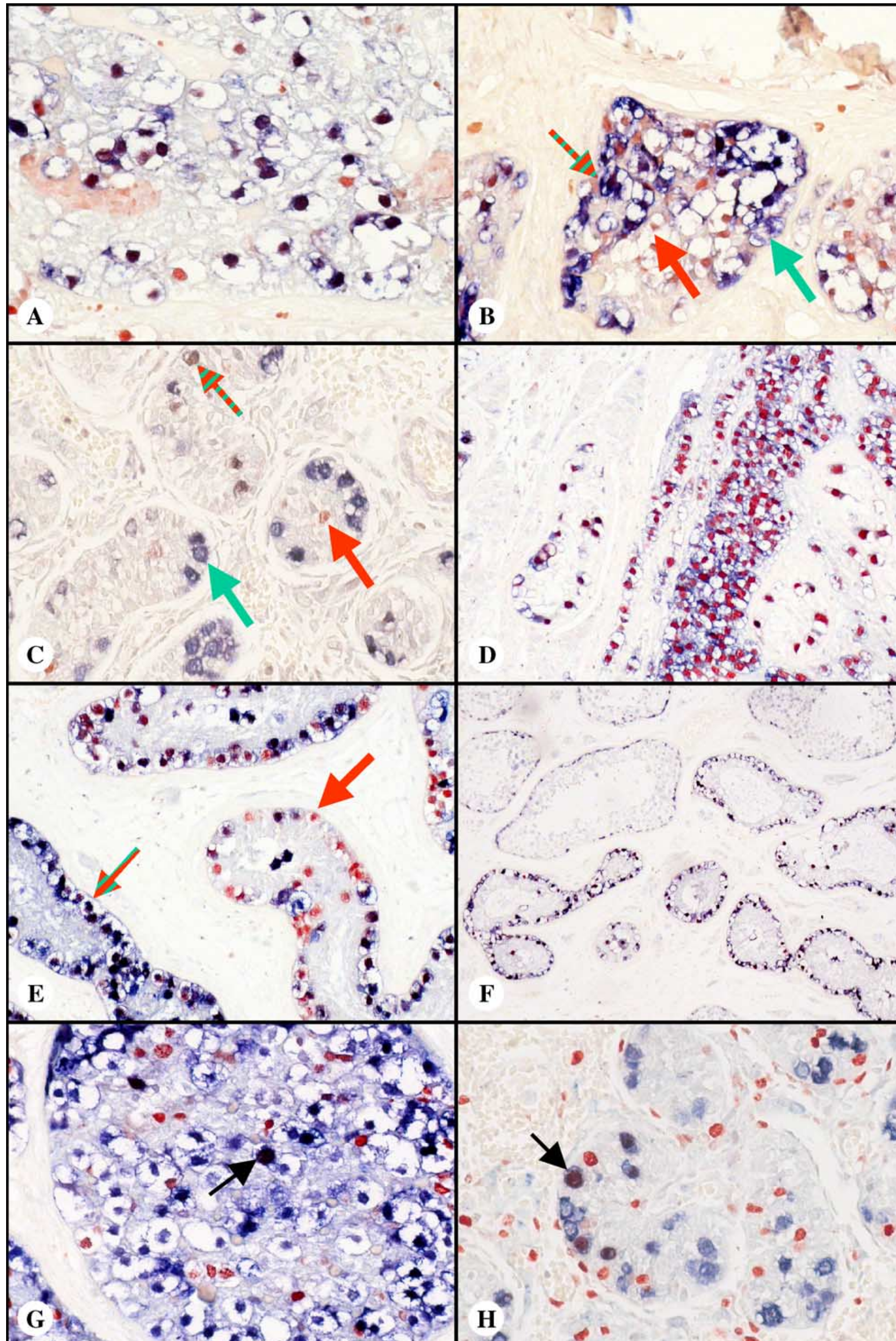
On the basis of morphology, the germ cells present in GBs were classified as immature or mature. The immature cells are characterized by their smaller size and a higher nuclear to cytoplasmic ratio. These cells are similar to normal gonocytes/oogonia. The mature germ cells, which are larger with a clear cytoplasm and which has a lower nuclear to cytoplasmic ratio, are similar to oocytes/prespermatogonia.

Fig. 1 Representative examples of histology and immunohistochemistry of a GB included in this study (A-D, case 4, Table 1). Parallel sections were used, allowing direct comparison of the different staining results. A, Hematoxylin and eosin staining. Note the presence of supportive cells, Sertoli/granulosa cells (indicated by a black arrow) as well as germ cells, with various stages of maturation; immature germ cells are small and show a high nuclear/cytoplasmic ratio (indicated throughout this figure with a red arrow), whereas mature germ cells show the opposite characteristics (indicated throughout this figure with a green arrow). B, PLAP staining: the red cytoplasmic signal in predominantly the immature germ cells, as well as in some stromal cells. C, OCT3/4 staining. Note the positive brown staining predominantly in the immature and not the mature germ cells. None of the other cell types shows a positive staining. D, TSPY staining: the red nuclear and cytoplasmic signal, predominantly in the mature compared to immature germ cells. In addition, supportive cells can be positive. All images are at a magnification factor of ×400. Representative example of immunohistochemistry for TSPY on a tissue section of (E) seminiferous tubules with CIS and invasive testicular seminoma and (F) seminiferous tubule with CIS and invasive EC. Note that the CIS cells are specifically stained, and note the absence of TSPY in the invasive tumor cells. G, Double staining for OCT3/4 (red) and TSPY (blue) in an invasive seminoma (same case as shown in E). Note the double-positive CIS cells and the loss of TSPY, but only of OCT3/4 in the invasive cells, that is, TSPY is lost upon invasive growth. Coexpression can result in a dark almost black signal. H, Representative example of immunohistochemistry for TSPY on a tissue section of testicular parenchyma, containing both seminiferous tubules with normal spermatogenesis (right part of the image) and CIS (left lower part of the image). Carcinoma in situ cells stain stronger than spermatogonia (hematoxylin and eosin, original magnification ×200).

2.2. Immunohistochemistry

Staining was performed as described before [26]. For immunohistochemistry, 3-μm-thick paraffin-embedded tissue sections were incubated with the primary antibodies overnight at 4°C: PLAP (1:200, Cell Marque, Hot Springs, Ark), c-KIT (1:500, Dako-Cytomation, Glostrup, Denmark), and TSPY (1:3000, provided by C. Lau). Ki-67 (1:100, Dako-Cytomation) were incubated for 1 hour and OCT3/4 (1:1000, Santa Cruz Bio-technology, Santa Cruz, Calif) for 2 hours at room temperature. All slides of the single-staining experiments were counterstained with hematoxylin. Alkaline phosphatase staining with new fuchsin was used for the detection of PLAP, c-KIT, and TSPY, resulting in a red signal of cytoplasmic, membranous, and cytoplasmic/nuclear localization, respectively. Diaminobenzidine was used as chromogen in the peroxidase staining for the detection of OCT3/4, resulting in a brown nuclear signal.

Double-staining experiments were performed using a combination of the same detection methods but with



different visualization methods: Fast Blue/Naphthol ASMX phosphate (F3378 and N500; Sigma, Steinheim, Germany) for a blue staining (TSPY) and 3-amino-9-ethyl-carbazole (A.5754 and D4254; Sigma)/H_2O_2 for a red staining (OCT3/4; Ki-67), without counterstaining. To reduce background signal, endogenous peroxidase activity and endogenous biotin were blocked using 3% H_2O_2 (for 5 minutes) and a blocking kit for endogenous biotin (Vector Laboratories, Burlingame, Calif).

2.3. Fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) was performed as previously described [27] on paraffin-embedded tissue sections using probes specific for the centromeric regions of chromosomes X (DXZ1) and Y (DYZ3). Only whole nuclei of intact cells were scored in 2 different tumor areas, both in regions containing GB and DG, if present. The mean number of spots per nucleus is indicated; minimum number of cells assessed was 117.

3. Results

c-KIT and PLAP are established markers suitable for the immunohistochemical detection of CIS cells of the adult testis (for review, see Ref [28]). This also counts for OCT3/4 [17], of which multiple confirmative studies have been reported [20-25], and for TSPY [29], although this is less well established. Here we compare the staining pattern of these markers in CIS with the findings in a series of GBs. In total, 6 GBs were included of 5 independent patients with a 46,XY karyotype (one was bilateral). In 4 cases, an adjacent invasive DG component was available for investigation. Three of the invasive tumors showed an early stage of invasiveness near the GB areas (termed *early invasive dysgerminoma*, Table 1), and 1 case also contained large invasive tumor areas at a distance from the precursor lesion (termed *progressed dysgerminoma*, Table 1). The results of the staining experiments are summarized in the Table 1, and representative examples of staining results are shown in Fig. 1. Double-staining experiments were performed for TSPY and OCT3/4, and TSPY and Ki-67, of which representative examples are shown in Figs. 1G and 2. To assess the presence of chromosome X and Y, FISH analysis was performed using centromere probes for these chromosomes.

Morphologically, GB lesions contain various cell types, including somatic cells and germ cells (Fig. 1A). The first show characteristics of Sertoli/granulosa cells (further supported by a weak staining for vimentin using immunohistochemistry, data not shown), whereas the germ cells are a mixture of cells at different stages of maturation. The smaller cells with a high nuclear to cytoplasmic ratio are immature germ cells, resembling gonocytes/oogonia. The bigger cells with a large clear cytoplasm are mature germ cells, which are similar to prespermatogonia/oocytes.

3.1. Results of single-staining experiments

c-KIT was the least consistent marker investigated in the GBs in this study. It showed no staining in the 3 GBs and the 2 matched DGs. In contrast, PLAP was detected in all GBs and adjacent invasive components, with a heterogeneous pattern in the most advanced DG. However, not all germ cells present in GBs were positive for PLAP (Fig. 1B). Although most mature germ cell were negative, the immature germ cells were mainly positive, although stromal cells could show staining. Most of the tumor cells in invasive DG showed a weaker signal intensity or were negative for PLAP (Table 1). This indicates that PLAP is present in GB but weaker to negative in invasive DG. A similar pattern has been observed in testicular CIS and seminoma, in which PLAP is down-regulated upon tumor progression (Table 1).

OCT3/4 was readily detectable as a nuclear staining in all GBs (Fig. 1C), in which the protein was restricted to the immature germ cells, although the more differentiated germ cells were negative. In contrast to the heterogeneity seen in GB, all tumor cells of DGs, both adjacent to and at distance from GB areas, showed a homogenous staining for OCT3/4 (see also Fig. 2). Therefore, a selection of OCT3/4-positive

Fig. 2 Representative examples of double immunohistochemical staining experiments. Shown are TSPY (blue cytoplasmic and nuclear signal) and OCT3/4 (red nuclear signal) in (A) early invasive DG (case 3, Table 1). Similar to seminoma, the vast majority of DG cells show expression of both markers. Original magnification ×400. B, Gonadoblastoma (case 1, Table 1). Note the heterogeneous staining pattern, only a minority of cells shows a positive staining of both factors. The germ cells only positive for OCT3/4 are indicated by a red arrow, and the germ cells only positive for TSPY by a green arrow. The double-positive cells are indicated by a red/green arrow. Original magnification ×400. C, Normal fetal germ cells, gestational age 21 weeks. Note that whereas TSPY is predominantly expressed in prespermatogonia (located on the basal membrane, green arrow), OCT3/4 is seen more frequently in gonocytes in a more central localization of the tubule (red arrow). Only a minority of germ cells is positive for both factors (green/red arrow). Original magnification ×400. D, CIS and early (micro-)invasive seminoma. The CIS and early invasive seminoma cells (left and middle panel of the image) show expression of both markers. Invasive seminoma cells at more distance (right panel of the image) show loss of TSPY but not of OCT3/4. Original magnification ×200. E, CIS adjacent to an invasive tumor. Note the presence of CIS cells with both markers (red/green arrow) as well as CIS cells without TSPY, but staining for OCT3/4 (red arrow). Original magnification ×400. F, CIS before invasiveness. All cells show coexpression of both factors. Original magnification ×100. G, Double-staining for TSPY (blue cytoplasmic and nuclear signal) and Ki-67 (red nuclear signal) in GB (case 5). Mature germ cells are TSPY positive, whereas Ki-67 is mainly positive in immature cells. Only a minority of the cells is positive for both factors, indicated by an arrow. Original magnification ×400. H, Normal fetal germ cells (same case as shown in C). Only a minority of germ cells (around 30%) is positive for both factors (indicated by a black arrow). Original magnification ×400.

cells takes place during development of an invasive tumor from GB. These data are in accordance to observations in CIS and TGCTs (see the following paragraphs and see Ref [17] for discussion).

A heterogeneous staining pattern was found in GB for TSPY. In contrast to OCT3/4, TSPY was detected in both a nuclear localization and in the cytoplasm (Fig. 1D). The protein was not detectable in somatic cells resembling granulosa/Sertoli cells. Tumor cells of early invasive DG closely adjacent to the GB areas consistently showed a strong and homogenous staining for TSPY, similar to the pattern seen for OCT3/4 (see Fig. 2 for examples). TSPY expression could be lost in invasive DG areas at distance from the preinvasive lesion. A similar pattern was also detected in the testis: TSPY was strongly expressed in CIS adjacent to invasive tumors, both seminoma and nonseminoma (see Table 1 and Fig. 1E-G). Intratubular seminoma was consistently positive, whereas invasive seminoma, like DG, can lose expression of TSPY. In contrast, all nonseminomas lack TSPY. Interestingly, even the intratubular EC component is already negative (data not shown). Because FISH analysis revealed that the Y chromosome is not lost in these tumors (data not shown), loss of TSPY protein is not due to gross loss of Y-genetic material on progression.

In normal spermatogenesis, TSPY staining was predominantly seen in spermatogonia and sometimes spermatocytes (see Fig. 1H, right panel). However, the staining intensity of TSPY in CIS was stronger than in spermatogonia (Fig. 1H, left panel).

3.2. Results of double-staining experiments

To further investigate the observed heterogeneity in expression of OCT3/4 and TSPY in GB, CIS and invasive tumor components, double-staining experiments were performed. The results for OCT3/4 and TSPY were in line with the results of single-staining experiments. The early invasive DG components showed a homogenous staining for both OCT3/4 and TSPY, in which most tumor cells were positive for both markers (Fig. 2A). In GB, however, the mature germ cells were positive for TSPY and negative for OCT3/4, whereas immature germ cells were mainly positive for OCT3/4 only (Fig. 2B). Only a subpopulation of germ cells in GB showed coexpression of both markers. To investigate whether coexpression of OCT3/4 and TSPY is a physiological event during normal intrauterine male germ cell development, we stained 2 normal testes of fetuses of 16 and 21 weeks of gestational age. Most of the germ cells were found to be positive for only one factor, either OCT3/4 or TSPY. A minority of OCT3/4-positive germ cells also showed expression of TSPY (Fig. 2C). Interestingly, these cells were located on the basal membrane of the seminiferous tubule. Whereas the overall number of OCT3/4-positive cells decreased from weeks 16 to 21 of gestational age, the fraction of germ cells showing expression of both markers remained unchanged.

These results were compared to the samples of testicular CIS and TGCTs, subjected to the same protocol for double staining. In accordance with the single-staining results, CIS and early invasive seminoma components were homogenously positive for both OCT3/4 and TSPY (Fig. 2D, left and middle panel). A more heterogeneous pattern was seen in later stages of invasiveness of seminomas, where expression of TSPY could be lost, but OCT3/4 remained positive (Fig. 2D, right panel, and Fig. 1G). In all cases of CIS adjacent to an invasive tumor, either seminoma or nonseminoma (which were all negative for TSPY), CIS cells without expression of TSPY but positive for OCT3/4 were found (Fig. 2E). In 2 cases of CIS without invasive tumor, all CIS cells expressed TSPY (Fig. 2F). These data indicate that TSPY is lost upon invasiveness of GCT cells. This step can already occur in the in situ stage (Fig. 2E).

Finally, to assess whether there is a correlation between TSPY expression and proliferation, we performed double-staining analyses for TSPY and Ki-67. In addition to CIS and GB, the 2 normal fetal testes were investigated. Most malignant germ cells (around 90%) of both CIS and intratubular seminoma were positive for both TSPY and Ki-67. Gonadoblastoma with an adjacent invasive tumor component showed a similar pattern as CIS, whereas GB without invasive tumor was much more heterogeneous (case 5, Fig. 2G). In fact, less double-positive cells were identified. In these cases, mature germ cells were mainly TSPY positive, whereas immature cells were mostly positive for Ki-67 only, and coexpression of both markers was rarely seen. This was similar to the pattern seen in fetal testes, where the 2 factors were not frequently coexpressed. Only around 30% (29% at 16 weeks; 32% at 21 weeks gestational age) of germ cells positive for TSPY also showed expression of Ki-67 (see Fig. 2H).

4. Discussion

The term *dysgenetic gonads* is used to describe the phenomenon of a disturbed gonadal sex determination or differentiation. This condition is associated with an increased risk for the development of malignant GCTs. The invasive tumor components, referred to as DGs and non-DGs, resemble those of the adult testis (TGCTs), seminoma, and nonseminoma of various histologies. The precursor lesion is known as GB [4]. Histologically, GBs consist of aggregates of germ cells and sex cord elements, including immature Sertoli and granulosa cells. Some of the germ cells show similarities to CIS, the known precursor of TGCTs [10]. Both CIS and GB are thought to be the result of a maturation arrest of early germ cells, possibly due to a disrupted gonadal environment or unknown pathogenetic hits. Because CIS is highly similar to the invasive TGCTs (ie, seminoma), and is suggested to be "only one step behind in the karyotypic evolution" [30,31], it might not be the best target for the identification of the earliest steps in the

development of TGCTs. This could, however, be the case for GB [8]. We undertook the present study to further understand the biology of this condition, and to compare similarities and differences between GB and CIS.

