Germ cell tumors in patients with disorders of sex development

Risk factors, initial developmental stages and targets for early diagnosis

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PhD thesis

Germ Cell Tumors in Patients with Disorders of Sex Development

Risk factors, initial developmental stages and targets for early diagnosis

Kiemceltumoren bij patiënten met stoornissen in de geslachtsontwikkeling:

Risicofactoren, initiële stadia en kenmerken voor een vroege diagnose

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Abbreviations

AFP: alpha1-fetoprotein

AIS: androgen insensitivity syndrome

AMH: anti-Müllerian hormone

CAIS: complete androgen insensitivity syndrome

CH: choriocarcinoma

CIS: carcinoma *in situ*

c-KIT: gene encoding the stem cell factor receptor which has tyrosine kinase activity

DG: dysgerminoma

DHT: dihydrotestosterone

DSD: disorders of sex development

EC: embryonal carcinoma

ESC: embryonic stem cell

FSH: follicle stimulating hormone

FISH: fluorescent *in situ* hybridization

GA: gestational age

GB: gonadoblastoma

GBY: gonadoblastoma region on the Y chromosome

GD: gonadal dysgenesis

hCG: human chorionic gonadotrophin

HE: hematoxylin-eosin

HSD: hydroxy steroid dehydrogenase

ITGNU: intra tubular germ cell neoplasia unclassified

LH: luteinizing hormone

non-FISH: non-fluorescent *in situ* hybridization

OCT3/4: octamer binding transcription factor 3/4, involved in the regulation of pluripotency, also referred to as POU5F1

PAIS: partial androgen insensitivity syndrome

PBL: peripheral blood

PCR: polymerase chain reaction

PGC: primordial germ cell

PLAP: placental/germ cell alkaline phosphatase

SIDS: sudden infant death syndrome

TE: teratoma

TDS: testicular dysgenesis syndrome

TGCT: testicular germ cell tumors

TSPY: testis specific protein – Y encoded

UGT: undifferentiated gonadal tissue

YST: yolk sac tumor

WHO: world health organization

Definitions

Complete gonadal dysgenesis / Pure gonadal dysgenesis: Normal female phenotype (without Turner stigmata) in the presence of bilateral streak gonads (devoid of germ cells) in a 46,XX or 46,XY individual.

Disorder of sex development: A congenital condition in which development of chromosomal, gonadal or phenotypic sex is atypical.

Dysgenetic male pseudohermaphroditism: (Mostly ambiguous) phenotype in the presence of bilateral dysgenetic testes.

Gonadal dysgenesis (GD): Incomplete or defective formation of the gonads, as a result of a disturbed process of migration of the germ cells and/or their correct organization in the fetal gonadal ridge.

Hypervirilization: The presence of ambiguous or male external sexual characteristics in a 46,XX individual, exposed to androgens (endogenous, due to genetic defects in enzymes involved in adrenal steroid hormone production or of exogenous origin) during fetal life or thereafter.

Incomplete gonadal dysgenesis / Partial gonadal dysgenesis: (Mostly ambiguous) phenotype in the presence of bilateral dysgenetic testes or a dysgenetic testis on one side and a streak on the other side.

Intersex: A condition of incomplete or disordered genital or gonadal development leading to a discordance between genetic sex, gonadal sex, and phenotypic sex.

Mixed gonadal dysgenesis: (Mostly ambiguous) phenotype resulting from the presence of a dysgenetic testis on one side and a streak on the other side.

True hermaphroditism: (Mostly ambiguous) phenotype in the presence of both testicular tissue consisting of seminiferous tubules, and ovarian tissue, containing germ cells that are all enclosed in primordial and eventually growing follicles in the same individual, either in a single gonad or in opposite gonads.

Undervirilization: The presence of an ambiguous or female phenotype in a 46,XY individual with bilateral (intra-abdominal, inguinal or scrotal) well-differentiated testes, in whom testosterone production or action is inadequate.

1

Introduction

1.1 Type II germ cell tumors

Germ cell tumors are often considered as a heterogeneous group of neoplasms - arising in a multitude of morphological variants at different anatomical sites of the human body, in men as well as in women, and covering a widespread age range - apparently they share only one common characteristic: a primitive germ cell as their cell of origin (1-3). Recently, a classification of these tumors into five major entities, based mainly on the biological characteristics of the originating germ cell (maturation stage and status of genomic imprinting), has been proposed (see (2) for a review) (Table 1-1). This thesis focuses on the origin and earliest developmental stages of the so-called Type II germ cell tumors, further on in this text simply referred to as germ cell tumors. They occur in the gonads and at specific extragonadal sites along the midline of the body: the anterior mediastinum (the thymus) and the pineal and hypothalamic-hypophyseal regions of the brain. These locations probably reflect the original migration route of the germ cells during early embryogenesis (see below) (4, 5), however, a stem cell origin for these extragonadal tumors is not excluded (6).

Although sharing a common precursor cell with similar biological characteristics, invasive germ cell tumors are divided into two major groups that are distinct from each other histologically and with regard to outcome: the seminomatous germ cell tumors, called differently according to their location (seminoma in the testis/dysgerminoma in the ovary and the dysgenetic gonad/germinoma in the brain) and the non-seminomatous germ cell tumors (2). Seminomas are homogeneously composed of pluripotent malignantly transformed early germ cells whereas the undifferentiated non-seminomatous embryonal carcinoma cells have the capacity to differentiate further into various extra-embryonal and embryonic tissue components. It is currently believed that seminomas can transform into non-seminomas by a process of chromosomal loss, also known as activation of pluripotency (see below) (3, 7), although this hypothesis was questioned recently (8).

Table 1-1: The five types of germ cell tumors (reprinted with permission from (2))

Type	Anatomical site	Phenotype	Age	Originating cell	Genomic imprinting	Genotype	Animal model
I	Testis/ovary/ sacral region / retroperitoneum/ mediastinum/ neck/midline brain/other rare sites	(Immature) teratoma/ yolk-sac tumor	Neonates and children	Early PGC/ gonocyte	Biparental partially erased	Diploid (teratoma), Aneuploid (yolk-sac tumor) : gain of 1q, 12(p13), and 20q, loss of 1p, 4 and 6q	Mouse teratoma
II	Testis	Seminoma/ non-seminoma	> 15 y (median: 35 and 25 y)	PGC/ gonocyte	Erased	Aneuploid (+/- triploid) : gain of X, 7, 8, 12p and 21, loss of Y, 1p, 11, 13 and 18	Not available
	Ovary	Dysgerminoma/ non-seminoma	> 4 y	PGC/ gonocyte	Erased	Aneuploid	Not available
	Dysgenetic gonad	Dysgerminoma/ non-seminoma	Congenital	PGC/ gonocyte	Erased	Diploid/tetraploid	Not available
	Anterior mediastinum (thymus)	Seminoma/ non-seminoma	Adolescents	PGC/ gonocyte	Erased	Diploid/tri-tetraploid	Not available
	Midline brain (pineal gland/ hypothalamus)	Germinoma/ non-seminoma	Children (median age 13 y)	PGC/ gonocyte	Erased	Diploid/tri-tetraploid	Not available
III	Testis	Spermatocytic seminoma	> 50 y	Spermatogonium /spermatocyte	Partially complete paternal	Aneuploid : gain of 9	Canine seminoma
IV	Ovary	Dermoid cyst	Children/ adults	Oogonia/oocyte	Partially complete maternal	(Near) diploid, diploid/tetraploid, peritriploid (gain of X, 7, 12 and 15)	Mouse gynogenote
V	Placenta/uterus	Hydatidiform mole	Fertile period	Empty ovum/ spermatozoa	Completely paternal	Diploid (XX and XY)	Mouse androgenote