Immunohistochemical analysis of both known (PLAP, c-KIT) and recent markers of early male germ cells (OCT3/4, TSPY) was performed in GBs and adjacent DGs. Gonadoblastomas have previously been reported to be positive for PLAP and c-KIT [9-11]. Our results are mainly in line with these findings. PLAP can be present in GBs and is weakly but homogenously positive in the invasive component. However, c-KIT was not always detected. We therefore conclude that c-KIT is not a reliable marker for the detection of GB. A heterogeneous staining pattern was found for both OCT3/4 and TSPY in all GBs investigated. Early invasive DG cells closely adjacent to the GB component were positive for both OCT3/4 and TSPY. A similar pattern was found in most CIS cells and could be confirmed by double-staining experiments. TSPY has previously been reported in a few cases of GB [12,13], although, to our knowledge, the presence of TSPY in early invasive DGs and the loss of expression on tumor progression are novel. These findings support a model in which progression from early GB to invasive growth is associated with expression of both TSPY and OCT3/4 in immature germ cells (see Fig. 3 for a schematic representation). A similar pattern is present in CIS and early stages of seminoma. This indicates that TSPY might be involved in the initial selection of tumorigenic

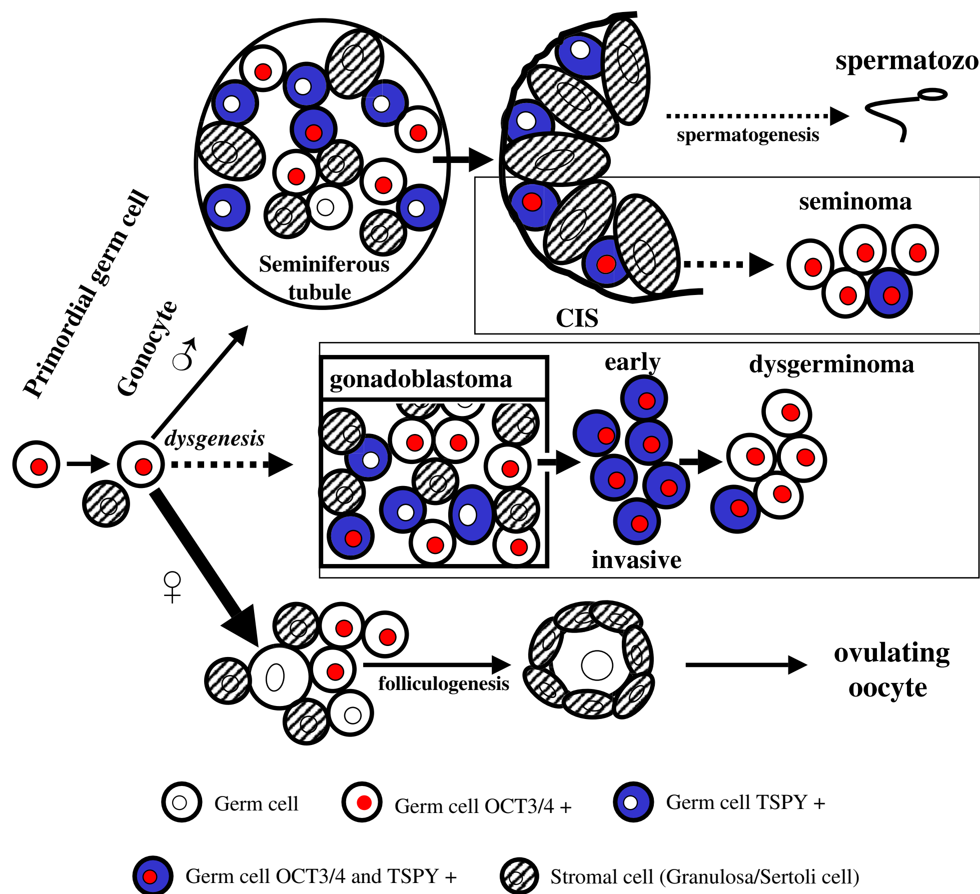


Fig. 3 Schematic representation of normal and neoplastic germ cell development. The earliest stage of maturation (starting with a primordial germ cell) is indicated on the left side of the illustration and the most mature (differentiated) stage at the right. Maturation of germ cells is graphically depicted by increasing size of cells and nuclei. Normal male development (top row) and female development (bottom row) lead to functional gametes (spermatozoa and ovulating oocyte, respectively). Note that in the absence of determining factors for male development, female differentiation occurs—a mechanism known as "default pathway" (see arrow sizes leading from gonocytes to normal male or female development). Red nuclei indicates germ cells positive for OCT3/4; cytoplasmic blue cells, germ cells positive for TSPY. The colors are consistent with the detection methods in the double staining as shown in Figs. 1 and 2. During normal male fetal germ cell development (and in GB), only a minority of cells show coexpression of OCT3/4 and TSPY (depicted by a red nucleus and a blue cytoplasm). A disturbed process of germ cell maturation can result in invasive GCTs (boxed areas, middle panel). Gonadoblastoma contains germ cells at different stages of development in a disturbed stromal environment, classifiable both morphologically and with regard to the presence of the immunohistochemical markers OCT3/4 and TSPY (middle panel). In contrast, CIS (upper boxed panel) and early invasive DG are much more homogeneous, both morphologically and with regard to the expression of OCT3/4 and TSPY. This suggests a process of selection and clonal expansion and supports the model that GB is an earlier stage in the process of GCT formation than CIS.

cells, possibly by playing a role in cell cycle regulation or cell division [13]. We therefore assessed the fraction of cells that were positive for both TSPY and Ki-67, a marker for proliferation. Interestingly, coexpression was found in the vast majority of CIS and early invasive seminoma cells, as well as in GB with an adjacent invasive tumor. This underlines the strong proliferative activity of both lesions. Whereas the proliferative activity of CIS has been reported before [32], little is known about proliferation in GB. TSPY and Ki-67 were less frequently coexpressed in one GB case that had not yet developed into an invasive tumor (case 5, Table 1). In our view, this could indicate that this specific GB case had not yet progressed to the next pathogenetic step, that is, the expansion of one premalignant clone. In fact, it could pathogenetically be one step behind CIS, which is the obvious result of a clonal process. Whatever the role of TSPY in preinvasive lesions, proliferation in the invasive GCTs is no longer dependent on TSPY, as is demonstrated by the loss of TSPY on tumor progression.

Like TSPY, OCT3/4 has been shown to be present in GBs [17,18]. In the present series, OCT3/4 was readily detectable in all GBs in the nuclei of cells showing morphological features of immature germ cells. Furthermore, as OCT3/4 remained positive throughout all stages of tumor development from GB to DG, it can serve as a marker to identify cells showing a high risk for malignant transformation. However, it is important to notice that the coexpression of OCT3/4 and TSPY does not per se confer neoplastic properties to germ cells. This is illustrated by the fact that normal male fetal germ cells of the second trimester can coexpress both TSPY and OCT3/4, although only in a minority of all cells.

Results of the double-staining experiments support the notion that GB consists of a heterogeneous group of mature and immature germ cells, of which the immature cells show the phenotype similar to premalignant cells, in line with earlier findings [10]. Interestingly, we recently observed that mature germ cells in GB resemble more the male germ cell lineage than the female [33]. The higher incidence of these tumors in males compared to females confirms the finding of a crucial role of part of the Y chromosome in the development of this tumor [14]. Our results are compatible with the model that after a process of selection and consecutive clonal expansion, immature germ cells in GB expressing OCT3/4, PLAP, and TSPY progress to the next pathogenetic step, eventually leading to invasive growth. Whether the presence of TSPY is needed to maintain the germ line commitment of these cells at an early stage, or confers a growth advantage during the critical step to invasiveness, remains to be elucidated.

In conclusion, our results show that GB consists of a heterogeneous population of germ cells. Both in DG in ovaries and in seminoma, its counterpart in the adult testis, the step to an invasive GCT seems to involve selection and clonal expansion of an immature germ cell, positive for OCT3/4 and TSPY. At later stages of invasiveness, TSPY can be lost, whereas OCT3/4 always remains present in tumor cells exhibiting germ line characteristics, that is, DG or seminoma. Our analysis places GB at a very early stage of GCT development, making it an interesting entity to elucidate changes involved in the early pathogenesis of this disease in the future.

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Chapter 5

GERM CELL LINEAGE DIFFERENTIATION IN NON-SEMINOMATOUS GERM CELL TUMOURS

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Original Paper

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.
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Keywords: **germ cell tumours; germ cell lineage; differentiation; totipotency; primordial germ cells**

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

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 re-activate totipotency and show properties of immature and more mature germ cells (see boxed area to the right)

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.

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.

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 counterstaining. Endogenous peroxidase activity and/or endogenous biotin was blocked using 3% H_2O_2 (for 5 min) and/or a blocking kit for

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

Antibody	Company	Clone, code	Pretreatment	Dilutions, incubation time and temperature	Secondary antibody (1 : 200) (biotinylated)	Visualization
PLAP	Cell Marque	CMC203	HIAR*	1 : 200, overnight (o.n.), 4 °C	Rabbit anti-mouse (Dako E0413)	ABCplx-ap[1]
c-KIT	DakoCytomation	A4502	HIAR	1 : 500, o.n., 4 °C	Swine anti-rabbit (Dako E0413)	ABCplx-ap
OCT3/4	Santa Cruz	sc-8629	HIAR	1 : 1000, 120 min, room temperature (RT)	Horse anti-goat (Vector BA9500)	ABCplx-hrp[2]
VASA	Provided by D. Castrillon		HIAR	1 : 2000, overnight (o.n.), 4 °C	Swine anti-rabbit	ABCplx-ap
TSPY	Provided by Y. Lau		None	1 : 3000, o.n., 4 °C	Swine anti-rabbit	ABCplx-ap
BMP4	Novocastra	3H2	None	1 : 100, o.n., 4 °C	Rabbit anti-mouse	ABCplx-ap
SCF	Santa Cruz Biotechnology	sc-1302	HIAR	1 : 400 o.n., 4 °C	Horse anti-goat	ABCplx-ap
CD30	DakoCytomation	Ber-H2	HIAR	1 : 100 o.n., 4 °C	Rabbit anti-mouse	ABCplx-hrp[2]
CD34	Neomarkers	QBEnd/10, MS-363-S	None	1 : 20, 30 min, RT	Rabbit anti-mouse	ABCplx-hrp[2]
CD61	Immunotech	SZ 21, 2116	HIAR	1 : 100, 30 min, RT	Rabbit anti-mouse	ABCplx-hrp[2]
Glycophorin C	DakoCytomation	M0820	HIAR	1 : 100, 120 min. RT	Rabbit anti-mouse	ABCplx-hrp[2]
Lysozyme	DakoCytomation	A0099	Pronase, 10 min	1 : 900, 30 min, RT	Swine anti-rabbit	ABCplx-hrp[2]
Myeloperoxidase	DakoCytomation	A0398	HIAR	1 : 5000, 30 min, RT	Swine anti-rabbit	ABCplx-hrp[2]

* Heat-induced antigen retrieval.
[1] ABCplx-ap, streptavidin–biotin–alkaline phosphatase complex.
[2] ABC-hrp, streptavidin–biotin–horseradish peroxidase complex.

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].

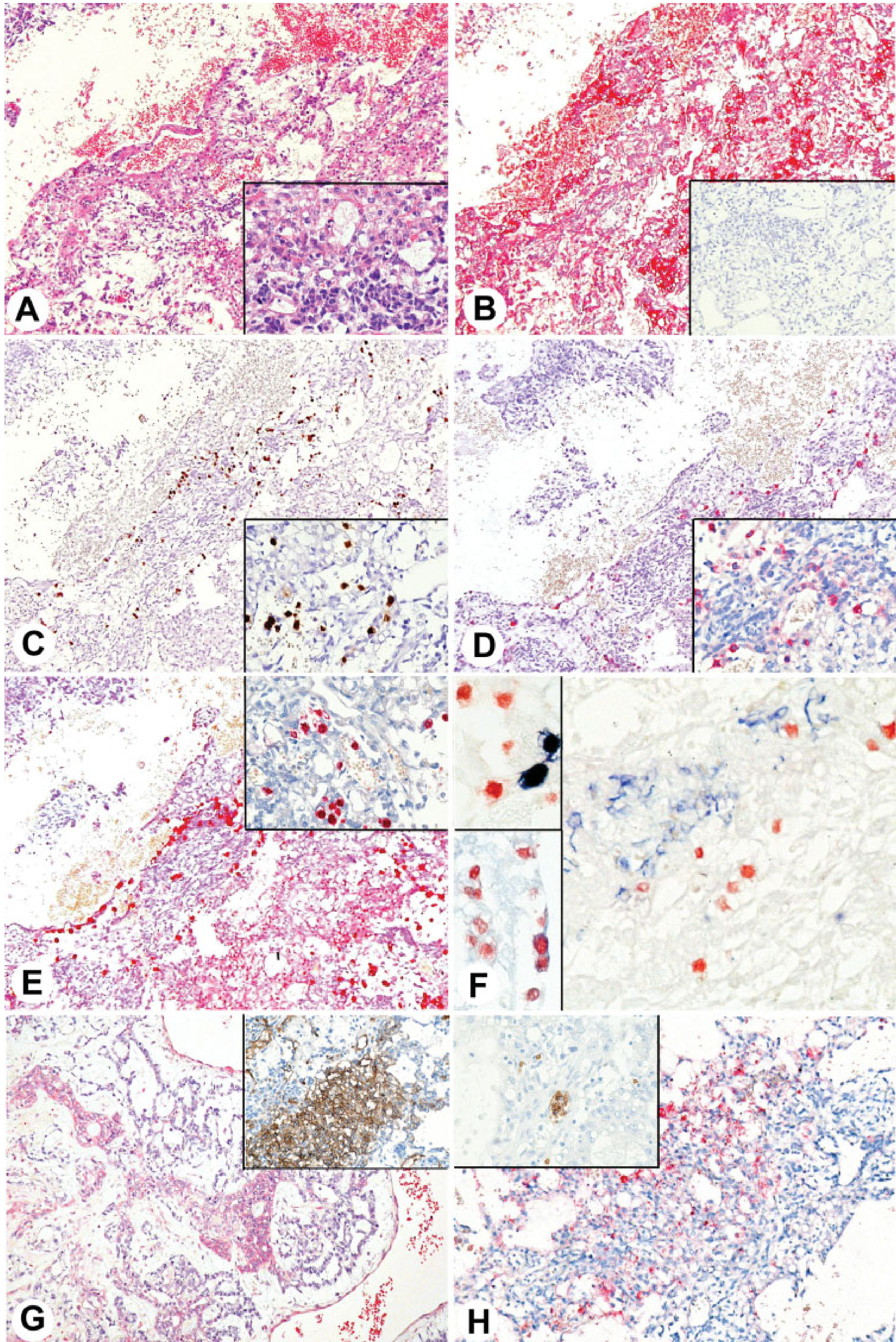
In two of the three cases, haematopoietic differentiation was found, underlining the close developmental relationship between germ cells and haematopoietic precursor cells. In fact, it has been hypothesized that primordial germ cells could be haematopoiesis-initiating cells [14], a process in which BMP4 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

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 ×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 ×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)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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.

Acknowledgements

We thank the urologists and pathologists in the south-western part of The Netherlands for their cooperation in collecting the tumour samples. This work was supported by the Dutch Cancer Society (H.S., J.W.O., L.H.J.L.) and the Deutsche Krebshilfe, Dr Mildred Scheel Stiftung (F.H.)

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Chapter 6

INVOLVEMENT OF E-CADHERIN AND β-CATENIN IN GERM CELL TUMOURS AND IN NORMAL MALE FETAL GERM CELL DEVELOPMENT

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Original Paper

Involvement of E-cadherin and β-catenin in germ cell tumours and in normal male fetal germ cell development

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Abstract

Intercellular contacts, mediated by E-cadherin, are essential for germ cell migration and maturation. Furthermore, it has been suggested that decrease or loss of E-cadherin correlates with tumour progression and invasive behaviour. β-catenin is involved in a number of different processes, including cell–cell interaction when bound to cadherins, and determination of cell fate in pluripotent cells when activated via the Wnt signal-transduction pathway. To shed more light on the role of these factors in normal fetal germ cell development and the pathogenesis of germ cell tumours (GCTs), the present study investigated the presence and localization of E-cadherin and β-catenin by immunohistochemistry. E-cadherin was only weakly expressed in or absent from fetal germ cells of the second and third trimesters, and was not expressed in carcinoma *in situ*/intratubular germ cell neoplasia unclassified (CIS/ITGCNU) and gonadoblastoma, the precursor of an invasive GCT in dysgenetic gonads. In GCTs, it was generally not expressed in seminoma and dysgerminoma, but was found in the vast majority of non-seminoma cells. β-catenin was found in the cytoplasm of fetal germ cells at all gestational ages and in spermatogenesis in post-pubertal testes. It was also present in CIS/ITGCNU and gonadoblastoma. Whereas seminomas and dysgerminoma were negative, non-seminoma cells were frequently found to express β-catenin. Expression of both factors therefore reflects the degree of differentiation of these tumours. No differences for either E-cadherin or β-catenin were observed between samples of tumours resistant or sensitive to chemotherapy, and E-cadherin expression did not correlate with vascular invasion. E-cadherin and β-catenin therefore play a role in both normal and malignant germ cell development and differentiation that warrants further investigation, but they seem to be of limited value as predictive or prognostic factors in GCTs.
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Keywords: E-cadherin; β-catenin; fetal gonads; germ cell tumours; carcinoma *in situ*/ intratubular germ cell neoplasia unclassified (CIS/ITGCNU); gonadoblastoma; chemotherapy resistance; vascular invasion

Introduction

Epithelial (E-) cadherin is a transmembrane glycoprotein that is involved in Ca^{2+}-dependent intercellular adhesion. Through a cytoplasmic domain, it binds via catenins (α-, β-, and γ-catenin) to the actin cytoskeleton. The cadherin–catenin complex is involved in signal transduction from the cell surface to the cytoskeleton and the nucleus. Little is known about E-cadherin expression during human intrauterine germ cell development. In tumour cells, E-cadherin has shown properties of a tumour suppressor, as cells become more invasive if E-cadherin expression is down-regulated [1].

Besides a role in cadherin–catenin complexes, β-catenin has been found to be involved in embryonic stem (ES) cell differentiation of the mouse via the Wnt signal-transduction pathway [2]. Stabilization of β-catenin by mutation or disturbed degradation leads to accumulation in the nucleus, a process implicated in cancer development [3].

An extensive immunohistochemical analysis was undertaken to assess whether the E-cadherin/β-catenin complex is involved in the pathogenesis of GCTs. Data from the literature on this topic are not conclusive [4,5]. Since testicular germ cell tumours (TGCTs) originate from CIS/ITCGNU, which is most likely composed of the malignant counterpart of an early germ cell [6], we also included normal fetal testes in

our investigations of both factors. Like CIS/ITGCNU, gonadoblastoma, the precursor lesion of the dysgenetic gonad, shows the potential to progress to an invasive GCT, mainly dysgerminoma [7]. The presence and localization of E-cadherin and β-catenin were examined both during normal fetal germ cell development and in GCTs of different stages and histological types, including CIS/ITGCNU, gonadoblastoma, intratubular and invasive seminoma, dysgerminoma, and intratubular and invasive non-seminoma. The results were correlated with treatment outcome (tumours refractory or sensitive to chemotherapy) and vascular invasion to assess the potential value of these factors as prognostic or predictive markers.

Materials and methods

Materials

The 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).

Fetal gonads

Human gonads of 11 males from the second and third trimesters were obtained after spontaneous miscarriages ($n = 8$; gestational ages 16, 17, 21, 2×24, 27, 32, 35 weeks) or from term neonates ($n = 3$) that had died shortly after birth. Tissues were collected within 24 h of death and post-mortem examinations were carried out in our department. Testes were dissected and fixed in 10% formalin and processed into paraffin wax. Cases showing conditions potentially affecting gonadal development, such as chromosomal aberrations, hydrocephalus, malformations of the genitourinary tract, 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.

Unselected GCT-patient group

To correlate vascular invasion and E-cadherin expression, fresh frozen or formalin-fixed, paraffin wax-embedded tissues that had been collected between 1991 and 2000 in close collaboration with urologists and pathologists in the south-western part of The Netherlands were retrieved from our archive. Forty patients with a GCT TNM stage 1 or 2 with the following histological diagnosis were included: 18 non-seminomas, 13 seminomas, seven combined tumours, and two only CIS/ITGCNU. The presence of vascular invasion (both lymphatic and angio-invasion) was assessed by an experienced pathologist (JWO). In addition, paraffin wax-embedded tissue from four patients with gonadoblastoma, the precursor lesion of an invasive GCT in dysgenetic gonads, was available for investigation. In two of the samples, a component of invasive dysgerminoma histology was also present.

Selected GCT-patient group

Tumour samples were obtained from patients treated according to protocols followed by the Department of Haematology/Oncology, University of Tübingen. This series has been reported before [8]. In the chemosensitive group, samples collected before therapy from 12 high-risk patients were investigated. Only patients with complete remission or a marker-negative partial remission after high-dose chemotherapy and a relapse-free follow-up of more than 1 year were included. The series consisted of 11 non-seminomas and one seminoma. In the refractory group, 23 samples from 22 patients with chemotherapy-refractory disease were investigated. Patients were considered refractory when progression or relapse occurred despite adequate initial and/or salvage treatment. The material from 14 patients was obtained at initial diagnosis; in nine cases, the material was sampled at relapse after exposure to chemotherapy. In one case, material from both the primary tumour and a metastasis at relapse was available. The series consisted of 22 non-seminomas and one seminoma. Table 1 summarizes the characteristics of the patients with refractory and chemo-sensitive tumours. All cases were reviewed and diagnosed by JWO according to the WHO classification.