Details on the classification of germ cell tumors can be found in the cited literature
y: years; PGC: primordial germ cell

1.2 Epidemiology of testicular germ cell tumors

95% of all germ cell tumors occur in the testis and are therefore referred to as testicular germ cell tumors (TGCT). Although overall a rare disease (the incidence is estimated at 6 - 11/100 000 (9), accounting for about 2% of all neoplasms in men (10)), a TGCT is the most commonly diagnosed neoplasm in young men and its incidence has shown a remarkable rise in Caucasian populations over the last decades (3, 11). However, the incidence of TGCT differs approximately 10-fold between European countries, with Denmark and Switzerland revealing the highest incidence numbers. Afro-Americans have a significantly lower risk than Americans of Caucasian origin and an increasing trend in the incidence of TGCT has been observed for a long time only in the latter group (11). However, morbidity rates for ethnic Africans living in the United States are higher than for those living in their nature countries (12), and recent data point at a rise in the incidence of TGCT specifically in Afro-Americans over the last decade (13). Among many theories, in addition to a genetic predisposition,

increased exposure to estrogens during fetal life is commonly mentioned as a determining etiologic factor (3, 12, 14-16) (see below).

1.3 Precursor lesions and progression towards invasiveness

1.3.1 Intra tubular germ cell neoplasia unclassified and progression-related changes

In the testis, seminomas as well as non-seminomas are always preceded by an *in situ* neoplastic precursor lesion, the so-called intra tubular germ cell neoplasia unclassified (ITGNU) also known as carcinoma *in situ* (CIS) (17, 18). Long term follow-up data suggest that the presence of ITGNU, if left untreated, will invariably result in the development of an invasive TGCT (19). ITGNU consists of a clonal expansion of early germ cells within the seminiferous tubule, more precisely on the basal lamina, where normally the spermatogonia reside, meanwhile the nuclei of Sertoli cells are displaced luminally (3, 20).

An ITGNU cell shares many biological (genomic imprinting), morphological (size, aspect) and immunohistochemical (expression of specific markers) characteristics with a normal early germ cell, i.e. the primordial germ cell or gonocyte, and is therefore believed to be its neoplastic counterpart. Numerous epidemiological data support the theory that the neoplastic transformation of an ITGNU cell is related to pathogenetic events during fetal life (see below) (2, 18, 21-25). The study of the genetic contents of ITGNU cells, seminomas and non-seminomas has led to the proposal of a model for the further progression of ITGNU towards invasiveness. Germ cells residing in an insufficiently supportive environment are delayed/blocked in their maturation at a pre-meiotic stage, which eventually allows continuous mitotic proliferation (see below) (25, 26). These developmentally delayed germ cells are prone to step-wise aneuploidisation and the occurrence of specific chromosomal aberrations, most likely involving (proto-) oncogenes and tumor suppressor genes. One of the earliest events in this process is polyploidisation of the germ cells, resulting in a (near) tetraploid DNA content of the ITGNU lesion. Subsequently, a consistent pattern of chromosomal gains and losses has been reported, mostly affecting the chromosomes 7, 8, 12, 17q and X (gains) and the chromosomes 4, 5, 11, 13, 18 and Y (losses) (7, 24, 27), thus leading to further aneuploidisation. The most consistent chromosomal gain that was

observed so far, i.e. the gain of 12p, most often by the formation of an isochromosome i(12p), represents the last step of the ITGNU lesion towards invasiveness (7, 24, 27, 28). The resulting seminomas, like the ITGNU cells are hypertriploid, non-seminomas, by further chromosomal loss, are hypotriploid (7, 22, 29-32).

A subgroup of patients with disorders of sex development (DSD), namely patients with undervirilization syndromes, reveals a high incidence of ITGNU, rapidly progressing towards invasiveness (around or soon after puberty). Therefore, a prophylactic gonadectomy is usually advised in these patients at the time of diagnosis or at the onset of puberty (33-38).

An *in situ* precursor lesion was hitherto not demonstrated for ovarian or extragonadal germ cell tumors. However, dysgerminomas of the ovary are tetraploid or aneuploid and show a comparable pattern of chromosomal gains and losses as TGCT, including the gain of 12p (27, 39-41). Though polyploidisation does not seem to be an essential step in the formation of extragonadal germ cell tumors, gain of 12p was also demonstrated at these sites (2, 27).

1.3.2 Gonadoblastoma and progression-related changes

It is widely accepted that patients with DSD and the presence of Y chromosomal material in their karyotype are at increased risk for the development of malignant germ cell tumors, namely seminomas/dysgerminomas and non-seminomatous tumors. Specifically in dysgenetic gonads, a precursor lesion which is morphologically different from the ITGNU lesion, the gonadoblastoma (GB), precedes the development of invasive tumors (33, 35, 42-46). GB is histologically defined as a tumor composed of two principal cell types: large germ cells similar to those of seminoma and small cells resembling immature Sertoli and granulosa cells; elements resembling Leydig or lutein-like cells may also be present (1). Thus, GB are *in situ* neoplastic lesions that are essentially composed of the same cell types as can be found in testicular tubules containing ITGNU, i.e. immature germ cells and Sertoli cells, but they are not organized within the context of a seminiferous tubule. Our actual knowledge regarding the chromosomal patterns and ploidy status of GB and the resulting dysgerminoma is limited and is hampered by the co-presence of relatively high numbers of non-malignant germ cells and Sertoli/granulosa cells within the GB lesion. Although tetraploidy has been described (47), GB are mostly found to be diploid. However, while progressing towards invasiveness,

they become aneuploid, with again the amplification of (parts of) 12p, eventually via the formation of an isochromosome i(12p), as a consistent finding (39, 40, 48).

1.4 Normal gonadal development

To gain insight into the pathogenetic mechanisms underlying DSD and tumor formation in this specific patient population, a profound understanding of the normal development of the genital system in males and females is required, and will therefore be described in detail in the following paragraphs.

The various precursor organs for sexual development in the developing embryo are bipotential, meaning that they contain the intrinsic capability to evolve either along the male or female pathway (49). In mammals, the accurate expression of the Y chromosomal gene *SRY* actively interferes with the intrinsic tendency of the bipotential sex organs to feminize, via the activation of a cascade of sex-determining genes. Thus, the genetic sex (the presence or absence of the Y chromosome) determines the gonadal sex (the development of the bipotential gonad into a testis or an ovary). The timely production of hormones by the male fetal gonad and their adequate action are essential to establish a concordant male phenotypic sex (masculinization of internal and external sexual characteristics). If not, the phenotypic sex of the embryo will be female (49, 50).

1.4.1 The bipotential gonad

The bipotential gonad develops as a thickening of the coelomic epithelium, in close contact with the kidney and the primitive adrenal. In humans, genes that have been clearly shown to be essential for the stabilisation and outgrowth of the undifferentiated gonad are *SF1* and *WT1*, in mice additional genes involved at this stage have been identified (*Lim1, Lhx9, M33, Emx2, Igf1r/Ir/Irr*) (49-51). A detailed overview of genes involved in the stabilisation of the bipotential gonad and in male and female gonadal differentiation (see below) is provided in Table 1-2. For background information, the reader is referred to the references cited in the text.