Immunohistochemistry

Immunohistochemistry was performed on 3-µm-thick paraffin wax sections. Sections were dewaxed in xylene and rehydrated; frozen tissue sections were fixed in acetone for 5 min and dried. Pressure cooking in 0.01 M citrate buffer, pH 6.0 (1.2 bar) was used for antigen retrieval on paraffin wax sections. All antibodies were diluted in phosphate-buffered saline (PBS), pH 7.4, with 1% bovine serum albumin (BSA). Primary antibodies [E-cadherin (HE-CADHERIN-1, 1:300; Zymed Laboratories, San Francisco, CA, USA), and β-catenin (CAT-5H10, 1:2000; Zymed Laboratories)] were incubated at 4 °C overnight or on frozen tissue sections for 1 h at room temperature. OCT3/4 (sc-8629, 1:1000; Santa Cruz Bio-Technology, Santa Cruz, CA, USA) was incubated for 2 h at room temperature. After washing in PBS, slides for E-cadherin and β-catenin were incubated with biotin-labelled rabbit anti-mouse (Dako-Cytomation, Glostrup, Denmark), and slides for OCT3/4 with biotin-labelled horse anti-goat (Vector BA9500; Vector Laboratories, Burlingame, CA, USA) immunoglobulins for 30 min. Subsequently, all slides were incubated with a biotinylated streptavidin horseradish peroxidase complex (Dako-Cytomation, Glostrup, Denmark) for 30 min. DAB solution (Fluka Chemie,

Table 1. Overall results of immunostaining and characteristics of patients with proven chemo-sensitive or -resistant GCTs (selected group)

	Chemo-sensitive ($n = 12$)	Refractory ($n = 22$)
Age, years		
Median	28	29
Range	20–47	17–56
Histology		
Seminoma	1	1
Non-seminoma	11	22
Stage at diagnosis (according to UICC)		
I	0	3
II	0	7
III	12	12
Initial treatment after surgery		
Surveillance	0	2
Chemotherapy	12	20
Follow-up, months		
Median	49	39
Range	12–67	11–180
Relapse-free survival, months		
Median	NR	7.1
Range		0–150
Response to initial treatment		
Complete remission	8	5
Partial remission, marker negative	4	8
Partial remission, marker positive	0	3
Progressive disease	0	3
Unknown	0	3
No of regimens in relapse		
Median	0	3
Range	0	1–9
% of cases showing β-catenin staining	NS: 92% SE:–	NS: 100% SE:–
% of cases showing E-cadherin staining	NS: 89% SE: –	NS: 80% SE: –
Co-localization of E-cadherin and β-catenin staining in >80% of tumour cells	5/8 (42%)	11/22 (50%)

UICC = International Union Against Cancer; NR = not reached; NS = non-seminoma; SE = seminoma.

Buchs, Switzerland) with H_2O_2 was used to visualize the immune aggregates. Slides were counterstained with Mayer's haematoxylin. Omitting the primary antibody served as a negative control in all experiments, whereas positive staining of epithelial structures (eg rete testis) served as a positive internal control.

Statistical analysis

A chi-squared test was performed to evaluate a possible correlation between E-cadherin expression and vascular invasion. Two-sided testing was applied and $p < 0.05$ was considered statistically significant.

Results

Expression of E-cadherin and β-catenin in normal fetal germ cells, adult germ cells, gonadoblastoma, and CIS/ITGCNU

During fetal development, weak membranous staining for E-cadherin was seen (see Figure 1A) in three of 11 cases containing germ cells. Two of the four gonadoblastomas were weakly positive for E-cadherin, but no expression was detectable at any stage of spermatogenesis in normal adult testis or in CIS/ITGCNU (data not shown). β-catenin was strongly positive at all stages during fetal germ cell development, showing a distinct staining pattern in germ cells: central perinuclear areas of the cytoplasm did not show any signal, but strong staining was detected in a submembranous location (see Figure 1B). In normal adult testis, β-catenin was mainly expressed in the cytoplasm of germ cells at earlier stages of spermatogenesis (ie spermatogonia A and B), but was no longer detectable in spermatids and spermatozoa. Both CIS/ITGCNU and gonadoblastoma showed a staining pattern comparable to that seen in fetal germ cells, with a clear cytoplasmic and submembranous signal location (cf Figures 1B and 1D).

Expression of E-cadherin and β-catenin in invasive GCTs

To assess a possible correlation between the presence of E-cadherin, β-catenin, and clinical outcome, we investigated samples of GCTs with known clinical course. Clinical information and staining results are summarized in Table 1. Overall, no differences were found between the two groups. Both E-cadherin and β-catenin were found in the vast majority of non-seminomas, but not in seminomas (see Tables 1 and 2). No expression of E-cadherin and β-catenin was seen in two dysgerminomas arising in dysgenetic gonads (data not shown). 92% of the chemo-sensitive non-seminomas and 100% of the chemo-resistant non-seminomas showed expression of β-catenin. Expression of E-cadherin was seen in 89% and 80% of the non-seminomas, respectively. Whereas intratubular seminoma and invasive seminoma were negative for E-cadherin, intratubular non-seminoma and embryonal carcinoma (EC) were strongly positive, and yolk sac tumour (YS) was moderately to strongly positive. In teratomas, strong signal intensity for E-cadherin was seen in components showing epithelial differentiation.

β-catenin was mainly seen in a membranous location with differing signal intensity in GCTs of different histological types and stages of invasion (see Figures 1E and 2B). Intratubular seminoma was weakly positive, whereas invasive seminoma was negative. Intratubular non-seminoma and EC showed moderate staining intensity, and YS moderate to strong staining intensity. Using OCT3/4 to identify EC cells in tumours with mixed histology, the signal intensity of β-catenin was found to be stronger in YS cells than

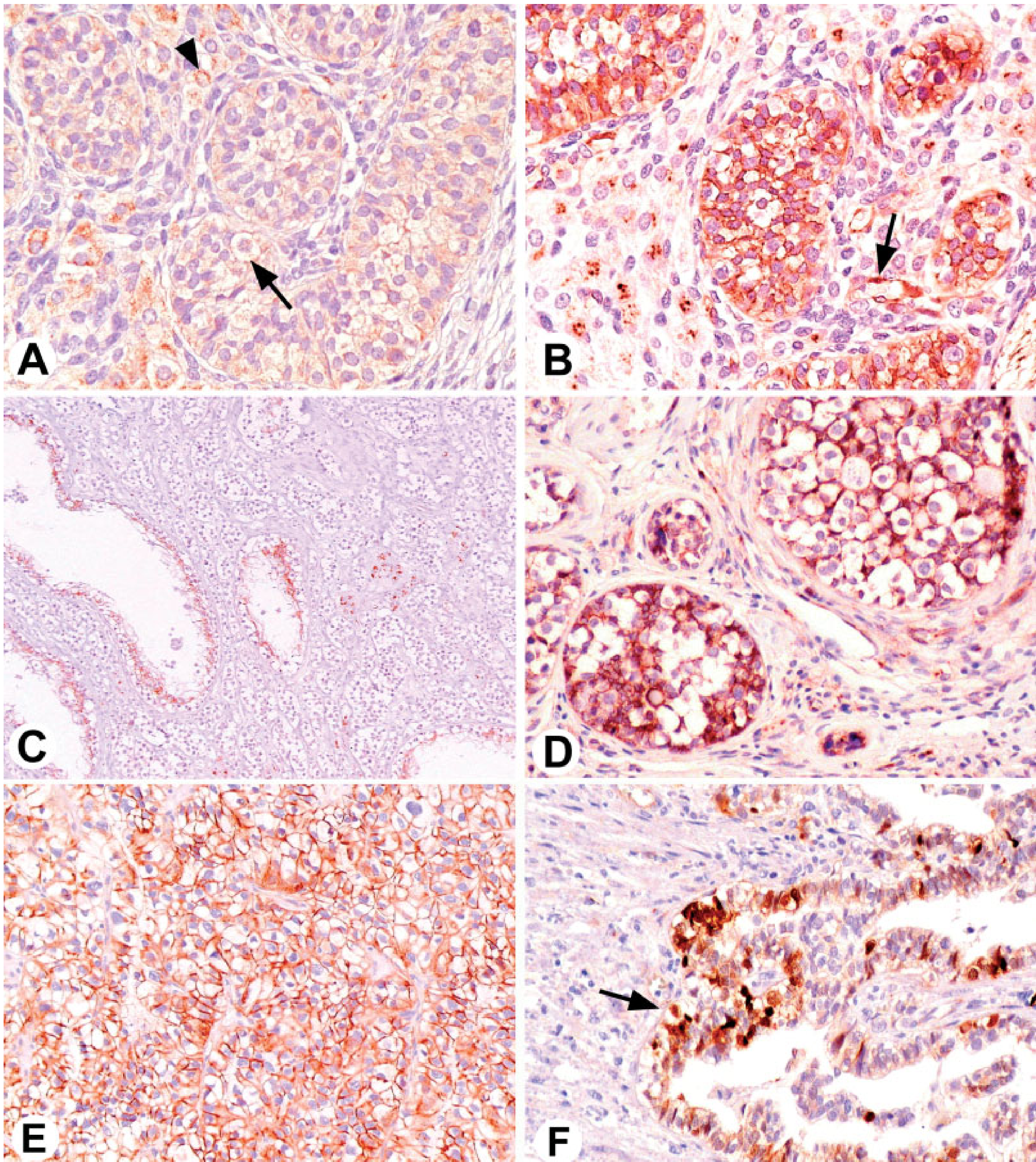

Figure 1. (A) Fetal testis (16 weeks of development); E-cadherin (brown signal) is seen as a very weak membranous signal in germ cells (arrow). Some staining is also seen in Leydig cells (arrow-head). (B) Same case, showing strong signal intensity for β-catenin in a cytoplasmic and membranous location in germ cells and Sertoli cells. Some signal is seen in Leydig cells. Note that endothelial cells are also positive for β-catenin (arrow). (C) Germ cell tumour showing seminoma and CIS/ITGCNU. Note the brown β-catenin signal in tubules containing CIS/ITGCNU, but absence of staining in the invasive seminoma component. (D) Gonadoblastoma, positive for β-catenin (brown membranous and cytoplasmic signal). Note the similarity of the staining pattern to that of fetal germ cells (cf B). (E) Yolk sac tumour with solid growth pattern; membranous staining for β-catenin is seen (brown signal). (F) Papillary yolk sac tumour showing mainly cytoplasmic and, in a minority of tumour cells, also nuclear β-catenin staining (brown signal, arrow)

Table 2. Results of immunostaining, differentiated by histological subtype; note that one tumour sample can contain more than one histological type

Histology	No	Overall E-cadherin staining (%)	>20% tumour cells positive (%)	Overall β-catenin staining (%)	>20% tumour cells positive (%)
Refractory series					
CIS/ITGCNU	7	0	0	100	100
ITSE	3	0	0	0	0
SE	2	0	0	0	0
YS	16	75	44	100	100
CC	3	100	100	100	100
TE	3	100	100	100	100
ITNS (EC)	2	100	100	100	100
EC	8	87.5	87.5	100	100
Sensitive series					
CIS/ITGCNU	4	0	0	100	100
ITSE	1	0	0	0	0
SE	3	0	0	0	0
YS	4	100	75	100	75
CC	0	0	0	0	0
TE	3	66	66	100	100
ITNS (EC)	1	100	100	100	100
EC	7	86	86	86	71

CIS/ITGCNU = carcinoma *in situ*/intratubular germ cell neoplasia unclassified; ITSE = intratubular seminoma; SE = seminoma; YS = yolk sac tumour; CC = choriocarcinoma; TE = teratoma; ITNS = intratubular non-seminoma; EC = embryonal carcinoma.

in EC cells (see Figures 2A–2C). Strong staining was also observed in teratomas, mainly in epithelial components.

In both groups, co-localization of E-cadherin and β-catenin in the majority of tumour cells (judged by comparing tumour areas in adjacent slides) was seen in approximately 50% of the cases (see Table 1). Nuclear localization of β-catenin was observed in a maximum of 10% of all tumour cells in two tumours from the refractory group, one before and one after exposure to chemotherapy, and in one tumour in the sensitive group, prior to chemotherapy treatment (see Figure 1F).

Correlation of E-cadherin expression and vascular invasion and metastasis

All of the chemo-sensitive cases and 19 of the 22 refractory cases (selected patient group) had metastases at the time the tumour sample was obtained. No correlation between vascular invasion or metastatic behaviour and E-cadherin expression was seen: of the three refractory cases without metastases, two showed E-cadherin expression, and one did not. In several cases, vascular invasion by non-seminoma (mostly EC cells) expressing E-cadherin was seen.

To extend our analysis of a possible correlation between E-cadherin expression and vascular invasion, a series of 40 tumour samples (unselected patient group) from patients with early-stage GCT was assessed. In the group without vascular invasion ($n = 29$), 13 samples were E-cadherin-negative and 16 samples were positive. In the group with vascular invasion ($n = 11$), E-cadherin expression was absent from three and present in eight samples ($p = 0.31$, statistically not significant).

Discussion

GCTs are at the crossroads of developmental and tumour biology, and are of interest from both points of view. Comparative analyses of tumour samples and normal counterparts can help to define events related to the pathogenesis of these tumours. This is also documented by our study investigating the expression and localization of E-cadherin and β-catenin in both normal early germ cells and GCTs of different histological types. Besides interpreting our findings from the point of view of development of germ cells, both normal and malignant, we will also discuss our results with regard to clinically important issues, including tumour invasiveness and chemotherapy resistance.

Aspects of developmental biology

Characteristics of early germ cell development

The cadherin–catenin complex plays an important role in the very early stages of embryonal development. E-cadherin has been described in early blastomeres [9] and on human EC cell lines, which closely resemble ES cells. E-cadherin is essential for the migration and homing of mouse primordial germ cells (PGCs) from the hindgut to the genital ridges [10,11]. Whereas little is known about the expression of E-cadherin on human migratory and post-migratory germ cells of

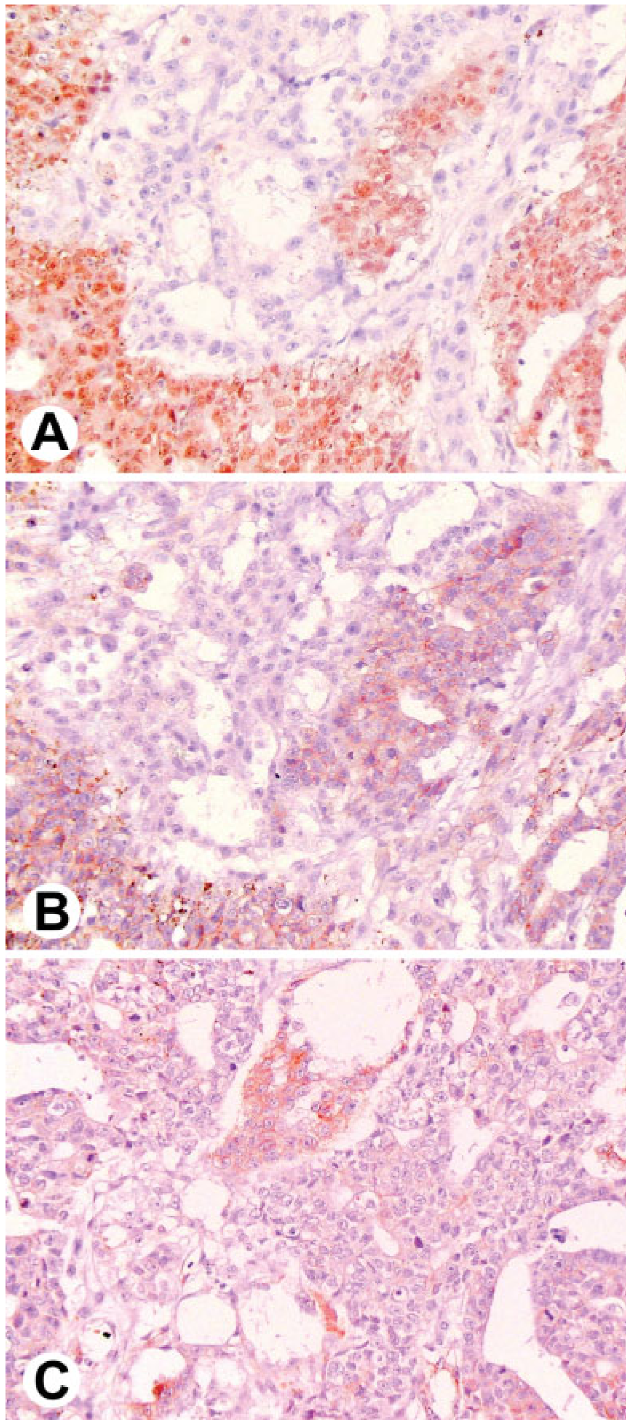

Figure 2. (A) Germ cell tumour showing a combination of embryonal carcinoma and yolk sac tumour. Note the brown nuclear signal for OCT3/4 in embryonal carcinoma, but not in the cells showing yolk sac features. (B) Same case; E-cadherin staining is seen in embryonal carcinoma, but not in yolk sac tumour. (C) Same case; β-catenin is seen predominantly in yolk sac areas and is much weaker in embryonal carcinoma

the male, we recently described the presence of E-cadherin in both early and maturing female germ cells (unpublished observations). β-catenin has been shown to play a major role in the stem cell compartment of germ cells in vertebrates and non-vertebrates [12,13].

Our data show that E-cadherin is only weakly expressed on or absent from the surface of male germ cells at different stages of fetal development and during spermatogenesis. In contrast, β-catenin is present throughout all stages of intrauterine germ cell maturation. Interestingly, the expression pattern and intensity of both factors seen in gonadoblastoma and CIS/ITGCNU show a close resemblance to male fetal germ cells. The weak expression of E-cadherin in gonadoblastoma present in dysgenetic gonads of XY individuals with a female phenotype resembles immature male germ cells more than immature female germ cells, as these are strongly positive for E-cadherin (unpublished observations). These results add further evidence to the model that both gonadoblastoma and CIS/ITGCNU originate early during fetal development [14,15]. An interesting, yet unexplained, observation is the phenomenon that β-catenin is present in all precursor lesions, but is lost consistently upon progression to an invasive tumour showing germ cell characteristics. This was observed in the progression of both gonadoblastoma to dysgerminoma and CIS/ITGCNU to seminoma.

Characteristics of early embryonal development

Seminoma and dysgerminoma cells, resembling early germ cells, are mainly negative for E-cadherin, whereas ES-like properties of EC are reflected by their strong expression of E-cadherin. This is illustrated by the similar expression pattern of OCT3/4, a marker specific for pluripotent germ cell tumour cells such as EC [16], and E-cadherin in tumours of different histological types (see Figures 2A and B). Low levels or the absence of E-cadherin in seminomas and some YS is not accompanied by nuclear translocation of β-catenin in the majority of the tumours investigated, in contrast to findings from functional knock-out studies of E-cadherin in ES cells [17]. Furthermore, the switch from pluripotent EC cells to extra-embryonic or somatic tissues such as YS and teratoma leads to an increase in the signal intensity of cytoplasmic β-catenin (see Figure 2C). In teratomas, both factors are strongly expressed in epithelial structures, most likely reflecting changes in gene expression induced by somatic differentiation. Our results confirm the earlier observation that the expression of E-cadherin and β-catenin in GCTs correlates with the degree of differentiation of these tumours [5].

Pathobiological implications

Correlation of E-cadherin and β-catenin expression with treatment outcome

A role for E-cadherin and β-catenin has been postulated in cancer invasion, aggressiveness of the tumour, and metastasis [18]. E-cadherin has been viewed as a tumour suppressor, mainly by maintaining cell–cell adhesion and anchoring β-catenin in a submembranous location [19]. Mutations of E-cadherin have been found in a number of tumours, including breast and gastric cancer, but not in testicular cancer [20]. Adriamycin, a cytotoxic drug, has

been shown to induce E-cadherin-mediated cell–cell adhesion *in vitro* [21], and in another *in vitro* model, expression of E-cadherin led to reduced BCL-2 expression and increased sensitivity to etoposide, an important drug in the treatment of GCTs. β-catenin, a target of the Wnt-signalling pathway, has been identified as a potential candidate for targeted therapy [22]. As little is known about these factors in treatment resistance in GCT, we investigated E-cadherin and β-catenin in samples from tumours both resistant and sensitive to chemotherapy.