Table 1-2: Update of the actually identfied genes involved in human gonadal sex differentiation, adapted from (47, 49-52)

Gene	Function	Human gene locus	Human phenotype	Mouse phenotype
BIPOTENTIAL GONAD				
WT1	TF	11p13	Heterozygous mutation in exons encoding zinc finger motifs: Denys-Drash syndrome (including gonadal dysgenesis or undervirilization); heterozygous mutation in splice site junction with loss of *+KTS* isoform: Frasier syndrome (including gonadal dysgenesis); deletion: WAGR syndrome	Null: absence of gonads and kidneys
SF1	TF	9q33	Heterozygous missense mutation: XY sex reversal + adrenal insufficiency; XX: adrenal insufficiency with apparently normal ovaries	Null: absence of gonads and kidneys
DAX1	TR	Xp21.3	Mutation: adrenal hypoplasia + hypogonadotrophic hypogonadism (normal testicular differentiation in XY), duplication: XY gonadal dysgenesis	XY null: infertile male; XX null: ovarian development; duplication: XY female
LIM1	TF	11p12-p13	Renal + gonadal developmental defects, brain abnormalities*	Null: absence of gonads and kidneys
LHX9	TF	1q25-q31	Gonadal developmental defects*	Null: gonadal agenesis
EMX2	TF	10q26.1	Schizencephaly (heterozygous mutation), no urogenital abnormality described yet	Null: absence of gonads and kidneys, brain abnormalities
MALE DEVELOPMENT				
SRY	TF	Yp11.3	Mutation: XY gonadal dysgenesis; translocation to X: XX male	Mutation: XY fertile female; translocation to X or autosome: XX male
SOX9	TF	17q24	Haploinsufficiency: campomelic dysplasia + gonadal dysgenesis (~ 70%); duplication: XX male	Null: campomelic dysplasia + gonadal dysgenesis; duplication: XX male
DMRT1	TF	9p24.3	Haploinsufficiency: gonadal dysgenesis in XY and XX, associated with mental retardation and craniofacial abnormalities in 9p24.3→pter deletion	Null: normal male sex differentiation with postnatal loss of germ cells and Sertoli cells
ATRX	Helicase	Xq13.3	Deletions and mutations: XY gonadal dysgenesis or undervirilization, mental retardation and α-thalassemia	Not determined
DHH	SM	12q13.1	Homozygous mutation: XY gonadal dysgenesis + minifascicular neuropathy	Null: XY gonadal dysgenesis
10q-	?	10q25.3-qter	Undervirilization, multiple congenital anomalies	Not determined
M33	TF	17q25	XY gonadal dysgenesis*	Null: XY gonadal dysgenesis, Sox9 reduced
FGF9	SM	13q11-q13	XY gonadal dysgenesis + lung defects*	Null: XY gonadal dysgenesis + lung abnormalities
FEMALE DEVELOPMENT				
WNT4	SM	1p35	Duplication: XY undervirilization; mutation: XX hyperandrogenism + Mayer-Rokitansky syndrome (absence of Müllerian derivatives)	Null: XX absence of Müllerian ducts, development of Wolffian ducts, testosterone synthesis
FOXL2	TF	3q23	Mutations: premature ovarian failure + BPES	Deletion: XX male (goat)

* Presumed phenotype, no genetic defect has been described in humans yet
TF: transcription factor; TR: transcription repressor; SM: signaling molecule; WAGR: Wilms' tumor aniridia genitourinary anomalies and mental retardation; BPES: blepharophimosis ptosis epicanthus inversus syndrome

The bipotential gonad consists of four different cell types: 1) the precursors for Sertoli and granulosa cells 2) the precursors for the steroid producing Leydig and theca cells 3) supporting cells and 4) germ cells, arriving in the developing gonad around the fourth and fifth week gestational age (GA) (see below) (50).

1.4.2 Origin and migration of primordial germ cells

Most studies concerning early germ cell development and migration are performed in mice and extrapolated to the human situation. Primordial germ cells (PGC) are not derived from the bipotential gonadal primordium but originate from pluripotent epiblast cells, under the control of extraembryonically expressed genes (*BMP4/BMP8b*) (4, 50). In a 24-day old human embryo, they can be recognized by their positive staining for placental/germ cell alkaline phosphatase (PLAP) or octamer binding transcription factor (OCT3/4) (2, 5, 50). From the proximal epiblast, PGC move to the yolk sac, and then, in the fourth and fifth week GA, they migrate both passively and actively, under precise control mechanisms along the midline, from the yolk sac through the hindgut, and leave the hindgut at its dorsal side to migrate laterally towards the developing genital ridges (Figure 1-1). Several genes play an important role in the survival and migration of the PGC at this stage (among them are the tyrosine kinase receptor *c-KIT* and its ligand *Steel factor/ stem cell factor*). PGC that do not reach the genital ridges timely are killed by Bax-related apoptosis (56) (for a review see (4, 5)). It seems reasonable to hypothesize that extragonadal germ cell tumors derive from ectopic PGC that failed to reach the gonadal ridges but escaped these apoptotic mechanisms (2, 27). However, other possibilities are not excluded (6).

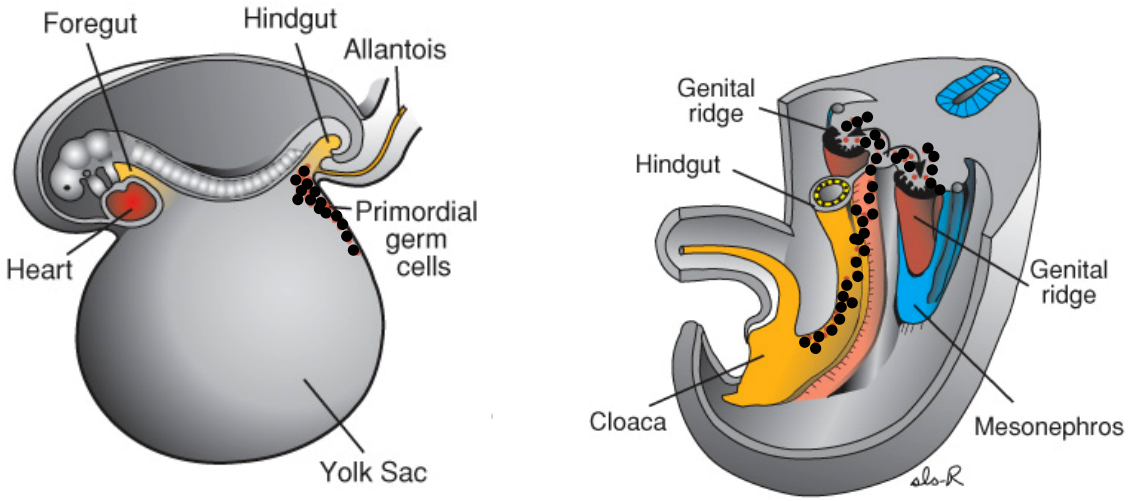

Figure 1-1: Migration route of the primordial germ cells. Left: Location of the primordial germ cells prior to migration into the embryo, at the base of the allantois within the yolk sac. Right: Migration route of the primordial germ cells through the embryo via the hindgut into the developing genital ridges. Adapted from (55).

Once germ cells reach the genital ridges, around the fifth or sixth week GA, and although their biological characteristics do not change at that time, they are referred to as gonocytes (2, 50). The further differentiation of the bipotential gonad into testis or ovary determines the evolution of the germ cells into the male or female direction, irrespective of their own karyotype (57).

1.4.3 Testis differentiation

Testis differentiation is initiated by the expression of the *SRY* gene, located on the distal part of the short arm of the Y chromosome (Yp) (59). The expression of *SRY* is controlled by upstream genes, among them are those encoding the transcription factors WT1+KTS (one of the two isoforms of WT1 gene product), GATA4 and FOG2 (49). Gonadal sex determination is not accomplished by the action of SRY. In contrast, SRY acts as a transcription factor, initiating the expression of a complex cascade of downstream genes, controlling and fine-tuning testicular differentiation by activating and inhibiting mechanisms and subtle gene-dosage effects (Table 1-2 and Figure 1-2) (49-52, 54). Immediately downstream of *SRY* and following shortly after its expression, *SOX9* is upregulated in the male gonad; in fact, the activation of SOX9 might even be the only target for SRY (49). *Sox9* is the main sex-determining gene in many vertebrates, contains a highly conserved DNA-binding domain which is very similar to the DNA-binding domain of *SRY* and can functionally substitute *SRY* expression (49-51).

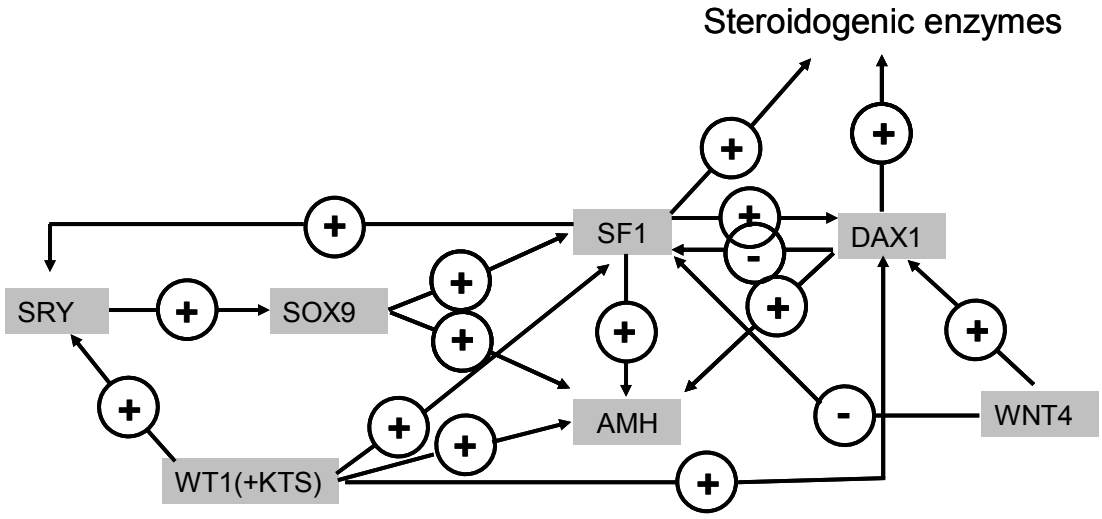

Figure 1-2: Possible model for the interaction of different genes involved in male sex differentiation

The expression of SRY is confined exclusively to the Sertoli cell precursor lineage (60), and is possibly reinforced by unknown paracrine stimuli (49). It is believed that the testis-determining cascade initiates first pre-Sertoli cell and later also interstitial cell proliferation, thereby increasing the expression of testis promoting genes, with the purpose of reaching a critical threshold that is able to irreversibly canalize the chosen male pathway. The next step is Sertoli cell differentiation, for which also a precisely regulated dosage of DAX1, downstream of or in parallel with the SRY - SOX9 tandem is important (49, 61). The primitive Sertoli cells orchestrate further testis development, requiring migration of the precursors for Leydig cells, peritubular myoid cells and endothelial cells from the mesonephros and the formation of a testis-specific vasculature. All the prerequisites are now present to accomplish the formation of testicular cords, the later seminiferous tubules, which represents the first microscopically visible sign of testicular differentiation around six weeks GA (49, 50, 62-64).

During fetal life, Sertoli cells produce hormones (Anti Müllerian Hormone (AMH) and Inhibin), and nurture the germ cells, possibly preventing them from entering meiosis (see below). Steroid synthesis by Leydig cells starts around nine weeks GA (50).

The immature germ cells reside in the centre of the developing seminiferous tubule. While maturing, they progress towards the basal lamina. Once in contact with the basal lamina, and during the whole fetal and postnatal period of testicular development prior to spermatogenesis, they are termed prespermatogonia (65, 66). In the spermatogonial niche they undergo a series of mitotic divisions in the fetal and early postnatal period, and remain then quiescent until puberty, when spermatogenesis starts (50, 65). It was suggested that the prespermatogonia are prevented from entry into meiosis by the action of a local factor, secreted by the Sertoli cells (67), whereas their survival is supported by the paracrine action of Insulin-like Factor 3 (INSL3), secreted by the Leydig cells, which binds on the LGR8 receptor (leucine-rich repeat-containing G-protein-coupled receptor 8), expressed in the germ cells (68, 69).

Around the seventh month GA the testis starts to descend into the scrotum (65). This process is considered to occur in two stages, comprising a transabdominal and a transinguinal phase (70). The mechanisms controlling testicular descent are still not fully elucidated. The gubernaculum is a ligamentous structure connecting the inferior pole of the testis to the base

of the scrotum. Its development is under the control of the INSL3/LGR8 complex (68, 69) and is thought to be a major actor in the process of testicular descent (71, 72). Among others are mentioned an increase in intra-abdominal pressure, other hormonal stimuli from the testis and the placenta (testosterone, AMH), the development of epididymis, vas deferens and testicular vessels and neural stimuli from the genitofemoral nerve (72). Based on studies in mouse models and in children with the androgen insensitivity syndrome (AIS, see below), it is currently believed that androgens are of major importance in the second, transinguinal phase of testicular descent, whereas the first, transabdominal phase is relatively androgen-independent, but is mainly controlled by the action of INSL3 and AMH (70, 71).

1.4.4 Ovarian differentiation

The bipotential gonad has an intrinsic tendency to evolve along the female pathway in the absence of testis promoting signals. However, at present it is believed that proper ovarian development does not only require the presence and survival of germ cells (in contrast to testicular development), but also the correct activation of its own ovarian promoting genes (49). The WNT signalling molecule WNT4, in concordance with its downstream target DAX1, prevents invasion of the developing female gonad by mesonephric cells (the precursors for Leydig cells, peritubular myoid cells and endothelial cells), and interferes with the formation of the testis-specific vasculature. Furthermore, WNT4 is essential for the stabilisation and development of the Müllerian tract (see below) (49, 50, 63, 73-75). FOXL2 is involved in follicle formation in human ovaries (49). Additional genes, involved in murine ovarian development have been identified (*Figla, Bmp2, Fst* (49, 63, 76).