Although expression of both factors correlated with different histological subtypes, and was seen in non-seminomas but not in seminomas, no differences in treatment outcome could be detected. Our data suggest that the detection of E-cadherin or β-catenin by immunohistochemistry has no prognostic or predictive value for patients with newly diagnosed GCTs. The overall prevalence of nuclear translocation of β-catenin was low in our series. Although the factors were not co-localized in approximately 50–60% of all tumours, events leading to nuclear translocation of β-catenin seem to be relatively rare in GCTs.

Correlation of E-cadherin expression and vascular invasion in GCTs

It has been speculated that loss of cell adhesion might help cells to detach from a primary tumour and become invasive. For GCTs, a correlation between loss of E-cadherin and lymph node metastases in stage I non-seminomas has been implicated [4]. Yet, given that E-cadherin is essential for the migration and homing of mouse PGCs, a different function for E-cadherin in GCTs is conceivable. In GCT cells showing characteristics of early germ cells, E-cadherin could actually enhance motility and the capacity to penetrate tissues, or enable circulating tumour cells to attach to vessel walls and 'home' in a new environment. In seminomas, which show no or only weak E-cadherin expression, vascular invasion is not a frequent event. On the other hand, ECs, which in the vast majority of cases show high levels of E-cadherin expression, are frequently angio-invasive [23]. In fact, the presence of EC is associated with an odds ratio of 2.9 for developing occult metastases [24].

Our results from the tumour samples from the selected group of patients suggest that neither vascular invasion nor the frequency of metastatic spread correlate with E-cadherin expression. Yet, as this series was highly biased towards later stages of GCTs and contained almost no cases without metastases at diagnosis, we expanded our series by including samples of tumours of earlier stages without metastases. In a cohort of 40 cases, 29 without and 11 with vascular invasion, no statistically significant correlation of E-cadherin expression and vascular invasion was seen, in line with the results of our selected patient group. This suggests that, unlike in other tumour entities, vascular invasion and E-cadherin expression do not seem to correlate in GCTs. However, our results must be interpreted with caution, as the sample size investigated was rather small. Further investigation of this topic should ideally be performed as part of a multivariate analysis of prognostic factors within large clinical trials.

In summary, our results allow a number of conclusions: (1) The majority of immature male germ cells of the second and third trimesters show no or very weak expression of E-cadherin, but consistently express β-catenin. (2) E-cadherin is not expressed in gonadoblastoma, CIS/ITGCNU, or intratubular and invasive seminoma; in contrast, β-catenin is expressed in gonadoblastoma and CIS/ITGCNU, but is lost during progression to intratubular seminoma and invasive seminoma or dysgerminoma. (3) Both factors are expressed in the vast majority of intratubular and invasive non-seminomas. Expression therefore seems to be regulated during differentiation. (4) Nuclear translocation of β-catenin is a rare event in GCTs. (5) No differences in the presence of either E-cadherin or β-catenin were observed between samples of tumours refractory or sensitive to chemotherapy, and E-cadherin expression did not correlate with vascular invasion.

E-cadherin and β-catenin therefore play a role in both normal and malignant germ cell development and differentiation that warrants further investigation, but they seem to be of limited value as predictive or prognostic factors in GCTs.

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Chapter 7

XERODERMA PIGMENTOSUM GROUP A PROTEIN AND CHEMOTHERAPY RESISTANCE IN HUMAN GERM CELL TUMORS

***Laboratory Investigation* 83: 1489-1495, 2003**

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Xeroderma Pigmentosum Group A Protein and Chemotherapy Resistance in Human Germ Cell Tumors

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SUMMARY: The exceptional sensitivity of germ cell tumors (GCTs) of adolescents and adults to chemotherapy, in particular to cisplatin, has been attributed to low levels of xeroderma pigmentosum group A protein (XPA), a crucial component of the nucleotide excision repair DNA repair pathway. In different types of solid tumors, resistance to cisplatin has been associated with enhanced expression of XPA. To assess the role of XPA levels in clinical sensitivity and resistance of GCTs to chemotherapy, immunohistochemistry was performed on tumor samples of both unselected patients before therapy and patients with fully documented clinical course before and after therapy. In the case of high XPA levels, fluorescent in situ hybridization was applied to assess the possibility of gene amplification. XPA protein levels were investigated by Western blot analysis after repeated exposure to cisplatin in different GCT-derived cell lines. Finally, XPA levels of both sensitive and cisplatin-resistant GCT cell lines were compared with cell lines derived from other neoplasms. We found that the presence of XPA protein as assessed by immunohistochemistry differs among the various histologies of GCTs. It is found more frequently and with a more homogenous staining pattern in histologic subtypes showing a more differentiated phenotype. Overall, no differences in the presence of XPA was observed between samples of tumors refractory or sensitive to chemotherapy. No XPA gene amplification was found. Interestingly, all tumors resected in relapse after chemotherapy in the refractory group stained positive for XPA. However, XPA was not induced by repeated courses of sublethal doses of cisplatin in GCT-derived cell lines in vitro, and no correlation between XPA protein levels and sensitivity to cisplatin in three GCT-derived cell lines was observed. We therefore conclude that XPA does not play a critical role in overall treatment resistance of GCTs. (*Lab Invest 2003, 83:1489–1495*).

Germ cell tumors (GCTs) of the testis are the most frequent solid tumor in Caucasian men between 20 and 45 years of age (Adami et al, 1994). Histologically, they display patterns that resemble stages of embryonal development (Looijenga and Oosterhuis, 1999, 2002). Seminomas show characteristics of early germ cells (primordial germ cells or gonocytes), whereas nonseminomas can contain different, both embryonic and extra-embryonic, elements. The embryonal carcinoma is the stem cell component of nonseminomas, which may differentiate into yolk sac tumor, choriocarcinoma, and teratoma.

Seminomas and nonseminomas are highly sensitive to chemotherapy, notably to cisplatin (Einhorn, 2002). Presently, more than 80% of patients with metastatic disease can be cured by cisplatin-based combination chemotherapy. However, mature teratomas are intrinsically resistant to chemotherapy. This histology is found in about 40% of residual lesions after chemotherapy and should be resected to prevent malignant transformation and development of secondary non–germ cell malignancies (Fizazi et al, 2001; Oosterhuis et al, 1983). The biological basis of the overall high sensitivity to chemotherapy and the infrequent but mostly lethal occurence of resistant phenotypes remains unclear.

Different mechanisms of cellular resistance to cisplatin have been described, including inhibition of drug uptake and increased efflux, inactivation by sulfur-containing proteins, enhanced replicative bypass of platinum DNA adducts, changes in concentrations of regulatory proteins, and enhanced repair of DNA crosslink lesions caused by cisplatin (Kartalou and Essigmann, 2001; Mayer et al, 2003; Reed, 1998). Nucleotide excision repair (NER) is believed to be the main mechanism by which damage caused by cisplatin through formation of bulky DNA adducts is repaired in mammalian cells (Chaney and Sancar, 1996; Zamble and Lippard, 1995). Xeroderma pigmentosum

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group A protein (XPA), involved in the NER system, is a zinc finger protein that is absent or aberrant in cells of patients with xeroderma pigmentosum complementation group A (Bootsma et al, 2001). Binding of XPA to the replication protein A is the initiating and rate-limiting step of NER (Matsuda et al, 1995). It subsequently recruits other factors to damaged substrates (Buschta-Hedayat et al, 1999). GCT-derived cell lines were found to have low XPA levels and a low NER capacity. This finding was proposed as the major reason for the high sensitivity of GCTs to chemotherapy (Koberle et al, 1999). The *XPA* gene maps to chromosome 9q22, one of the regions found to be specifically amplified in GCTs showing chemotherapy resistance (Rao et al, 1998).

The objective of the present study is to investigate the presence of XPA in GCTs and clarify its role in sensitivity and resistance to cisplatin-based chemotherapy. XPA status was assessed by immunohistochemistry in tumor samples from patients with histologically defined GCTs without information on clinical outcome. To correlate immunohistochemical findings with clinical outcome, tumors from patients with fully documented clinical course, including chemotherapy-sensitive and refractory cases, were investigated. In addition, the correlation between XPA protein levels and cisplatin sensitivity was investigated by immunoblotting in different cell lines, including GCT cell lines with defined sensitivity and resistance to cisplatin.

Results

The presence of XPA protein was investigated in different histological elements of GCTs, including carcinoma in situ (CIS), their obligatory precursor lesion in the testis. Immunohistochemistry was performed on paraffin-embedded tissue sections of 85 unselected tumors of all histologic variants (Table 1). XPA showed a nuclear localization in the tumor cells with differing frequency and intensity in the different subtypes and histologic elements. Figure 1 shows representative examples, including normal spermatogenesis. Embryonal carcinomas showed staining in 26% of cases (4 of 15), followed by seminomas (16 of 33, 48%), choriocarcinoma (2 of 4, 50%), and yolk sac tumors (11 of 18, 61%). Interestingly, all teratomas (19 primary cases and 4 residual mature teratomas) were strongly positive. The overall percentage of positive tumor cells varied between different subtypes and histologies. Seminomas and embryonal carcinomas displayed a heterogenous staining pattern with only a few tumors (n = 8/33 and 2/15, respectively) showing more than 50% positivity. Yolk sac tumors, choriocarcinomas, and teratomas revealed a much more homogenous staining, with up to 100% of nuclei being positive. About 25% of the CIS cells, as present in adjacent testicular parenchyma of invasive seminomas or non-seminomas (n = 5), showed a positive staining for XPA. This positivity was confirmed using a double-staining for c-KIT and XPA (Fig. 1B).

To assess a possible correlation between the presence of XPA and clinical outcome, we investigated samples of GCTs from patients with chemotherapy-sensitive (n = 12) and refractory (n = 23) tumors. Clinical information is given in Table 2. The immunohistochemical results demonstrated no difference between the two groups: 9 of 12 (75%) tumors in the chemosensitive and 16 of 23 (70%) tumors in the refractory group stained positive for XPA (Table 2). However, all tumors sampled after chemotherapy (n = 9) in the refractory group were positive for XPA. The histologies found in these cases were yolk sac tumor (n = 7), teratoma (n = 2), and choriocarcinoma (n = 2; some of the tumors exhibited more than one histologic subtype). No gene amplification of XPA was found using double-color fluorescent in situ hybridization (FISH) on tumors showing strong XPA expression in the refractory group (n = 9 cases).

To investigate possible mechanisms related to the consistent presence of XPA in the refractory GCTs after cisplatin exposure, we investigated the total amount of XPA protein in three well-characterized GCT-derived cell lines (NT2, 2102Ep, and NCCIT) by Western blotting. Whereas NCCIT showed a low and 2102Ep an intermediate expression level, NT2 contained XPA at a level comparable to that of various other tumor cell lines (Fig. 2A). Furthermore, in cytotoxicity assays no correlation between XPA level and cisplatin sensitivity was observed (Fig. 2B). Continuous treatment with 0.5 μM and 1.5 μM cisplatin, respectively, for up to 48 hours did not result in up-regulation of XPA in NT2 or 2102Ep (Fig. 2C). Subsequently, NT2 and 2102Ep cells were repeatedly treated with sublethal doses of cisplatin, resulting (after 18 cycles of treatment) in cell lines relatively resistent to cisplatin. The NT2 subline (NT2/CDDP) showed a 2.2-fold and the 2101EP subline (2102EP/CDDP) a 2-fold resistance to cisplatin at the IC50 (Fig. 2B). However, this was not accompanied by an increase in the level of XPA in these sublines (Fig. 2A).

Table 1. Immunohistochemical detection of xeroderma pigmentosum group A protein in CIS and in GCTs of different histologies (unselected tumor samples)[a]

Histology	Cases showing any positivity, %	Cases with > 50% positive nuclei, %
EC (n = 15)	26	13
SE (n = 33)	48	24
CC (n = 4)	50	50
YS (n = 18)	61	44
TE (n = 23, including 4 RMT)	100	95
CIS containing samples (n = 5)[b]		

CC, choriocarcinoma; CIS, carcinoma in situ; EC, embryonal carcinoma; GCTs, germ cell tumors; RMT, residual mature teratoma; SE, seminoma; TE, teratoma; YS, yolk sac tumor.

[a] The majority of cases showed more than one histological subtype.

[b] 25% positivity of all CIS cells.

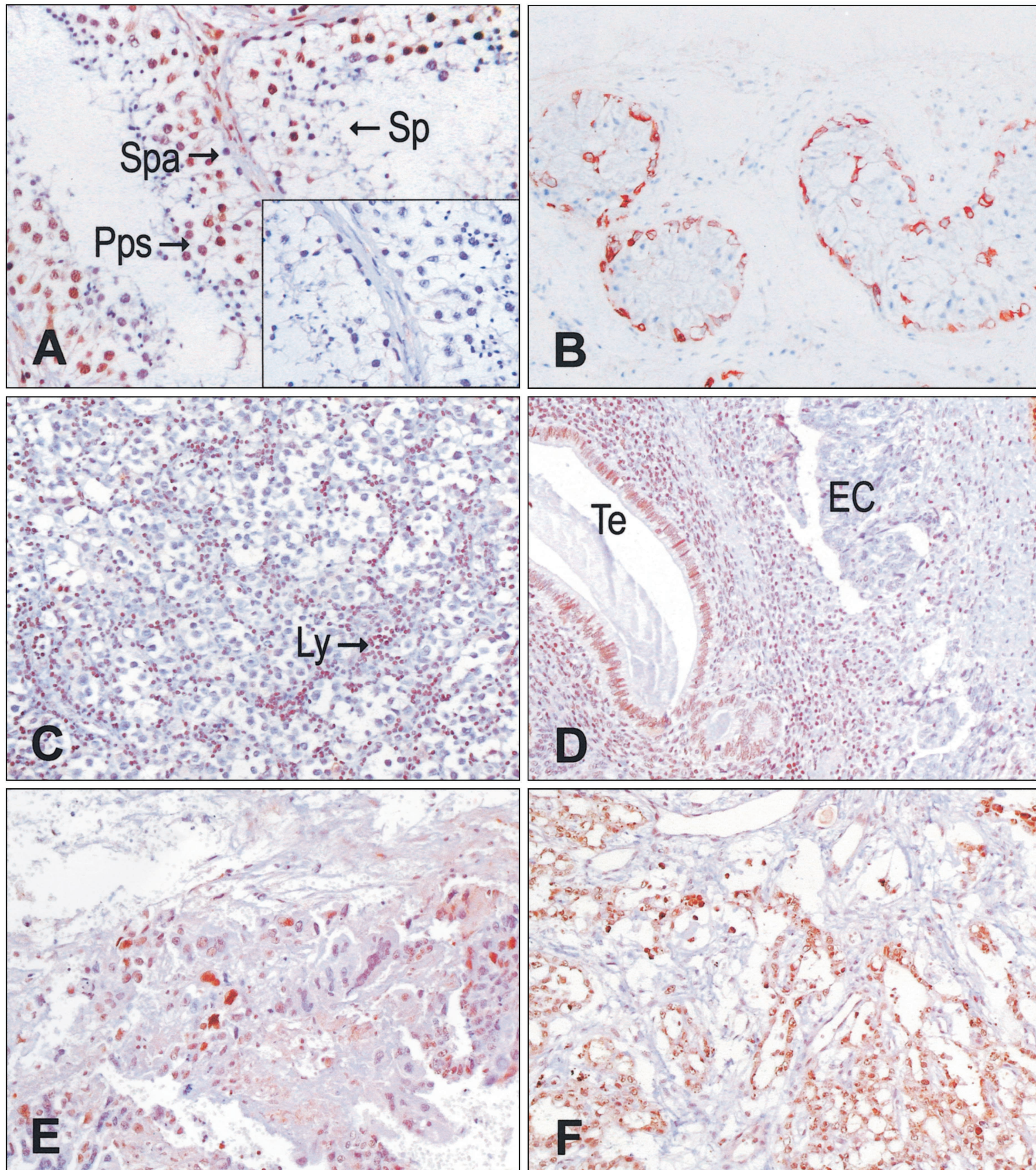

Figure 1.

Representative examples of the immunohistochemical stainings for xeroderma pigmentosum group A protein (XPA) in normal testis and germ cell tumors (GCTs) of different histologic subtypes. All sections have been lightly counterstained with hematoxylin with exception of B (double-staining), which was not counterstained to allow identification of both stainings. (A) Normal testicular parenchyma. Note positivity of germ cells at various stages of maturation as reported before (Stoop et al, 2001). Primary and pachytene spermatocytes (*Pps*) are XPA positive, whereas spermatogonia (*Spa*) and spermatids (*Sp*) are negative. Insert: Negative control (primary antibody omitted). (B) Seminiferous tubules containing carcinoma in situ (CIS) cells. Double-staining for c-KIT (red membrane-bound staining) and XPA (blue nuclear staining). All CIS cells show positivity for c-KIT and can be either positive or negative for XPA. (C) Seminoma, negative for XPA. Note positivity of infiltrating lymphocytes (*Ly*). (D) Nonseminoma containing both teratoma (*Te*) showing epithelial differentiation positive for XPA and embryonal carcinoma (*EC*) negative for XPA. Note that stromal cells and infiltrating lymphocytes can be positive for XPA. (E) Choriocarcinoma showing a heterogeneous staining for XPA. Note the varied staining reaction of the multinucleated syncytiotrophoblasts. (F) Yolk sac tumor positive for XPA.

Discussion

The unique chemosensitivity of GCTs is poorly understood on a molecular level so far (Mayer et al, 2003). Based on studies on the mouse teratocarcinoma cell line P19, it has been assumed that a high level of wild-type p53 results in a low threshold for induction of apoptosis (Lutzker and Levine, 1996; Lutzker et al, 2001). However, this model differs from human GCTs in various parameters, and we recently demonstrated that treatment outcome in patients with GCTs does not correlate with p53 status (Kersemaekers et al,

Table 2. Characteristics of patients with proven chemosensitive or refractory germ cell tumors

	Chemosensitive (n = 12)	Refractory (n = 22)
Age, years		
Median	28	29
Range	20–47	17–56
Histology		
Seminoma	1	1
Nonseminoma	11	21
Stage at diagnosis (per UICC)		
I	0	3
II	0	7
III	12	12
Initial treatment after surgery		
Surveillance	0	2
Chemotherapy	12	20
Follow-up, months		
Median	49	39
Range	12–67	11–180
Relapse-free survival, months		
Median	NR	7.1
Range		0–150
Response to initial treatment		
Complete remission	8	5
Partial remission, marker negative	4	8
Partial remission, marker positive	0	3
Progressive disease	0	3
Unknown	0	3
No. of regimens in relapse		
Median	0	3
Range	0	1–9
Cases showing XPA positivity	75% (9/12)	70% (16/23)

NR, not reached; UICC, International Union Against Cancer; XPA, xeroderma pigmentosum group A protein.

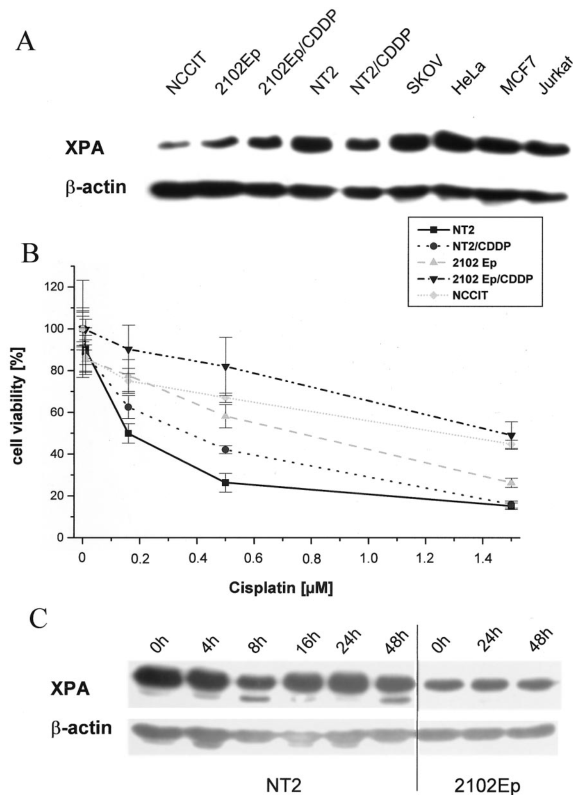

Figure 2.