Whereas in the male, the first morphological changes, i.e. the formation of testicular cords, are visible around six weeks GA, in the female embryo, no apparent changes occur in the bipotential gonad until the 12th week. At that time, the actively proliferating germ cells, termed oogonia at this stage, enter meiosis and are then referred to as oocytes. They proceed to the various stages of the first meiotic division and are arrested at the diplotene stage, terminating the first meiotic division only at the time of ovulation, under the influence of LH induced INSL3 expression in ovarian theca cells (50, 65, 69, 77, 78). The presence of meiotic oocytes is required for the differentiation of pre-follicular cells into follicular cells. In case meiosis fails to occur or oocytes are lost, pre-follicular cells degenerate, resulting in the

formation of a streak gonad (50). Folliculogenesis (the formation of primordial follicles) begins around the 15th - 16th week GA, whereas primary follicles are visible from the 20th week on. Around birth, virtually all oocytes are enclosed in follicles and all the different stages of follicular development are present in normal ovaries (65, 77, 79). The oocyte population and follicle formation in the ovaries reaches a maximum of 6 to 7 million around 20 weeks GA, and is followed by a rapid degeneration of oocytes and follicles from then onwards, whereas the formation of new oogonia in the ovarian cortex ceases around 7 months GA. This results in a pool of several hundred thousand follicles at birth, gradually decreasing thereafter (50, 65).

1.4.5 Development of the male and female genital ducts

In the mammalian embryo, anlagen for the male as well as the female genital ducts (the Wolffian and the Müllerian ducts respectively), both structures derived from the mesonephros, have developed around the seventh week GA (50). In mice, several genes involved in both murine Wolffian and Müllerian duct development have been identified (*Pax2, Lim1, Emx2*) (80).

In males, regression of the Müllerian duct and differentiation of the Wolffian duct requires the action of two different hormones. One of the earliest functions of the primitive Sertoli cell is the production of AMH which promotes regression of the Müllerian duct during the 9th to 12th week GA; however, this structure is only sensitive to AMH action between this narrow time interval (50). The AMH induced regression of the Müllerian duct occurs in cranio-caudal direction via programmed cell death. AMH receptors, exclusively located on the Müllerian duct mesenchyme, transfer the apoptotic signal to the Müllerian epithelial cells presumably via paracrine actors, although the exact mechanisms remain to be elucidated (50, 80). A physiologic role for AMH in males after regression of the Müllerian duct is yet unknown (50). In females, AMH is not expressed prenatally (81), but has been shown to be important in regulating follicle recruitment and responsiveness of growing follicles to follicle stimulating hormone (FSH) by inhibitory effects (82).

Stabilisation and differentiation of the Wolffian duct into epididymis, vas deferens and seminal vesicles is AMH independent, but occurs under the influence of testosterone, secreted by the fetal Leydig cells and acting on receptors located on the Wolffian duct

mesenchyme. Again, the signal is transferred to the epithelial cells by fibroblast growth factors and other mediators (50).

In females, in the absence of testosterone, the Wolffian ducts degenerate, and in contrast to males, the Müllerian ducts develop irrespective of the presence of a (functional) ovary or the action of hormones (50, 80). It is widely accepted that the Müllerian ducts give rise to the oviducts or Fallopian tubes, the uterus and the upper part of the vagina, although recently it was suggested that the entire vagina might derive from the Müllerian ducts (80, 83). The uterus and vagina are formed by fusion of both Müllerian ducts at the posterior region (80). Essential for the stabilisation and further development of the Müllerian duct is the action of WNT4; mutations in *WNT4* or in its downstream target *DAX1* cause absence of Müllerian duct derivatives in females (74, 75, 80, 84). In mice, several other genes (*Wnt7a, Hoxa10, Hoxa11, Hd, Ovo1*) were shown to be additionally involved in the further differentiation of Müllerian derived structures (80).

1.4.6 Establishment of external sexual characteristics

Until eight weeks GA, the urogenital sinus in males and females is identical and has the capacity to differentiate in either direction. It is separated from a common cloaca during early embryogenesis and contains the urogenital slit, the genital tubercle, the urethral folds and the labioscrotal swellings. The enzyme 5α-reductase that converts testosterone into dihydrotestosterone (DHT) is expressed in the urogenital sinus already before testosterone is even produced by the primitive testis. The androgen receptor has a 10-fold greater affinity for DHT as compared to testosterone and its binding to DHT results in a more stable complex. Since levels of the locally produced DHT are much higher than those of testosterone, the development of the urogenital primordia is exclusively induced by DHT action (50, 71).

In males, binding of DHT to the androgen receptors of the urogenital sinus causes growth of the genital tubercle, which develops as a penis; fusion of the urethral folds to form the corpus spongiosum and the penile urethra, fusion of the labioscrotal swellings to form the scrotum and the ventral epidermal covering of the penis and differentiation of the prostate and bulbourethral glands. Furthermore, it inhibits growth of the vesicovaginal septum (see below).

Complete masculinization of the external genitals is accomplished at 14 weeks GA, however, penile growth continues after that period. Since the genital tubercle consists of corpora cavernosa and glans, the distinction between a penis and a clitoris is primarily based on size and whether the urethral folds have fused to form the corpus spongiosum (50).

As for the genital duct, in the absence of androgens, the urogenital sinus has an intrinsic tendency to feminize. The genital tubercle develops as a clitoris, the urethral folds form the labia minora and the labioscrotal swellings give rise to the labia majora. Proliferation of the vesicovaginal septum pushes the vagina posteriorly so that the urinary and vaginal openings become separated. Proper vagina formation additionally requires the contact and interaction of the urogenital sinus with the fused Müllerian structures (50).

The sensitivity of the urogenital sinus to DHT action is, except for the genital tubercle, limited to a critical time period between eight and 12 weeks GA. This is - at least in part - due to downregulation of the androgen receptors in the urethral folds and labioscrotal swellings after this period. Therefore, in females, exposure to androgens after this critical period can only result in clitoral hypertrophy and not in fusion of the labia minora and majora (50).

1.5 Mechanisms of disturbed gonadal development

In recent years it has become apparent that many disorders involving the male reproductive tract and male fertility reveal an increasing incidence in many Western countries. Moreover, mainly on the basis of epidemiological data, the etiology of these different disorders has been linked to disturbances in gonadal development during fetal life. This finding led the group of Skakkebæk and co-workers to the hypothesis that these different disorders have a common origin which affects fetal gonadogenesis and might represent the various symptoms of a single underlying syndrome, which he termed the “testicular dysgenesis syndrome” (TDS) (85, 86).

The origin of the ITGNU lesion already during fetal life has been explained above. The rising incidence of TGCT (see above) has been attributed to prenatal exposure to (xeno)estrogens and antiandrogenic pollutants (12, 15, 87), low or high birth weight (14, 15, 88), neonatal jaundice, high placental weight and high maternal socio-economic status (88), increased maternal age and primiparity (both resulting in higher circulating estrogen levels) (14).

Cryptorchidism, i.e. the incomplete descent of the testis into the scrotum, is associated with an increased incidence for the development of TGCT and shows a rising incidence over the last decades (3, 9, 14, 15, 33, 88-92). As stated above, testicular descent, which starts around the seventh month GA relies partially on the action of testicular hormones (INSL3, AMH, testosterone) and fails to occur in situations of diminished or absent testosterone action, e.g. in the AIS (71). The development of the testicular gubernaculum (see above), is disrupted in mice with a target deletion of *Insl3*, on the other hand, it was shown that prenatal exposure to estrogens downregulates this gene (85). Suboptimal fetal growth and prematurity also interfere with testicular descent (14, 91). Additionally, the occurrence of TGCT in spite of early orchidopexy of the cryptorchid testis led to the suggestion that both disorders may result from a common underlying pathogenetic mechanism early in life (3, 14, 89-91).