(A) Immunoblot analysis for xeroderma pigmentosum group A protein (*XPA*) and β-actin on different cell lines derived from germ cell tumors (GCTs), including NT2/CDDP and 2102Ep/CDDP cell lines with acquired resistance to cisplatin following repeated sublethal exposure. SKOV, HeLa, MCF7, and Jurkat are cell lines derived from ovarian cancer, cervical cancer, breast cancer, and leukemia cells. (B) Cytotoxicity assay for selected GCT-cell lines. Note the lack of correlation between CDDP sensitivity and XPA level. (C) Immunoblot for XPA and β-actin on NT2 and 2102Ep cells during initial treatment with cisplatin (NT2: 0.5 μM cisplatin, 2102Ep: 1.5 μM cisplatin).

2002). It has also been suggested that the unique treatment sensitivity of GCTs could be explained by a defective repair of cisplatin-induced DNA damage related to low XPA (Koberle et al, 1999). Several observations seem to support this model. For example, mammalian cells deficient in NER are more sensitive to cisplatin than corresponding wild-type cells (Dijt et al, 1988; Poll et al, 1984). Expression of a truncated XPA protein exerting a dominant-negative effect sensitizes human tumor cells to UV light and cisplatin (Rosenberg et al, 2001). Moreover, an enhanced XPA expression is associated with resistance to cisplatin treatment in human ovarian cancer (Dabholkar et al, 1994). However, studies correlating NER with clinical data result in conflicting data for different tumor entities. For example, expression of several NER factors in patients with chronic lymphocytic leukemia did not correlate with resistance to nitrogen mustard (Bramson et al, 1995), while an increased NER capacity in patients with non–small-cell lung cancer may be associated with poorer survival (Bosken et al, 2002). It is of specific interest that a link between NER and differentiation has been observed in normal tissues; somatic stem cells are highly sensitive to the effects of physical and chemical mutagens (Ijiri and Potten, 1987; Potten, 1977) and avoid certain forms of potentially error-prone DNA damage repair (Roth and Samson, 2002). It has been suggested that the choice of death rather than defective DNA repair in case of damage serves to avoid accumulation of replication errors with fatal consequences for the progeny (Cairns, 2002). So far, however, no data are available regarding NER and clinical outcome in GCTs.

The aim of this study is to assess the role of XPA in clinical resistance of GCTs. Immunohistochemistry in the unselected group of patients revealed that the staining pattern of XPA correlated with histology. In seminomas and in embryonal carcinoma, only a minority of cells contained detectable levels of XPA. In contrast, the majority of cells from choriocarcinomas,

yolk sac tumors, or teratomas were positive for XPA. Three teratomas and yolk sac tumors of infants and neonates that were included in this series showed a similar staining pattern as the tumors in histologic counterparts found in testis of the adults (data not shown). No differences in the presence of XPA was evident comparing tumors of patients with cisplatin-sensitive and refractory disease. The overall picture was heterogenous, as high expression of XPA was observed in several tumor samples of chemosensitive cases on the one hand, and lack of expression of XPA was seen in some refractory cases on the other hand. Results were in the same range as in the unselected group. Therefore, we conclude that XPA detection by immunohistochemistry has no prognostic or predictive value for patients with newly diagnosed GCTs.

None of the resistant tumors with detectable levels of XPA showed amplification of the respective locus using FISH analysis. This excludes amplification of the *XPA* gene as a common mechanism of chemotherapy resistance in GCTs. However, it is noteworthy that all tumors sampled at relapse in the refractory group, ie, after exposure to chemotherapy, were positive for XPA. This could simply be due to the particular histology (yolk sac tumors, choriocarcinoma, and teratoma) found in this limited series. Alternatively, induction of XPA or selection for XPA-positive cells could have occurred during treatment. To test this hypothesis, an in vitro model was used, which showed no correlation between XPA levels and sensitivity to cisplatin in the three GCT-derived cell lines investigated. We found a low level of XPA only in NCCIT, the cell line with the lowest sensitivity to cisplatin, whereas the levels of 2102Ep and NT2 were only slightly lower or comparable to that found in cell lines derived from other neoplasms. In addition, XPA expression was not induced in GCT cells that acquired cisplatin resistance by repeated exposure to the drug, differing from findings reported from ovarian cancer–derived cell lines (Hector et al, 2001). In this context it is of interest that even a low level of XPA expression in deficient cells is sufficient for total complementation of cellular sensitivity and DNA repair activity (Muotri et al, 2002). In accordance with this, it has been reported that sensitivity to 170 different compounds, including cisplatin, tested in a cytotoxicity assay did not correlate with XPA levels in 60 human tumor cell lines (Xu et al, 2002).

In conclusion, we demonstrated that the presence of XPA protein as assessed by immunohistochemistry differs between the various histologies of GCTs. XPA is found more frequently and with a more homogenous staining pattern in the histologic subtypes with a more differentiated phenotype. Overall, no differences in the presence of XPA were observed between samples of tumors refractory or sensitive to chemotherapy. Furthermore, we did not find a correlation between XPA protein levels and sensitivity to cisplatin in three GCT-derived cell lines. We therefore conclude that XPA does not play a critical role in overall treatment resistance of GCTs. Further research is needed to elucidate the mechanisms of chemotherapy sensitivity and resistance in these tumors.

Materials and Methods

Patient Material

Unselected Group: Formalin-fixed paraffin-embedded tissue blocks were retrieved from the archive of the Laboratory for Experimental Patho-Oncology, Department of Pathology, Erasmus MC, Rotterdam. The material was collected between 1991 and 2001 in close collaboration with urologists and pathologists in the Southwest of the Netherlands. Eighty-one tumors were collected before therapy. In four cases residual mature teratomas were removed after chemotherapy. No data on the clinical course of the patients were available.

Chemosensitive Group: Formalin-fixed, paraffin-embedded samples collected before therapy from 12 patients diagnosed between 1995 and 1998 were investigated. Only patients with a complete remission or a marker negative partial remission after high-dose chemotherapy and a relapse-free follow-up of more than 1 year were included. The series consisted of 11 nonseminomas and one seminoma.

Refractory Group: Twenty-three formalin-fixed paraffin-embedded samples from 22 patients with chemotherapy-refractory disease diagnosed between 1991 and 1998 were investigated. Patients were considered refractory when progression or relapse occurred despite adequate initial or salvage treatment, including high-dose chemotherapy in some cases. The material of 14 patients was obtained at initial diagnosis; in nine cases, the material was sampled after exposure to chemotherapy from metastatic lesions in relapse. In one case, material from both the primary tumor and a metastatic tumor at relapse was available. The series consisted of 22 nonseminomas and one seminoma. Table 2 summarizes the characteristics of the patients with refractory and chemosensitive tumors. All cases were reviewed and diagnosed by J.W.O. according to the World Health Organization classification, and the fully documented clinical course was available for these patients.

Cell Lines and Culture Conditions

The GCT-derived cell lines NT2 and NCCIT were purchased from the American Type Tissue Culture (Manassas, Virginia). 2102Ep was a kind gift from M. Pera. MCF-7 (breast cancer), SKOV-3 (ovarian cancer), and HeLa (cervical cancer) were gifts from P. Brossart. Jurkat (human T cell leukemia) was a gift from T. Brümmendorf (University of Tübingen Medical Center). Cells were grown as monolayer and maintained at 37° C in a humidified cell culture incubator with 5% carbon dioxide. NT2 were cultured in DMEM/glutamax supplemented with 10% FCS (Gibco BRL, Paisly, United Kingdom), penicillin (Biochrom), and streptomycin (Biochrom). NCCIT and 2102Ep were cultured in DMEM/nut mix F12 supplemented with 10% FCS, pencillin, streptomycin, and glutamin. All

other cell lines were kept in HEPES-buffered RPMI (Gibco).

Immunohistochemistry

Paraffin sections of 3-μm thickness were mounted on Starfrost slides (Knittel Gläser, Germany), dried at 50° C overnight, deparaffinized, and rehydrated. For antigen retrieval, pressure cooking at 1.0 bar in citrate buffer 0.01 mol/L pH 6.0 was used. All antibodies were diluted in PBS with 1% BSA (Sigma, Zwijndrecht, the Netherlands). The primary antibody anti-XPA (mouse mAb, Ab-1 Clone 12F5; NeoMarkers, Freemont, California) was used in a dilution 1:100, and incubation time was 60 minutes at room temperature. Biotin-labeled rabbit-antimouse immunoglobulins and an avidin-biotin-HRP complex (both DAKO A/S, Glostrup, Denmark) were subsequently applied for 30 minutes each. DAB (Fluka, Switzerland) was used as a chromogen, and slides were lightly counterstained with Mayer's hematoxylin stain. Double-staining for CIS cells was performed incubating slides sequentially with c-KIT (rabbit polyclonal antibody, C-19, Santa Cruz Biotechnology, California) after pretreatment in a dilution 1:300 overnight at 4° C and XPA as described above. XPA was visualized using avidin-biotin-alkaline phosphatase complex with Fast blue (Sigma) as chromogen. Detection of the c-KIT specific antibody was done using the peroxidase-anti-peroxidase method with 3-amino-9-ethyl-carbazole (AEC) (Sigma) as chromogen. For a negative control, the primary antibody was omitted; lymphocytes and normal spermatogenesis (pachytene spermatocytes) as present in the histological sections under investigation were used as positive control. Two investigators (F.H. and F.M. or H.S.) assessed samples independently. For evaluation of percentage of positive cells, 200 cells were scored in three randomly selected high-power fields. Only clearly nuclear staining of intact cells was considered positive.

Western Blotting and Amplification Analysis for XPA Using FISH

Protein harvest, Western blotting procedure, and FISH were performed as previously described (Kersemaekers et al, 2002). The XPA probe for FISH was derived from BAC clone RP11–414C23. A cut-off level of ≥ 10 signals per nucleus was chosen to assess amplification of the gene locus. Only intact nuclei of a minimum of 20 cells were scored.

Cisplatin Treatment and Toxicity, Resistance Assay, and Cytotoxicity Assay

In vitro cytotoxicity was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide-(MTT-)assay (MTT-assay), which was performed according to standard protocols. One-thousand cells/well were seeded in 96-well plates 8-fold, left to attach overnight, and were exposed to cisplatin in increasing concentration continuously for further 72 hours. The cisplatin-containing medium was carefully removed, replaced by medium containing MTT (Sigma), and incubated for 4 hours at 37° C. The tetrazolium salt was dissolved in DMSO, and the extinction was measured at 570 nm on a Bio-Rad ELISA-reader. The results are expressed relative to the extinction of the cells grown in the absence of cisplatin.

Induction of Drug Resistance

Cells were treated in increasing sublethal doses of cisplatin (ranging from 0.6 μM to 2.0 μM) for 2 hours, transferred to normal medium, and grown for 2 weeks before re-exposure to cisplatin. Exposure at each dose level was repeated three times.

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CHAPTER 8

GENERAL DISCUSSION

8. GENERAL DISCUSSION

Human GCTs are fascinating tumours, as they can be regarded as naturally occurring experiments revealing aspects of both developmental and tumour biology. However, with regards to some aspects, this can also be a limiting factor, as the observed findings can be either due to the process of tumourigenesis, or simply reflect normal development. Depending on their histology, GCTs can show significant similarities with early germ cells. Comparative analysis of the tumours and representative normal foetal counterparts, both female and male, can therefore help to define events related to the pathogenesis of this cancer. In the present work, therefore, a thorough analysis of changes in the marker profile during foetal germ cell development is presented (Chapters 2, 3, 4, and 6). Furthermore, the potential of GCTs to differentiate is explored, and the surprising finding of germ cell lineage differentiation in non-seminomas is presented (Chapter 5). Finally, aspects of treatment sensitivity/resistance of GCTs, most likely reflecting the intrinsic characteristics of the cell of origin and their derivatives, are discussed (Chapters 6 and 7).

8.1. Defining normal germ cell development

The common model of germ cell development in mammals holds that embryonic precursors of the gametes known as PGCs are set aside to an extra-embryonic localization during early embryonic development, and migrate to the area where the genital ridge will be formed around the 5th and 6th week of human development (1). Mammalian germ cells are known to first originate in the proximal region of the epiblast. In mouse experiments, cells that normally never would give rise to the germ lineage can differentiate into germ cells and extra-embryonic mesoderm when grafted to the proximal epiblast (2). This indicates that germ cell differentiation, rather than being an intrinsic quality of a predetermined progenitor cell, can be induced by extracellular factors and/or cell-cell interactions (3). Which of these factors are indispensable to induce commitment of an undetermined human ES cell to germ line fate is largely unknown. Recent findings indicate that co-aggregate culture of mice ES cells on feeder cells expressing bone morphogenetic protein 4 (BMP4) greatly enhances the population of germ cells (2).

In humans, the female germ cell pool expands until the second trimester, and then begins to decline in number until birth (4). Postnatal development and expansion of female germ cells has largely been ruled out. Recently, however, this dogma has been challenged, and oocyte generation in adult ovaries in the mouse has been described (5), although this needs to be confirmed. A pool of stem cells with germline characteristics has been identified in the bone marrow and in the peripheral blood (6). Interestingly, germ cell markers and markers of pluripotency like NANOG have been isolated from human adult bone marrow and peripheral blood (7, 8, 6). This could indicate that a respective pool of

committed germline stem cells also exists in humans beyond the foetal period in extragonadal localizations.

Even though male and female foetal germ cells show a number of differences, such as the timing of the onset of meiosis, there are also remarkable similarities. In both sexes, immature germ cells (called gonocytes in males and oogonia in females) can morphologically be distinguished by their large size, big nuclei, and clear cytoplasm. These cells eventually become more mature germ cells called prespermatogonia and oocytes. In humans, the process of maturation is not synchronized, and germ cells at different developmental stages can be detected at the same time in prenatal gonads. This suggests that the individual pace of maturation is driven by unknown intracellular mechanisms, rather than by external, e.g. hormonal, stimuli (9).

In addition to mere histological criteria, a number of markers for early germ cells have been described (10 for review). These could be confirmed and further characterized by our studies (Chapters 2 and 3). In particular, the exact timing of the regulation of these markers had previously not been described. In our analyses, we could distinguish an immature and a more mature phenotype of human germ cells, both in males and females. Immature germ cells are characterized by strong expression of PLAP (placental/germ cell alkaline phosphatases), the transcriptional regulator and marker of pluripotency POU5F1 (OCT3/4), and the stem cell factor receptor KIT. Furthermore, they show weak expression of the germ line specific protein VASA. PLAP is the first factor to be down-regulated during maturation, followed by OCT3/4 and then KIT in male germ cells, whereas in female germ cells, expression of KIT remains high in oocytes and primordial follicles.

Female germ cells are characterized by strong expression of E-cadherin and β-catenin during maturation, which persists throughout folliculogenesis. It is important to note that the pattern of marker expression shows significant differences between human and mouse foetal germ cells, as has been described in our study on human foetal germ cells in females (Chapter 2). This should be kept in mind when interpreting data from animal studies, and underlines the importance of analyses on human tissue.

Male foetal germ cells lack XPA (11), show weak expression of E-cadherin, but strong expression of β-catenin (Chapter 6). They can show expression of TSPY (testis-specific protein, Y-encoded), which is detected mainly in prespermatogonia. In both sexes, the pool of immature germ cells expands through strong mitotic activity until week 22 to 24. Transition to a more mature germ cell is accompanied by stronger expression of VASA and loss of OCT3/4 in both sexes. As this transition takes place in males when the gonocyte gets attached to the basal membrane of the seminiferous tubule, and in females when the germ cells engage in folliculogenesis, a crucial role of external factors like cell-cell contacts is postulated in the course of normal germ cell maturation.

Recently, the existence of a third population of germ cells showing an intermediate phenotype has been described in males (12). Obviously, within a continuous, yet unsynchronized process of maturation, different combinations of markers will be found at different time points. However, as there

is little necessity to further subdivide maturing germ cells for biological reasons, we and others have not made this distinction (13).

8.2. Disturbed germ cell development and malignant transformation

More than 30 years after the identification of carcinoma *in situ*/intratubular germ cell neoplasia unclassified (CIS/IGCNU) as an obligatory precursor lesion in the testis (14), we are still far from having a clear concept of events mandatory in the process of malignant transformation. Impaired cell maturation and retention of pluripotent cells in a phase vulnerable to further hits has been postulated as one possible pathogenetic mechanism eventually leading to GCT development (15, 16). Chromosomal instability leading to aneuploidy with specific gains and losses has been implicated as relevant factors in malignant transformation (17 for review). Moreover, the sex chromosomes X and Y seem to play a particular role in germ cell transformation (18 for review).

Having defined normal germ cell development, we studied cells showing delayed or disturbed maturation. Several proteins, like PLAP, KIT, or OCT3/4, have been found both in normal immature and malignant germ cells, called CIS/IGCNU in the testis, and gonadoblastoma in dysgenetic gonads. We undertook an extensive immunohistochemical analysis in CIS/IGCNU and gonadoblastoma and could confirm PLAP, OCT3/4 and TSPY as suitable makers for the diagnosis of preinvasive malignant germ cells in postpubertal gonads (Chapter 4). Of these, OCT3/4 shows the clearest and most robust staining pattern and is therefore recommended as a marker for the clinical routine. Although de-differentiation and consecutive re-expression of early markers cannot be ruled out as a mechanism for development of CIS/IGCNU, 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 GCTs.

An interesting, yet little understood factor, TSPY, is worth a more detailed look. Early invasive dysgerminoma cells consistently show staining for TSPY, similar to the pattern seen for OCT3/4. In contrast, TSPY expression can be lost in invasive dysgerminoma areas at distance from the pre-invasive lesion, even though the corresponding Y-chromosomal material is still present. A similar pattern is also detected in the testis: TSPY is strongly expressed in CIS/IGCNU. Intratubular seminoma is consistently positive, whereas in invasive seminoma, like in dysgerminoma, down-regulation of TSPY can be observed. Interestingly, TSPY has previously emerged among the top 10 up-regulated genes in CIS in a microarray analysis (19), supporting the idea that this protein plays a role in the pathogenesis of early malignant germ cells. Our results are compatible with the model that, following a process of selection and consecutive clonal expansion, immature germ cells in gonadoblastoma and CIS/IGCNU expressing OCT3/4 and TSPY progress to the next pathogenetic steps, eventually leading to invasive growth. Whether the presence of TSPY is needed to maintain the germ line commitment of these cells at an early stage, or confers a growth advantage during the critical step to invasiveness, remains to be elucidated.

In addition to a better definition of the emerging phenotype of CIS/IGCNU and gonadoblastoma, light was shed on diagnostic limitations of these markers in high-risk neonates and infants. Two

observations from our analyses are particularly noteworthy in that respect. First, the presence of markers such as PLAP, KIT, and OCT3/4 is not restricted to early stages of germ cell maturation, but can still be found in neonates during the first year of life. In contrast to the situation in the testis of adolescents and adults, these markers can be unreliable for the detection of malignant germ cells in very young children. Second, gonocytes showing expression of these markers are hardly ever found on the basal membrane, but are localized more centrally in the seminiferous tubules. This distinguishes them from CIS/IGCNU cells, which are always in contact with the basal membrane and phenotypically and ultrastructurally resemble prespermatogonia. This indicates that CIS/IGCNU cells, possibly due to a maturation arrest, show expression of markers that are inappropriate regarding their localization.