Hypospadias - the ectopic position of the urethral opening on the ventral surface of the penis or on the scrotum - results from incomplete fusion of the urethral folds. An increasing trend in the incidence of hypospadias was observed in the USA and in several European countries (93-95). Apart from mutations in genes involved in male reproductive tract development and androgen production or action, the occurrence of hypospadias has been linked to high maternal age and primiparity, paternal subfertility, low birth weight, prenatal exposure to estrogens and the presence of environmental pollutants with an antiandrogenic effect (94). Moreover, the occurrence of hypospadias is overrepresented in series of men with testicular cancer (85).

A decrease in sperm counts and semen quality has been observed over the past five decades, specifically in countries with a high incidence of TGCT (96, 97). Moreover, poor semen quality, sub- or infertility and impaired Leydig and Sertoli cell function are frequently diagnosed in men with TGCT, suggesting a common etiology (98, 99). Low birth weight has also been associated with subfertility (100).

The "TDS" links this variety of epidemiological observations to a disturbed testicular development as a common etiologic factor in genetically predisposed individuals: under the influence of yet unknown "endocrine disruptors" - environmental pollutants with estrogenic or antiandrogenic properties that are ubiquitously present in small amounts - the normal

development and function of the primitive Sertoli and Leydig cells are impaired. As a consequence, Sertoli cells are unable to induce proper and timely germ cell maturation and differentiation, eventually resulting in impaired spermatogenesis and subfertility in adult life. Leydig cells produce decreased amounts of testosterone, thereby increasing the risk for incomplete testicular descent (resulting in cryptorchidism) and incomplete fusion of the urethral folds (resulting in hypospadias) (85, 87). A developmentally delayed germ cell, by the prolonged expression of survival factors such as c-KIT, may be prone to neoplastic transformation and give rise to ITGNU and overt cancer later in life. Intersex disorders, characterized by a high incidence of germ cell tumors, are considered as the most pronounced form of the "TDS" (26, 85, 101).

In our view, this model is powerful in correlating in a logic way a broad variety of observational data, but needs yet confirmation in an experimental setting. Also, to what extent maturation delay of germ cells is the common end result of impaired Sertoli cell function in different disorders and how it is precisely related to neoplastic transformation of germ cells remains to be examined. Why the male fetus, continuously bathing in estrogens of placental origin, and with significant levels of circulating estrogens throughout pregnancy is - according to this model - particularly sensitive to microdoses of xenoestrogens is unclear at present. An experimental rat model has recently been proposed as a suitable model to further test the "TDS" hypothesis (102).

1.6 Disorders of sex development (Intersexuality)

The sex to which an individual belongs comprises four different aspects: the genetic sex (the presence or absence of the Y chromosome) determines the gonadal sex (the differentiation of the bipotential gonad into a testis or an ovary). The production and action of hormones by the functional gonad leads to the establishment of the phenotypic sex (the development of unambiguous male or female external sexual characteristics) in concordance with the genetic and gonadal sex. At puberty, the development of sex-specific secondary sexual characteristics confirms the established sex and reinforces the existing sexual dimorphism between individuals (50). The behavioural sex or sexual identity adds a fourth dimension to the complex process of human sex development (103).

"Intersex is a condition of incomplete or disordered genital or gonadal development leading to a discordance between genetic sex, gonadal sex, and phenotypic sex" (104). Together, DSD form a complex entity, of heterogeneous etiology, and affecting the four different dimensions of sex development. Many classification systems try to offer a comprehensive overview (50, 104). From a clinical as well as a pathologic point of view, the distinction between disorders of gonadal dysgenesis (GD), affecting the level of gonadal sex on the one hand, and syndromes of hypervirilization and undervirilization, affecting the level of phenotypic sex on the other, seems to be the most valuable and will therefore be used for the purpose of this thesis. However, it is important to realise that a given phenotype can be the manifestation of very different underlying conditions and that the same (genetic or other) defect can result in a broad variety of clinical presentations.

In the following paragraphs, hypothetical examples of patients will be presented to illustrate the various forms of intersex conditions.

1.6.1 Gonadal dysgenesis

GD is defined as an incomplete or defective formation of the gonads, as a result of a disturbed process of migration of the germ cells and/or their correct organization in the fetal gonadal ridge. It is caused by structural or numerical anomalies of the sex chromosomes or by mutations in one of the genes involved in the formation of the urogenital ridge and in sex determination of the bipotential gonad (see Table 1-2) (50, 52, 54, 105-107).

A typical patient with GD presents as an unambiguously phenotypic girl, referred to the pediatric endocrinologist during adolescence for delayed puberty (characterized by the absence of breast development, menstruation and growth spurt, whereas pubic and axillary hair growth is present). A diagnostic work-up reveals hypergonadotrophic hypogonadism, a 46,XY karyotype, intra-abdominal streak gonads, Fallopian tubes, a uterus and a vagina. The underlying defect in this hypothetical patient could be a mutation in the *SRY* gene. The bipotential gonad failed to differentiate as a testis. The absence of testosterone (and DHT) and AMH caused involution of the Wolffian ducts, proper differentiation of Müllerian structures, and the development of female external sexual characteristics, whereas the gonads failed to descent. Germ cells were unable to survive in this inappropriate environment, resulting in Sertoli/granulosa cell death and the formation of a streak. The

physiologic rise in gonadotrophins at puberty was not followed by “ovarian” estrogen production, however, adrenal steroid production led to the development of secondary sexual characteristics.

It is however important to realize that the phenotype and gonadal differentiation pattern of a patient with GD can be anywhere on the axis connecting the two opposite poles of normal male and female development. The patient’s karyotype can be 46,XY (containing a mutation in one of the different genes involved in gonadal sex differentiation, see Table 1-2), or it can contain a structural or numerical anomaly of the sex chromosomes, e.g. mosaicism for a Y bearing cell line, eventually only detectable in the gonad and not in peripheral blood (the peripheral blood (PBL) karyotype being 46,XX in that case).

1.6.2 Undervirilization

Undervirilization is characterized by the presence of an ambiguous or female phenotype in a 46,XY individual with bilateral (intra-abdominal, inguinal or scrotal) well-differentiated testes, in whom testosterone production or action is inadequate. Undervirilization syndromes are caused by errors in testosterone or DHT biosynthesis (due to enzymatic defects), by testicular unresponsiveness to stimulation from the pituitary (due to the absence or impaired function of the LH receptor on the Leydig cells) or by defects in androgen-dependent target tissues (due to androgen receptor defects) (see Table 1-3, Figure 1-3 and Figure 1-4) (50, 54).

A typical undervirilized patient presents as an unambiguously phenotypic girl, referred to the pediatric endocrinologist during adolescence for delayed puberty (characterized this time by breast development in the absence of pubic and axillary hair growth and without menstruation). A diagnostic work-up reveals normal or high testosterone levels, a 46,XY karyotype, bilateral inguinal testes, absence of Fallopian tubes and uterus, absent Wolffian duct derivatives and a blind ending short vagina. The underlying defect in this hypothetical patient could be a completely inactive androgen receptor (complete androgen insensitivity syndrome, CAIS). During embryonic life, bilateral testes developed in this patient and adequately produced hormones. AMH caused involution of the Müllerian ducts, however, due to the androgen receptor defect, testosterone binding is impossible or fails to induce signalling. Thus, Wolffian duct derivatives degenerated, and no external virilization occurred.

The transabdominal testicular descend, mainly testosterone independent, took place but the transinguinal descend was interrupted. At puberty, under the influence of increasing gonadotrophins, testosterone production rose, followed by the conversion of testosterone into estradiol (P450 aromatase action), hence the noticed breast development.

However, again it is of major importance to realize that the phenotype of an undervirilized individual can be anything between the normal female phenotype, as is the case in CAIS, and male, as is the case for example in subtle partial androgen receptor defects (PAIS). Of note is also that some enzymatic defects can be overcome at puberty, inducing severe virilization of a phenotypically female individual at that time (see Table 1-3) (50).

1.6.3 Hypervirilization

Hypervirilization is characterized by the presence of ambiguous or male external sexual characteristics in a 46,XX individual, exposed to androgens (endogenous, due to genetic defects in enzymes involved in adrenal steroid hormone production or of exogenous origin) during fetal life or thereafter.

Table 1-3: Identified genes involved in male phenotypic sex differentiation (adapted from (47). Some of these genes also play a role in female phenotypic sex differentiation.

Gene	Function	Gene locus	Human phenotype
AMH	AMH	19p13.3	46,XY: persistent Müllerian duct syndrome, cryptorchidism
AMH type2 receptor	AMH receptor	12q13	46,XY: persistent Müllerian duct syndrome
hCGβ	β subunit of hCG	19q13.32	Not reported, probably lethal
hCG/LH receptor	hCG/LH receptor	2p21	46,XY: undervirilization
StAR	Enzyme (cholesterol transport)	8p11.2	46,XY: undervirilization, adrenal insufficiency 46,XX: progressive postpubertal gonadal failure, adrenal insufficiency
CYP11A1	Enzyme (P450scc)	15q23-24	46,XY: undervirilization, late onset adrenal insufficiency
HSD3B2	Enzyme (3β-HSD2)	1p13	46,XY: undervirilization, adrenal insufficiency 46,XX: clitoromegaly, adrenal insufficiency (CAH)
AR	Androgen receptor	Xq11-12	46,XY: undervirilization
SRD5A2	Enzyme (5α-reductase)	2p23	46,XY: undervirilization (only external), virilization at puberty
HSD3B1	Enzyme (3β-HSD1)	1p13	Not reported, probably lethal (HSD3B1 is expressed in placenta)
CYP17	Enzyme (P450c17: 17α-hydroxylase and 17, 20 lyase)	10q24-25	46,XY, 17α-hydroxylase deficiency: undervirilization, hypertension 46,XY, 17, 20 lyase deficiency: undervirilization 46,XX, 17α-hydroxylase deficiency: hypertension, primary gonadal failure 46,XX, 17, 20 lyase deficiency: not reported, probably primary gonadal failure
HSD17B3	Enzyme (17β-HSD3)	9q22	46,XY: undervirilization, virilization at puberty
DHCR7	Enzyme (3β-HSR: cholesterol synthesis)	11q12-13	46,XY: Smith-Lemli-Opitz syndrome, severe form, lethal 46,XX: Smith-Lemli-Opitz syndrome

AMH: anti Müllerian hormone; hCG: human chorionic gonadotrophin; StAR: steroidogenic acute regulatory protein; scc: side chain cleavage; HSD: hydroxy steroid dehydrogenase; CAH: congenital adrenal hyperplasia; HSR: hydroxy steroid reductase