Recently, two studies from our group have revealed the value of our analyses on normal germ cell development as a baseline to which germ cells in different pathological conditions can be compared. In a study of diagnostic markers in gonads of young patients with undervirilization syndromes and maturation delay of germ cells, abnormal expression of OCT3/4 and TSPY seemed of pathogenetic relevance. Position of OCT3/4 positive cells within the seminiferous tubule could be used to distinguish maturation delay from CIS/IGCNU in young patients (20). The second study revealed a serious delay in germ cell maturation in male foetuses with trisomy 21, a condition associated with a higher risk of the development of a GCT. Compared to normal foetal germ cells, prolonged expression of OCT3/4 and increased expression of TSPY were observed, adding further evidence that these factors are likely to be involved in early malignant transformation (16).

8.3. Stem cells and germ cells

The histiogenesis of GCT is a reflection of the normal processes of gametogenesis, fertilization, and finally embryonic differentiation. CIS/IGCNU have been deemed a very good example of cancer stem cells (21). On the other hand, embryonal carcinoma (EC), which is believed to be the stem cell compartment of all non-seminomas, has been seen as a “caricature” of ES cells, pluripotent cells found in the inner cell mass of the blastocyst (22 for review). Whereas the relationship of teratomas, ES cells, and EC cells has been widely studied in the mouse, the situation in humans is less clear, and for obvious ethical and legal issues, there are many experimental limitations. However, when comparing human ES and EC cell lines directly, only few differences are seen. For example, it has been speculated that the greater repertoire of cell types developing from ES cells in response to differentiation could be due to “higher levels of expression/and or a greater repertoire of *Wnt* genes” (23). EC cell lines have been believed to be more developmentally restricted than ES cells. EC cells can to contribute to all three germ layers in transplantation experiments. However, in contrast to ES cells, they fail to contribute to the germ cell-lineage in chimeric mice, which has been seen as a consequence of limitations to differentiate.

In this light, our finding of germ cell-lineage differentiation is an amazing demonstration of developmental potential (Chapter 5). For the first time, human GCT must be regarded as truly

totipotent tumours whose developmental potential is seemingly unlimited. Several aspects of that study are noteworthy. First, the strict regulation of the markers in the tumour cells at different stages of differentiation are in perfect agreement with the pattern seen during normal development (Chapter 3). Particularly interesting is the re-activation of OCT3/4, possibly as a germ cell survival factor, in yolk sac tumour cells. The fact that the maturing germ cells show down-regulation of OCT3/4 and up-regulation of VASA argues strongly against a role of OCT3/4 as a driving force in the pathogenesis of all GCT cells, as has been suggested recently (24). Furthermore, the realization of the fascinating inherent potential of these tumour cells in a competitive tumour environment casts doubts on the popular thesis that in tumours, the selection pressure of a postulated "micro-evolution" is the main defining biological factor. Rather, in GCTs, aggressive tumour growth and differentiation do not seem to be mutually exclusive. Which, in consequence, seems an argument against the mechanistic idea of interfering with pluripotency as a promising means of controlling tumour growth in human GCTs (24). On a more practical note, the finding should stipulate endeavours to cultivate germ cell like cells from pluripotent EC cell lines. If successful, such a system could provide us with valuable information on growth factor requirements of human germ cells and equip us with a readily accessible experimental tool to study differentiation events without the ethical issues and legal restrictions associated with the use of human embryos, human ES cells, or human spermatogonial stem cells.

8.4. Nest or egg: intrinsic and extrinsic factors in germ cell tumour development

Tumour development is not necessarily and uniquely the consequence of the clonal expansion of a disturbed cell in an otherwise normal organism. Alternatively, tumours can result from non-mutated pluripotent cells that are localized in an inappropriate environment, possibly lacking important growth-inhibiting signals from their neighbouring cells. In others words, in analogy to the puzzling question of "chicken or egg", the question regarding tumour development could be coined as "nest or egg", where obviously the nest is the microenvironment (sometimes referred to as the "niche") and the egg is the premalignant cell. For GCTs, it has been proven experimentally that both intrinsic and extrinsic factors have a role to play in tumourigenesis. In mouse experiments, for example, teratomas and teratocarcinomas could be obtained by ectopic transplantation of early embryos (25). Maybe more surprisingly, injection of pluripotent EC cells into a blastocyst resulted in the development of a chimeric mouse with normal tissues, derived from both the blastocyst and the EC cells (26, 27). Regarding the role of pluripotency of these tumours, nowadays easily detectable by immunohistochemistry for OCT3/4, the phenomenon of total loss of tumourigenicity of human EC cells after terminal differentiation *in vitro* is noteworthy (28, 29). This suggests that epigenetic changes, e.g. executed via promoter methylation, regulated by master switches like OCT3/4, can be sufficient to ultimately alter the histology and the biological behaviour of GCT.

In vivo, however, the situation is much less clear. One experimental problem is that CIS/IGCNU, which clinically is regarded as the earliest tumour stage, represents a relatively late stage of tumour development (30). Finding a proper model for early GCT development with no or only few

chromosomal and genetic abnormalities is a difficult task. Both the comparatively young age at presentation (31), and their genomic constitution (32) suggest that gonadoblastoma could be the earliest accessible stage in the development of a malignant germ cell tumor (Chapter 4). Interestingly, when comparing gonadoblastoma and CIS/IGCNU on the one hand and normal gonocytes on the other hand (Chapters 3, 4, 6), not one single factor, neither intrinsic, nor extrinsic, could be identified whose presence would have been unique for GCT development. Rather, the combination of markers and the localization of the suspicious cells is indicative of (pre)malignancy. Quantitatively, a stronger expression of TSPY is seen in malignant cells; however, the underlying pathogenetic mechanism remains to be elucidated.

More recently, we detected stronger staining of the KIT ligand stem cell factor (SCF) when comparing normal foetal and malignant germ cells at various stages, including gonadoblastoma, CIS/IGCNU, and invasive GCTs (Stoop & Honecker, unpublished results). SCF could in fact be one of the first significantly altered extrinsic factors in GCT development. Even though preliminary, these results are worth further investigation, as activation of the KIT signalling pathway is regarded one of the first steps affecting malignant germ cell development (32; 34, 17 for reviews).

Studying germ cell development in conditions that either affect intrinsic properties of germ cells, like chromosomal constitution in trisomy 21, or lead to altered extrinsic factors, like the inappropriate environment of ill determined stromal cells in patients with undervirilization syndromes, has unequivocally revealed delay in germ cell maturation as a common risk factor for later tumour development (20, 16). Recently, the umbrella term “testicular dysgenesis syndrome” (TDS) has been introduced, combining different testicular abnormalities like GCTs, genital malformation, undescended testis, and infertility (35). However, so far the concept is rather descriptive, and can intellectually be criticized for not differentiating between intrinsic and extrinsic factors as the real driving forces behind GCT development. Therefore, the question “nest or egg?” (or both) remains open to debate.

8.5. Differentiation and chemotherapy response

Seminomas are highly sensitive to both radiation and chemotherapy. Nonseminomas are less susceptible to radiation but show an overall high sensitivity to combination chemotherapy. This observation might be related to the finding that in general, ES cells show an efficient defense mechanisms for DNA damage, resulting in either repair or induction of apoptosis (36, 37). Loss of this embryonic feature might in fact be related to the development of treatment resistance, which occurs in about 10-30% of patients diagnosed with a metastatic nonseminomatous GCT. A number of reviews on this issue have been published recently (38, 39, 40, 41). Here, our findings on involvement of OCT3/4, E-cadherin, β-catenin (Chapter 6), and XPA (Chapter 7) in treatment resistance will briefly be discussed.

In our extensive analysis on OCT3/4 in GCTs and other tumours, we also included a series of well-characterized GCTs with known clinical outcome (42). The results indicate that the staining pattern

was strictly determined by histology, irrespective of sensitivity to chemotherapy. Therefore, existence of a pool of pluripotent cancer stem cells expressing OCT3/4 can be ruled out as a mechanism of treatment resistance in GCTs. Doubts regarding the recently proposed concept of down-regulation of OCT3/4 as a promising target in GCT treatment (24) have already been expressed above. In fact, intrinsic treatment resistance seems to be a feature of more differentiated GCTs that have lost embryonic features.

The same series has been used to assess possible involvement of E-cadherin and β-catenin in treatment resistance and sensitivity. Although expression of both factors was correlated with different histological subtypes, and was seen in nonseminomas but not in seminomas, no differences in treatment outcome could be detected. Our data suggest that detection of E-cadherin or β-catenin by immunohistochemistry has no prognostic or predictive value for patients with newly diagnosed GCTs. The overall incidence of nuclear translocation of β-catenin was low in our series. Although the factors were not co-localized in around 50 to 60% of all tumours, gross disturbances of β-catenin signalling leading to a nuclear translocation of the protein seem to be relatively rare events in GCTs, in contrast to other tumours like colorectal cancers. Two findings from that analysis, even though not relevant in the light of treatment resistance, are noteworthy from a developmental point of view. First, β-catenin was always found in pre-invasive malignant germ cells, but never in invasive seminomas, suggesting that this factor is differentially regulated in these two cell populations that otherwise seem so similar. Second, expression of β-catenin is low in undifferentiated carcinoma cells (EC cells) and is consistently up-regulated during differentiation. This is in line with findings of others, who have described changes in WNT signalling during differentiation (43, 19). Recently, regulation of E-cadherin during differentiation of CIS/IGCNU and GCTs has been described, and is in accordance with our findings (44, 45).

Finally, our series has also been examined to elucidate the role of XPA in treatment resistance in GCTs (Chapter 7). Based on data from cell lines, it has been suggested that due to low levels of XPA, GCTs are not able to efficiently repair DNA damage by activating the NER pathway (46). Recently, the same group has presented an extended analysis, showing that in addition to XPA, also ERCC1 and XPF, other factors involved in DNA repair, are reduced in testis tumour cell lines (47). In our series of GCTs of different histologies, XPA could be demonstrated immunohistochemically in the majority of tumours. Seminomas and EC components showed the lowest rate of XPA-positivity. Yolk sac tumours and choriocarcinomas were found to have positive staining in a higher number of samples, and all mature teratomas showed a strong signal for XPA. In other words, less differentiated histologies showed less XPA expression than more differentiated tumour components. No differences were detected between responding and non-responding cases. Even though GCT-derived cell lines showed a slightly weaker XPA signal than other cell lines in our analysis, the differences did not reach the extent that has been reported for different GCT cell lines. Our results cast doubt on the model that a defect in NER caused by low XPA level is solely responsible for the exquisite chemosensitivity of

GCTs. Two findings, however, could indicate a possible relation of XPA to treatment response: all mature teratomas were strongly positive for XPA. Furthermore, all seven nonseminomas that were resected in relapse after previous treatment, showed a clear XPA signal, possibly due to induction of XPA during exposure to cisplatin, or a selection of a XPA-positive subclone of tumour cells. In this context, it is noteworthy, that XPA was not induced in GCT-derived cell lines treated repeatedly with CDDP. However, given the distribution of histologic subtypes in the group of pretreated tumours, the difference between the previously treated refractory samples compared to untreated tumours of the same histology is statistically not significant. None of the investigated cases positive for XPA by immunohistochemistry harboured an amplification of the gene, which might have been expected based on earlier chromosomal analyses (48). Taken together, our data on XPA demonstrate that from a clinical point of view, differences in XPA levels detectable by immunohistochemistry have a limited impact on treatment outcome at best. Yet, XPA can be regarded as another example of a factor whose expression is regulated during differentiation of GCTs.

8.6. Main findings and future perspectives

The presented investigations add to the understanding of normal and malignant germ cell development. As the investigations presented in this thesis touch on a number of different aspects of normal and malignant germ cells, the main findings are summarized before sketching future perspectives of human germ cell biology. The results include

- a presentation of the time frame during which the regulation of selected markers of normal human foetal germ cells takes place
- a comparison of normal and malignant germ cells, highlighting OCT3/4 and TSPY as important factors in both normal and disturbed development
- the finding of germ cell lineage differentiation in nonseminomas, establishing GCTs as truly totipotent tumours
- the description of the involvement of both external and intrinsic factors in the process of malignant transformation
- the analysis of factors putatively involved in responsiveness of GCTs to chemotherapy, and which are best interpreted in view of the embryonic properties of their cells of origin

It lies in the nature of any scientific progress, that any question successfully resolved will be replaced by at least one new question, arising from what has been achieved by an investigation. To name just a few, the following questions are unanswered so far:

Does OCT3/4 expression in human germ cells serve as a factor of pluripotency, or as a survival factor? Does OCT3/4 merely influence the susceptibility of germ cells to malignant transformation, or is it a driving force behind tumour development? What is the role of TSPY in normal and disturbed germ cell development? Could forced expression of human TSPY in mouse germ cells help to establish an animal model of type II tumours, providing the urgently needed tool for the investigation of GCTs?

What is the exact role of extrinsic and intrinsic factors in malignant transformation? Or in other words, what is the contribution of the nest, and what of the egg? Could SCF be a factor that allows distinction between normal and malignant germ cell development at an early stage? Will it be possible to cultivate germ cells from pluripotent EC cells using special cell culture conditions, as has been demonstrated for ES cells?

Regarding investigations of factors involved in treatment resistance, future research should focus on whole pathways and interactions of many factors, rather than looking at isolated factors. Determining the overall pattern of cell death after exposure to cytotoxic agents will help our understanding of pathways involved in chemotherapy response. The identification of factors that play a role in chemotherapy response of GCTs should involve the use of global screening instruments, like gene microarrays, or whole proteome analyses. These investigations are currently under way, and will hopefully contribute to our understanding of these fascinating tumours over the next few years.

8.7. References

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9 SUMMARY/SAMENVATTING

Defining normal and malignant germ cell development is crucial for understanding the biology and behaviour of GCTs. Comparison of tumours and normal counterparts can help to define normal and disturbed development of germ cells. Most likely related to their cell of origin, which resembles a primordial germ cell (PGC)/gonocyte, GCTs can show significant similarities with immature germ cells. Seminomas are uniform tumours, composed of cells sharing characteristics with PGCs/gonocytes. Nonseminomas are more heterogenous and can contain one or more histologic subtypes representing various differentiation lineages and stages of normal intra-uterine development. The embryonal carcinoma cells are the pluripotent stem cell component, which have the potential to differentiate towards extra-embryonal tissues and embryonic tissues with mesenchymal, epithelial and neuronal appearance. There is sufficient evidence to hypothesize that treatment sensitivity of GCTs reflects the intrinsic characteristics of the cell of origin, and their derivatives. The aim of this thesis was to identify factors involved in the development of normal and malignant human germ cells. The particular marker profile of male and female germ cells during normal foetal development could be established, and compared to the profile found in dysgenetic gonads. The intrinsic developmental potential of nonseminomatous germ cell tumours as totipotent tumours was established. Furthermore, the role of extracellular factors like the microenvironment was addressed. Finally, links between pluripotency, cellular differentiation, and treatment response, were investigated.

By analysing formalin-fixed, paraffine-embedded material, a number of markers for early germ cells could be confirmed and further characterized in our studies (Chapters 2 and 3). In particular, we describe for the first time the exact timing of the regulation of these markers during normal germ cell maturation. We could distinguish an immature and a more mature phenotype of human germ cells, both in males and females. Immature germ cells of both sexes are characterized by strong expression of PLAP, OCT3/4, and the stem cell factor receptor KIT. Weak expression of the germ line specific protein VASA was seen in these cells. PLAP is the first factor to be down-regulated during maturation, followed by OCT3/4 and then KIT in male germ cells. In female germ cells, the same pattern can be observed for PLAP and OCT3/4, occurring at the same time during foetal development as in males (second trimester). However, expression of KIT remains high in oocytes and primordial follicles. Furthermore, female germ cells are characterized by strong expression of E-cadherin and β-catenin during maturation, which persists throughout folliculogenesis.

Male foetal germ cells show weak expression of E-cadherin, but strong expression of β-catenin (Chapter 6). They can show expression of TSPY (testis-specific protein, Y-encoded), mainly in prespermatogonia. Double-staining experiments revealed that this factor is not restricted to cells in mitosis. In both sexes, the pool of immature germ cells shows strong mitotic activity until week 22 to 24 gestational age. Human germ cell maturation is characterized by an increase in signal intensity for VASA and loss of OCT3/4 expression in both sexes. This transition takes place in males when the

gonocytes get attached to the basal lamina of the seminiferous tubule, and in females when the germ cells engage in folliculogenesis. This led us to conclude that external factors like cell-cell signalling create a niche that is essential to ensure normal germ cell development.

Using our knowledge from studies defining normal development, we turned to cells showing delayed or even disturbed maturation. An extensive immunohistochemical analysis in CIS/IGCNU and gonadoblastoma could confirm PLAP, OCT3/4 and TSPY as suitable makers for the diagnosis of preinvasive malignant germ cells in postpubertal gonads (Chapter 4). 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 GCTs. Interesting results suggest a pathogenetic role of TSPY, whose role in normal and malignant germ cell development is largely elusive at this point. Our results are compatible with the model that, following a process of selection and consecutive clonal expansion, immature germ cells in gonadoblastoma and CIS/IGCNU expressing OCT3/4 and TSPY progress to the next pathogenetic steps, eventually leading to invasive growth. Whether the presence of TSPY is needed to maintain the germ line commitment of these cells at an early stage, or confers a growth advantage during the critical step to invasiveness, remains to be elucidated.

In addition to a better definition of the emerging phenotype of CIS/IGCNU and gonadoblastoma, light was shed on diagnostic limitations in high-risk neonates and infants. It must be noted that the presence of markers such as PLAP, KIT, and OCT3/4 is not restricted to early stages of germ cell maturation, but can extend into the first year of life. Therefore, these markers can be unreliable for the detection of malignant germ cells in very young children. Of diagnostic importance could be the observation that gonocytes showing expression of early markers are hardly ever found on the basal lamina, but are localized more centrally in the seminiferous tubules. This distinguishes them from CIS/IGCNU cells, which are always in contact with the basal lamina.

An amazing finding was the detection of germ cell-lineage differentiation in nonseminomatous GCTs in a limited number of cases (Chapter 5). Human GCT must therefore be regarded as truly totipotent tumours with unrestricted developmental potential. Interestingly, the strict regulation of the germ cell markers observed in the tumour cells at different stages of differentiation was perfectly mimicking normal development. The fact that the maturing germ cells show down-regulation of OCT3/4 and up-regulation of VASA can be seen as an argument against a role of OCT3/4 as a driving force in the pathogenesis of all GCT cells. Rather, in GCTs, aggressive tumour growth and differentiation do not seem to be mutually exclusive. From a technical point of view, the finding of germ cell development in GCTs should encourage efforts to cultivate germ cell like cells from pluripotent EC cell lines. Such a system could help to characterize human germ cell development in a readily accessible cell system without the ethical issues and legal restrictions associated with the use of normal primordial germ cells derived from healthy human sources.

Finally, our investigations present findings of different factors supposedly involved in chemotherapy resistance in a series of well-characterized GCTs with known clinical outcome. Neither E-cadherin or

β-catenin (Chapter 6), nor XPA (Chapter 7) were found to be clearly associated with treatment resistance. Interesting, however, is the finding that expression of E-cadherin, β-catenin and XPA correlated with different histologies, irrespective of sensitivity of the tumours to chemotherapy. Therefore, all these proteins can be regarded as examples of factors that are differentially regulated during GCT development. This illustrates nicely that in GCTs, it is particularly important to be able to interpret results in view of the underlying aspects of developmental biology, in order to avoid misinterpretation of experimental findings.

Samenvatting

Het definiëren van normale en kwaadaardige kiemcelontwikkeling is essentieel voor het kunnen begrijpen van de biologie het gedrag van kiemceltumoren (KCT). Hierbij kan de vergelijking van tumoren en de normale tegenhangers waardevol zijn voor het beschrijven van normale en afwijkende kiemcel ontwikkeling. Vermoedelijk op grond van een gemeenschappelijke oorsprong, namelijk een primordiale kiemcel/gonocyte, kunnen KCT gelijkenis vertonen met ongedifferentieerde kiemcellen. Seminomen zijn uniforme tumoren, opgebouwd uit cellen die overéénkomsten vertonen met primordiale kiemcellen/gonocyten. Nonseminomen zijn meer heterogeen en kunnen meer dan één histologische variant bevatten. Deze vertegenwoordigen de verschillende lijnen van differentiatie in de embryonale en foetale ontwikkeling. De embryonaal carcinoomcellen worden beschouwd als het stamcelcompartiment van de nonseminomen en hebben het vermogen om zowel extra-embryonaal en embryonaal weefsel te vormen. Er zijn duidelijke aanwijzingen dat chemotherapie-gevoeligheid en – resistentie van KCT een afspiegeling zijn van de eigenschappen van de cel van oorsprong en haar afstammelingen. Het doel van dit proefschrift was het identificeren van factoren die een rol spelen in de ontwikkeling van normale en kwaadaardige kiemcellen. De karakteristieke opéénvolgende aanwezigheid van eiwit merkers voor mannelijke en vrouwelijke kiemcellen tijdens de normale ontwikkeling wordt beschreven, en dit patroon wordt vergeleken met het profiel in abnormaal aangelegde gonaden. Tevens wordt het ontwikkelingsvermogen van nonseminomen bestudeerd, en hun totipotentie wordt voor het eerst beschreven. De rol van omgevingsfactoren, zoals het micromilieu, wordt geanalyseerd. Tenslotte worden connecties tussen pluripotentie, cellulaire differentiatie en behandelingsrespons gelegd.

Met gebruik van paraffine-ingebed weefsel wordt een aantal merkers voor vroege ontwikkelingsstadia van kiemcellen bevestigd en verder gekarakteriseerd (Hoofdstukken 2 en 3). Hierbij wordt vooral de volgorde van de aanwezigheid van eiwit-merkers voor normale kiemcellen binnen de zich ontwikkelende gonaden beschreven. Deze studies maken het mogelijk om onrijpe en meer uitgerijpte kiemcellen in zowel de mannelijke en vrouwelijke gonaden te onderscheiden. In beide situeaties brengen onrijpe kiemcellen de eiwitten PLAP, OCT3/4, en de stamcel factor receptor KIT tot expressie. De desbetreffende cellen vertonen een zwakke expressie van VASA, een specifieke merker voor kiemcellen. In mannelijke kiemcellen is PLAP de eerste factor die afname in expressie laat zien, gevolgd door OCT3/4 en tenslotte KIT. Vrouwelijke kiemcellen vertonen hetzelfde patroon voor PLAP en OCT3/4, en veranderingen vinden plaats op hetzelfde tijdstip als in mannelijke gonaden tijdens de foetale ontwikkeling (tweede trimester). Anderzijds blijft de expressie van KIT hoog in oocyten en primordiale follikels. Voorts zijn vrouwelijke kiemcellen gekarakterisieerd door E-cadherin en β-catenine, die tijdens de gehele ontwikkeling tot de folliculogenese een sterke expressie tonen.

Mannelijke fetale kiemcellen laten een zwakke expressie van E-cadherin en een sterke expressie van β-catenine zien (Hoofdstuk 6). Daaréntegen vertonen ze een wisselende expressie van TSPY (testis-specific protein, Y-encoded), meestal in prespermatogonia. Dubbelkleuringen bewijzen dat deze factor niet alleen beperkt is tot cellen die in deling (mitose) zijn. In beide geslachten tonen onrijpe kiemcellen een sterke mitotische activiteit tot en met week 22 tot 24 van de zwangerschap. Rijping van kiemcellen gaan gepaard met een versterking van het signaal van VASA en het verlies van OCT3/4. Deze overgang vindt bij de mannelijke ontwikkeling plaats op de tijdstip wanneer de gonocyten terechtkomen op de basaallamina van de zaadbuisjes, en bij de vrouwelijke ontwikkeling in het begin van de folliculogenese. Dit leidt tot de veronderstelling dat uitwendige factoren zoals cel-cel contacten essentieel zijn om de juiste mico-omgeving voor een normale ontwikkeling van kiemcellen te vormen.

Met de verkregen kennis omtrent de regulatie van de normale ontwikkeling worden gonaden met een vertraagde of gestoorde rijping bestudeerd. Een uitgebreide studie van immuunhistochemische merkers op carcinoma in situ/intratubular germ cell neoplasia unclassified (CIS/IGCNU) toont de waarde van PLAP, OCT3/4 en TSPY aan als diagnostische merkers voor het aantonen van de preinvasieve *in situ* letsels in postpubertaire gonaden (Hoofdstuk 4). De resultaten zijn in overeenstemming met het model dat rijpingsremming één van de eerste stappen is in de ontsporing van kiemcellen op weg naar een KCT. De resultaten betreffende TSPY zijn met name interessant en suggereren een belangrijke rol in de pathogenese van KCT, hoewel tot nu toe weinig bekend is over de mogelijke mechanisme. Een model is dat als gevolg van selectie en klonale expansie onrijpe kiemcellen in gonadoblastoom en CIS/ITGCNU, die OCT3/4 en TSPY tot expressie brengen, uiteindelijk progressie tot een invasief groeipatroon plaats vindt. Of de aanwezigheid van TSPY in deze cellen noodzakelijk is om een kiemcel-differentiatie te bepalen, ofwel een groeivoordeel tijdens de evolutie van de (nog) niet invasieve cellen oplevert moet worden onderzocht.

Op grond van een betere definitie van het fenotype van CIS/IGCNU en gonadoblastoom, wordt een diagnostische dilemma bij neonaten een jonge patiënten aangetoond. Hierbij moet benadrukt worden dat expressie van merkers zoals PLAP, KIT en OCT3/4 niet beperkt is tot vroege ontwikkelingsstadia van kiemcellen, maar tevens in het eerste jaar na de geboorte aanwezig kunnen zijn. Om die reden kunnen de merkers onbetrouwbaar zijn in het diagnostiseren van kwaadaardige kiemcellen in jonge patiënten. In dit verband is het feit dat normale gonocyten die nog expressie van de vroege merkers vertonen bijna nooit op de basaallamina gelocaliseerd zijn, maar in een meer centrale positie in de buis aangetroffen worden, van diagnostische waarde. Dit onderscheidt deze cellen van CIS/IGCNU, die per definitie in contact zijn met de basaallamina.

Een verbazingwekkende observatie was de detectie van kiemceldifferentiatie in enkele gevallen van nonseminomen (hoofdstuk 5). Op grond van deze bevinding moeten humane KCT van nu af aan als echte pluripotente tumoren (dus met een onbeperkt ontwikkelingspotentiaal) worden beschouwd. Opmerkelijk was de strikte opéénvolgende expressie van kiemcelmerkers in de tumorcellen die een

perfecte afspiegeling vormden van het normale proces. Het gegeven dat tijdens uitrijping van deze kiemcellen verlies van OCT3/4 plaats vindt en een toename van VASA, kan gezien worden als argument tegen de gedachte dat OCT3/4 een drijvende rol speelt in de pathogenese van deze tumoren. Een agressieve tumorgroei en differentiatie in KCT is niet wederzijds uit te sluiten. Vanuit een biotechnologisch perspectief zou deze observatie inspanningen aanmoedigen om kiemcellen uitgaande van pluripotente KCT cellijnen te kweken. Dit zal kunnen leiden tot een model systeem voor het bestuderen van de humane kiemcel ontwikkeling, zonder de ethische en legale beperkingen in verband met het gebruik van primordiale kiemcellen, afkomstg van humane embryo’s of humane ES cellen.

Ten slotte presenteren Hoofdstukken 6 en 7 de resultaten van studies op factoren die een mogelijke rol spelen in het mechanisme van chemotherapie-resistentie. De analyses zijn uitgevoerd op tumoren van goed gedefinieerde patiëntenpopulaties. Het expressiepatroon van zowel E-cadherin als β-catenine (Hoofdstuk 6) als ook XPA (Hoofdstuk 7) geeft geen duidelijke relatie met resistentie. Het is interessant dat E-cadherin, β-catenine en XPA wel verschillende expressiepatronen vertonen in de verschillende histologische subgroepen van KCT, onafhankelijk van chemotherapie-gevoeligheid. Deze eiwitten zijn in feite voorbeelden van factoren die differentieel gereguleerd worden tijdens KCT ontwikkeling. Dit is een informatief voorbeeld dat bij het interpreteren van resultaten van studies aan KCT altijd de ontwikkelingsbiologie betrokken moet worden om misinterpretaties te vermijden.

10 Abbreviations

BCRP	breast cancer resistance protein
BMP4	bone morphogenetic protein 4
CDKN1A	cyclin-dependent kinase inhibitor 1A (p21)
CDKN1B	cyclin-dependent kinase inhibitor 1B (p27)
CDKN2A	cyclin-dependent kinase inhibitor 2A (p16)
CDKN2B	cyclin-dependent kinase inhibitor 2B (p15)
CDKN2C	cyclin-dependent kinase inhibitor 2C (p18)
CDKN2D	cyclin-dependent kinase inhibitor 2D (p19)
CIS/IGCNU	carcinoma *in situ*/intratubular germ cell neoplasia unclassified
DNA	deoxyribonucleic acid
EC cell	embryonal carcinoma cell
ES cell	embryonic stem cell
GCT	germ cell tumor
GST	glutathione S-transferase
KCT	kiemceltumor
LRP	lung resistance protein
MDR	multidrug resistance
MMR	DNA mismatch repair
MRP1/2	multidrug resistance related protein
PGC	primordial germ cell
PLAP	placental/germ cell alkaline phosphatases
POU5F1	POU domain, class 5, transcription factor 1 (OCT3/4)
RB	retinoblastoma gene protein
RNA	ribonucleic acid
TDS	testicular dysgenesis syndrome
TP53	tumor protein p53
TSPY	testis-specific protein, Y-encoded
SCF	stem cell factor
XPA	xeroderma pigmentosum complementation group A protein

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12 CURRICULUM VITAE

Name:	Friedemann Honecker
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Undergraduate education

1980 - 1989	Hohenstaufengymnasium Göppingen
1976 - 1980	Elementary School in Wäschenbeuren and Göppingen

Medical school

1995 - 1999	Berlin Free University Medical School, Germany
1998	Auckland University Medical School, New Zealand McGill University Medical School, Canada
1994 - 1995	University of Newcastle Medical School, Great Britain
1991 - 1994	University of Lübeck Medical School, Germany

Internship/Residency:

01/2005 - present	Internal Medicine Department of Oncology, Hematology, Bone Marrow Transplantation and Pneumology, University of Hamburg Medical Center, Germany
04/2004 - 12/2004	Internal Medicine Department of Oncology, Hematology, Imunology and Rheumatology, University of Tübingen Medical Center, Germany
02/2002 - 03/2004	Research Fellow, Department of Pathology Laboratory for Experimental Patho-Oncology, Josephine Nefkens Institute, Erasmus MC, University Medical Center Rotterdam, Daniel den Hoed Cancer Center, The Netherlands supervisor: Prof. Dr. L Looijenga
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13 LIST OF PUBLICATIONS

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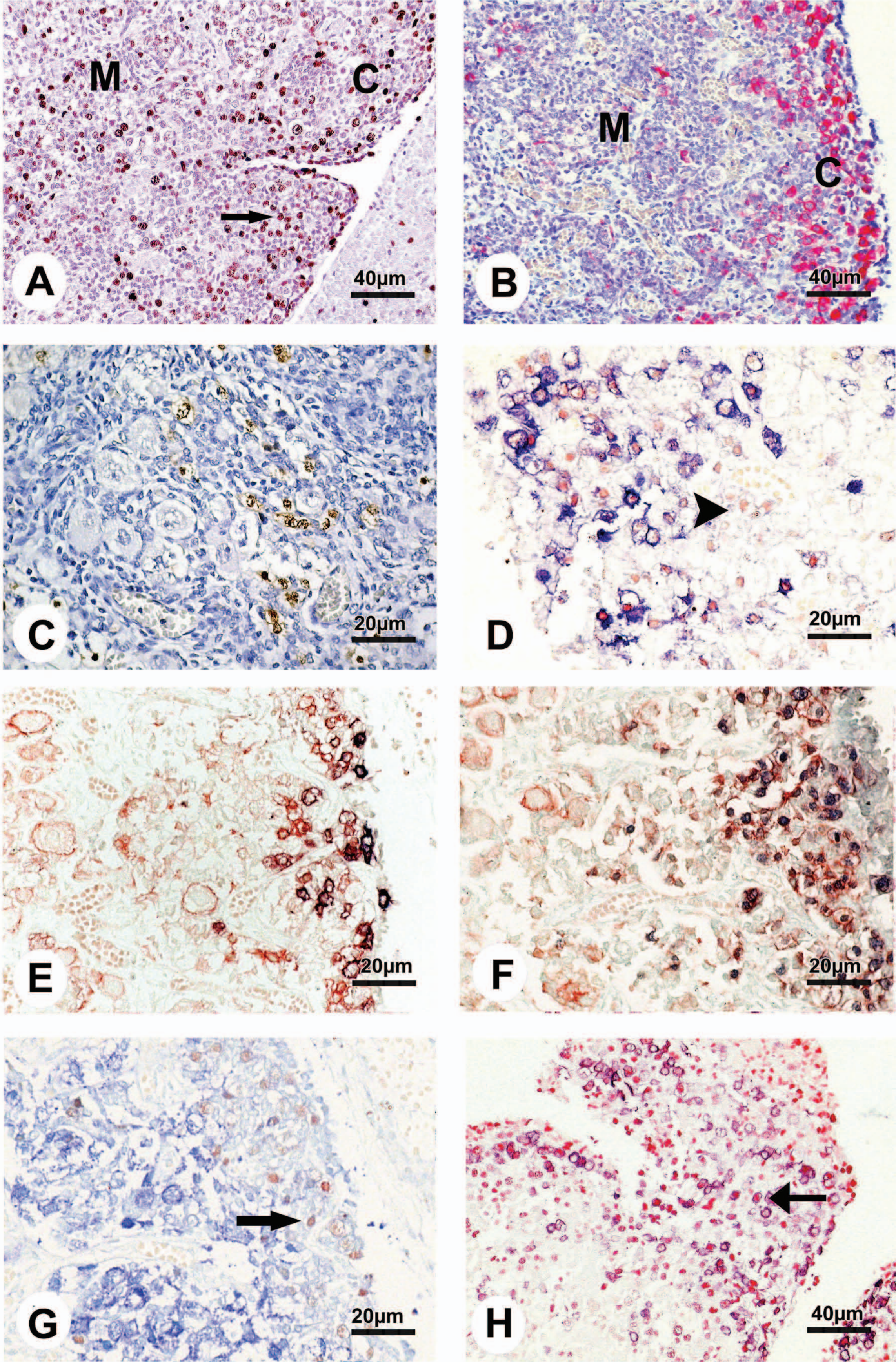
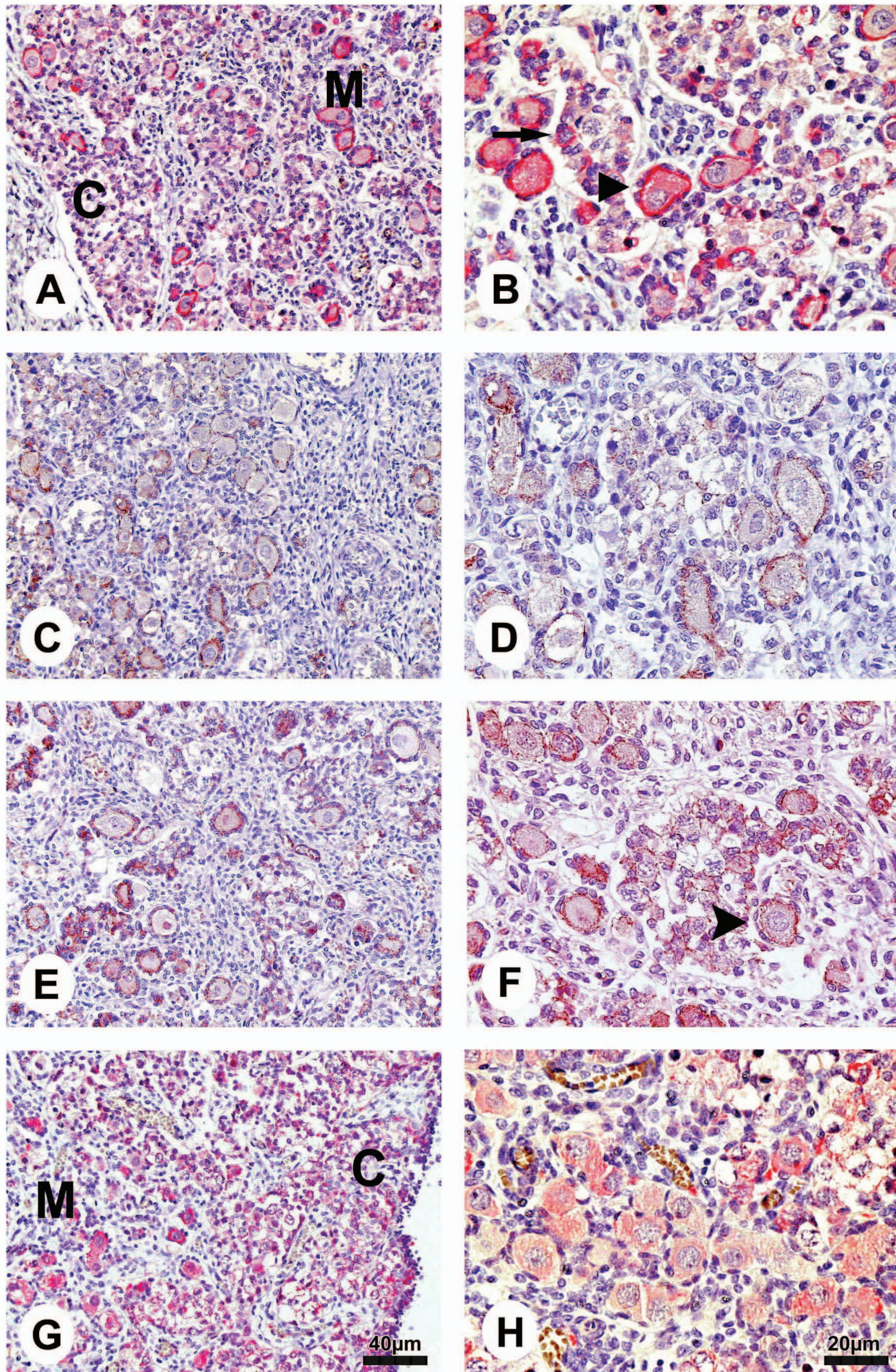
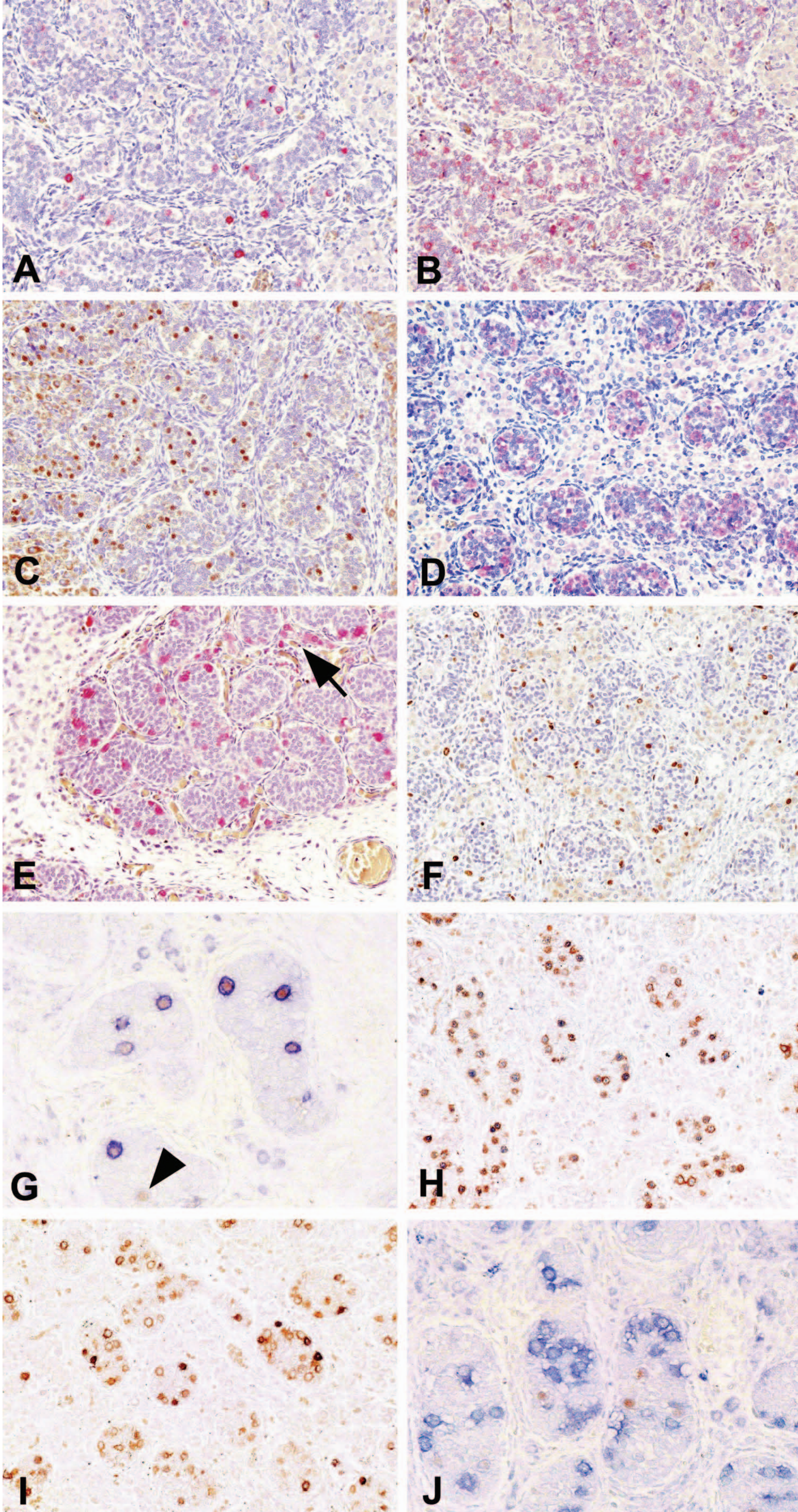
Chapter 2: Figure 2



Chapter 2: Figure 3



Chapter 3: Figure 1



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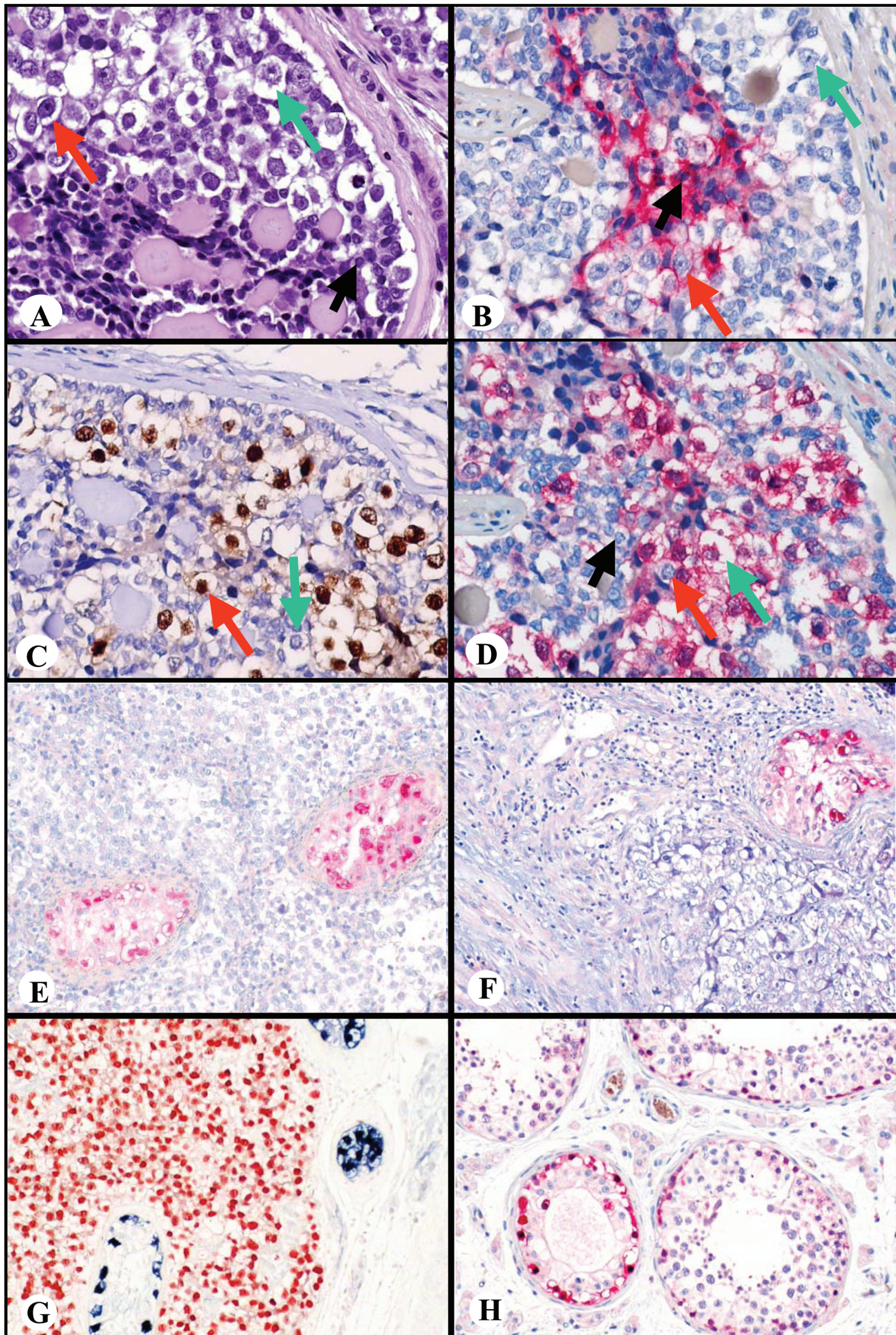

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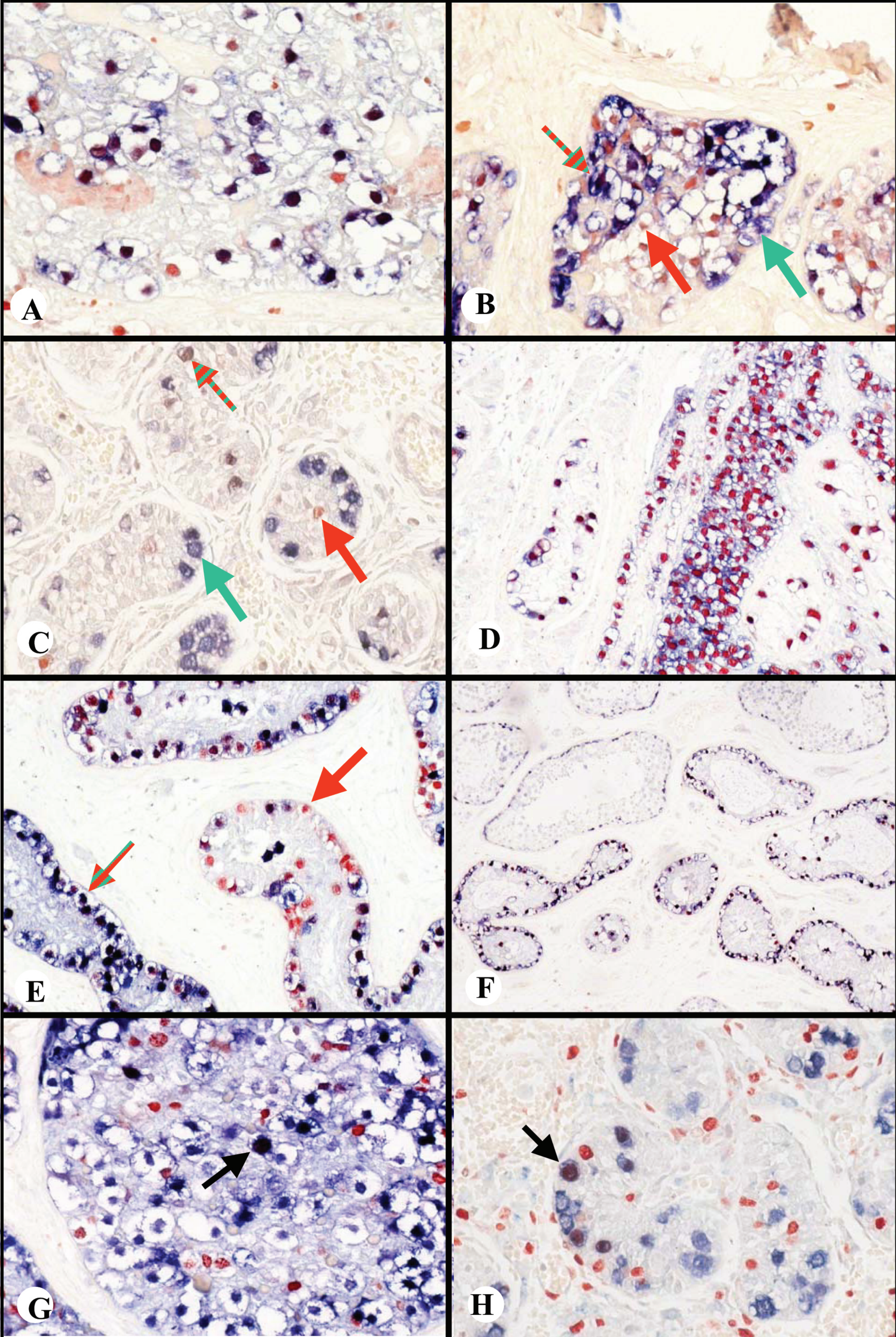

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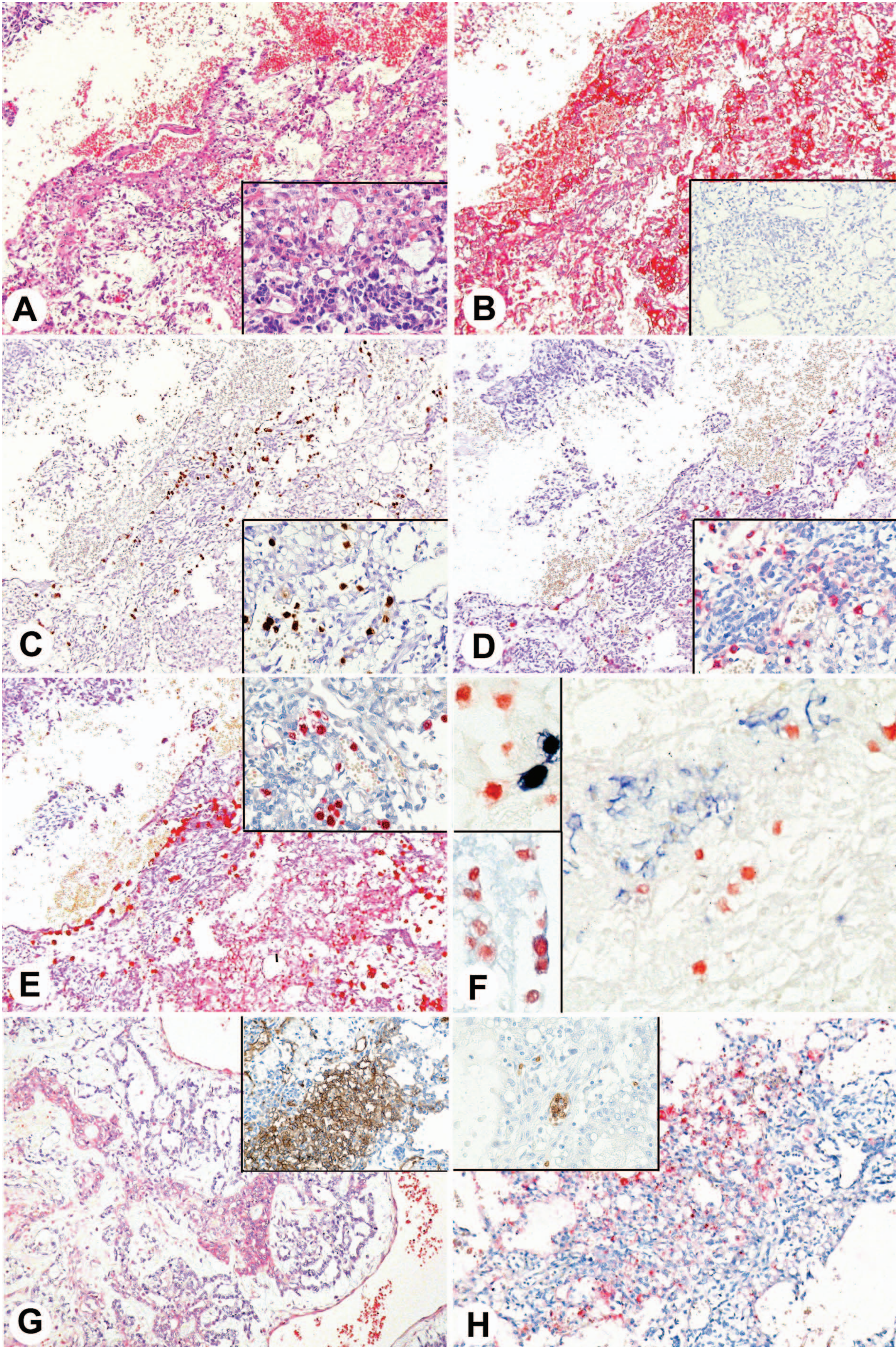
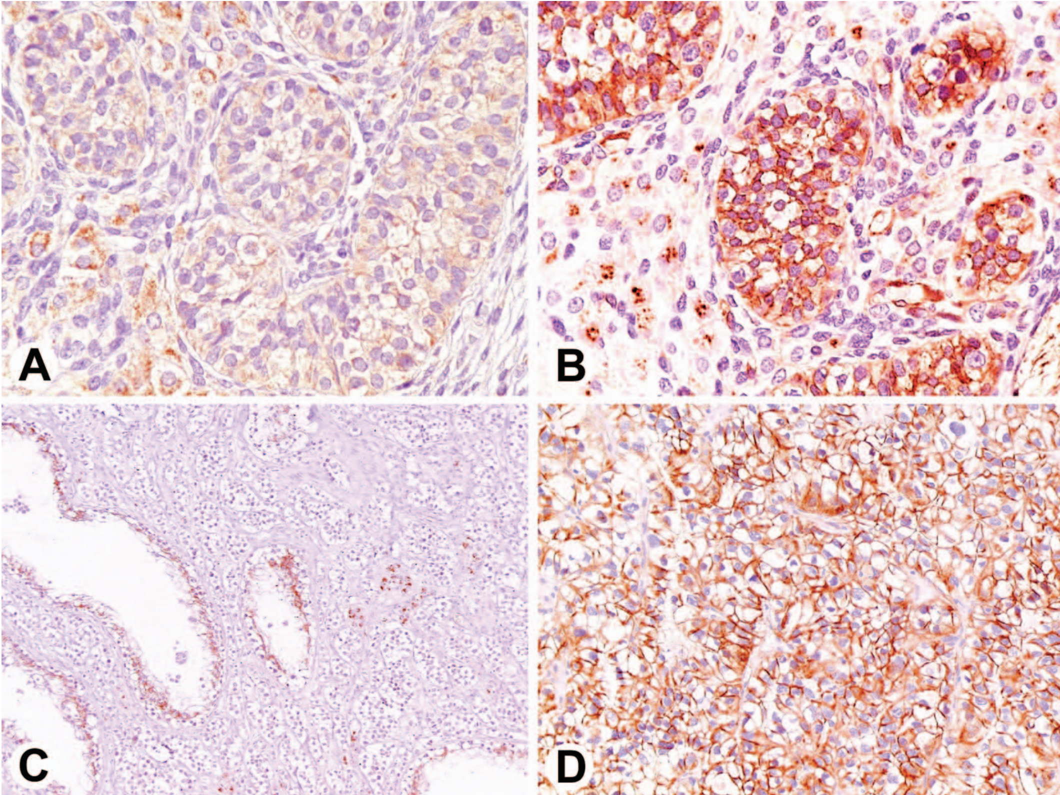


Chapter 6: Figure 1



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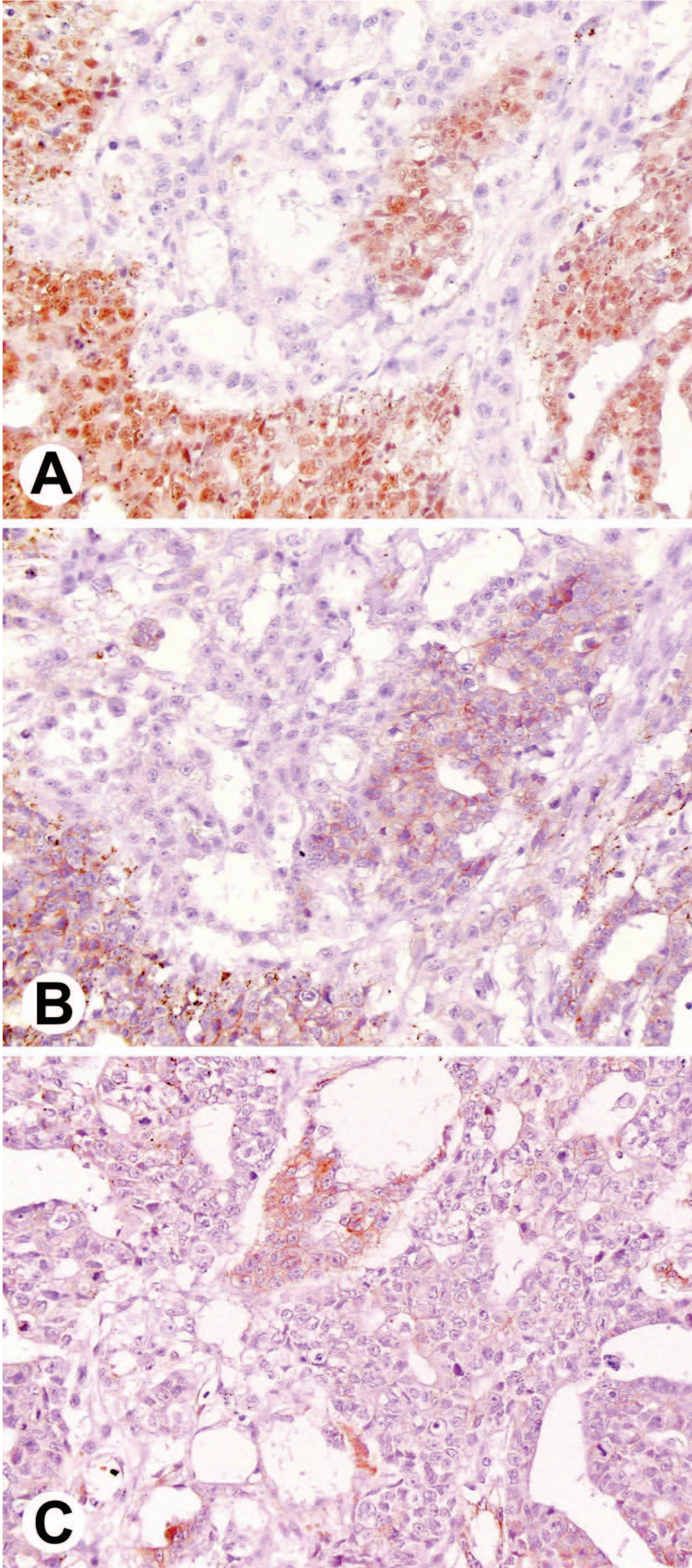

Chapter 7: Figure 1