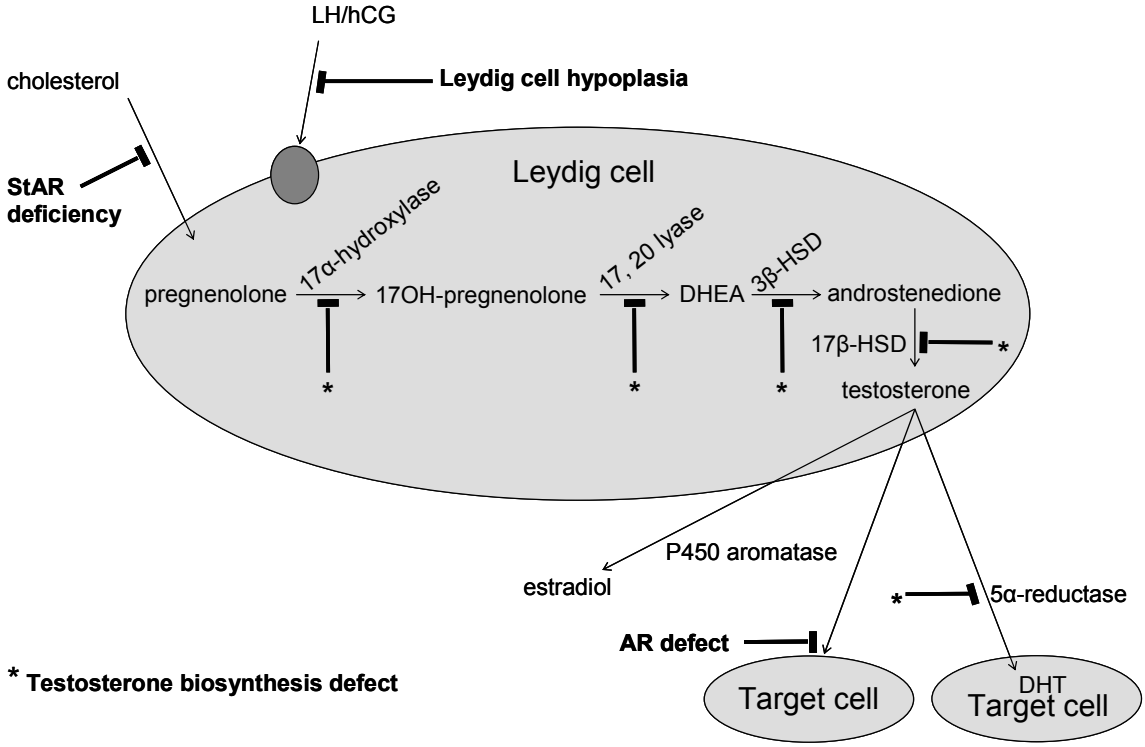

Figure 1-3: Schematic representation of testicular androgen synthesis

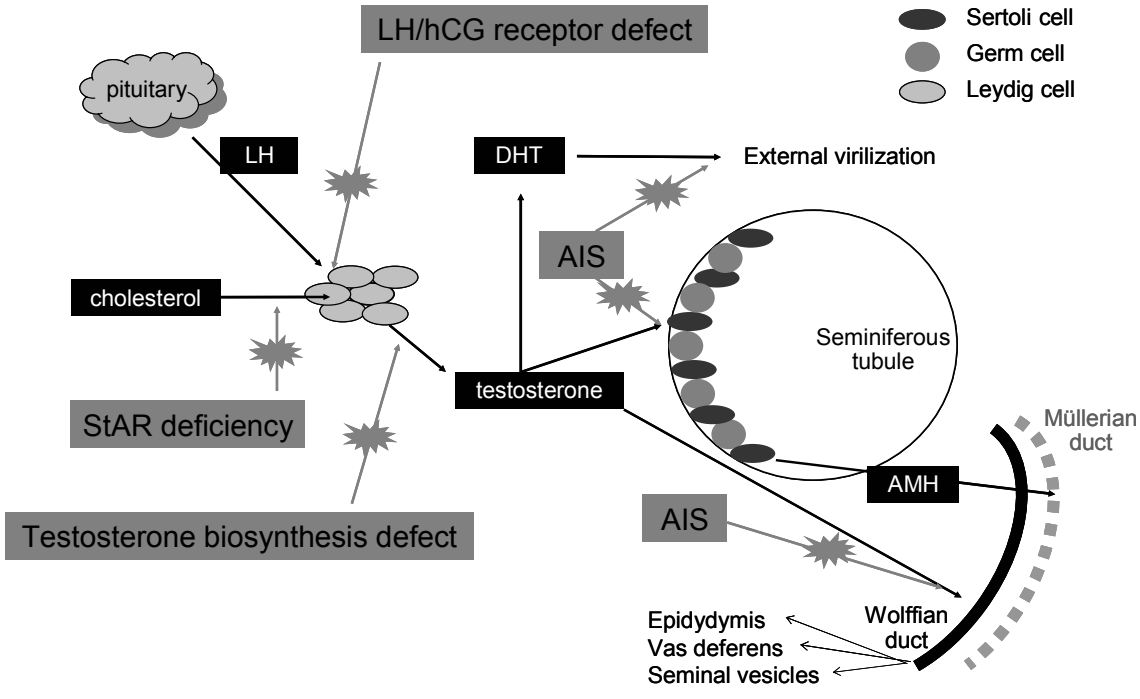

Figure 1-4: Schematic representation of the pathogenetic mechanisms in undervirilization syndromes

A typical hypervirilized patient is a newborn with male external sexual characteristics (but bilateral cryptorchydism), admitted to the neonatal intensive care unit with dehydration and vascular collapse during the second or third week of life. A diagnostic work-up reveals severe electrolyte disturbances (hyponatremia and hyperkalemia) and acidosis, increased plasma renine and ACTH, high androgen levels and a 46,XX karyotype. Genetic analysis reveals a mutation in the *CYP21* gene, encoding the 21-hydroxylase enzyme. Due to this enzymatic defect, the adrenal cortisol and aldosterone synthesis is blocked, leading to a rise in ACTH by a negative feedback mechanism. High ACTH levels cause overstimulation of the only functional adrenal steroid synthesis pathway, the production of androgens. Increased androgen synthesis, exceeding the capacity of P450 aromatase (converting testosterone into estradiol), started early in embryonic life (before 12 weeks GA), and led to virilization of the urogenital sinus. However, gonadal (ovarian) development and differentiation of the Müllerian ducts occurred normally in this patient.

Early intrauterine androgen exposure (before 12 weeks GA) can lead to a variable degree of labioscrotal fusion and clitoral enlargement, after week 12, androgen exposure causes isolated clitoromegaly (see above) (50).

1.6.4 Germ cell tumors in patients with disorders of sex development

For a long time it is recognized that germ cell tumors frequently occur in patients with DSD, and that the risk for tumor development is related to the presence of the Y chromosome. Therefore, a prophylactic gonadectomy is generally advised in patients at risk (33, 35, 42-46). The gonadoblastoma region on the Y chromosome (GBY), shared in common by all patients who developed a germ cell tumor, was proposed as being responsible for tumor development in this patient population, and a gene within this region, encoding the testis specific protein - Y encoded (TSPY) was identified as the main candidate gene (108-112) (Figure 1-5). The presence of SRY or other sex determining genes is irrelevant in this context. As in normal males, the development of invasive germ cell tumors in patients with DSD is preceded by the presence of *in situ* neoplastic lesions: ITGNU and GB. However, there are some important differences. The mean age of occurrence of TGCT in normal males is 25 years for the non-seminoma and 35 years for the seminoma (2). The adjacent ITGNU lesion, if present in the gonadectomy sample, is diagnosed at the same time. In view of the fetal

origin of ITGNU (see above), the morphologic and genetic changes in the malignant cells occurred over a time period of about three decades. Due to the practice of systematic prophylactic gonadectomy in patients with DSD, GB and ITGNU lesions are in this patient population usually diagnosed at a young age or even at birth. Moreover, the progression towards invasive tumors takes place much more rapidly (35, 44, 45, 113-115). Therefore, GB and ITGNU in patients with DSD represent the earliest accessible stages in the evolution of malignant germ cell cancer, and therefore, they are an excellent model to study early changes in germ cells that precede and can eventually lead to their malignant transformation.

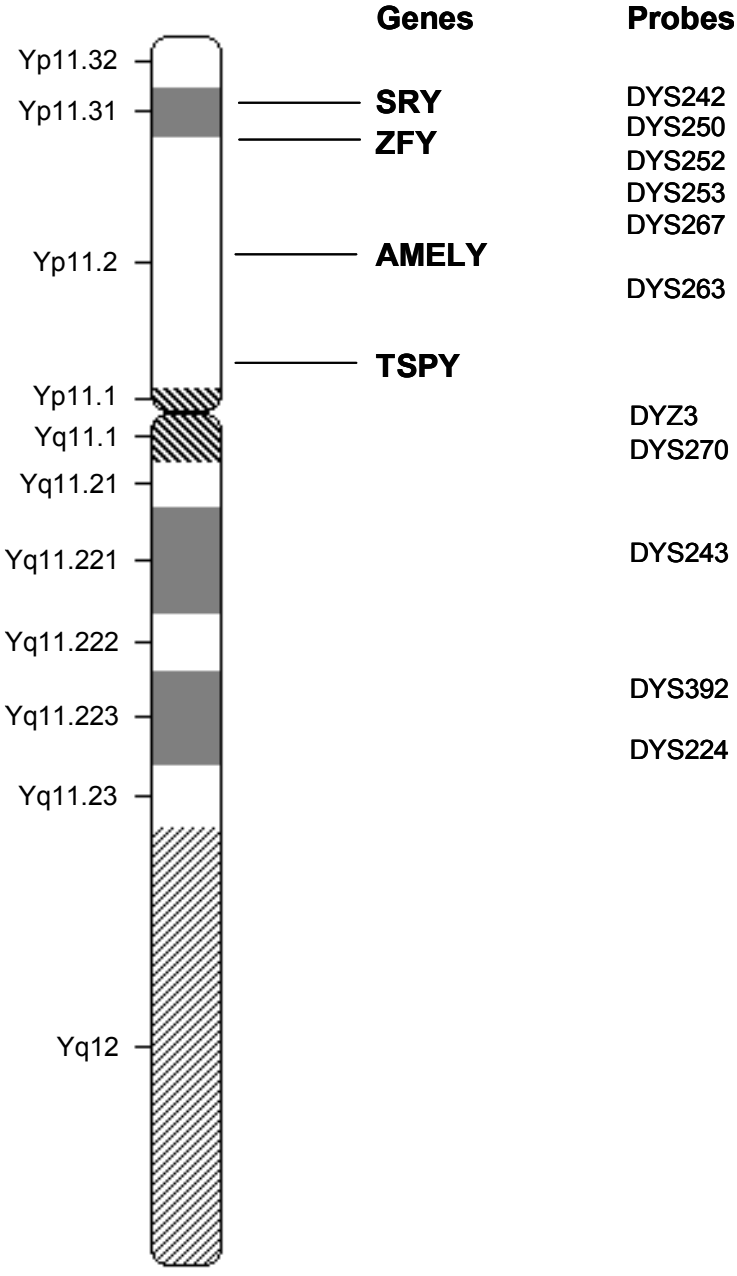

Figure 1-5: Position of TSPY on the Y chromosome

1.7 Scope of the thesis

This thesis aims to get an understanding of the earliest changes that take place in germ cells in their process towards the formation of an *in situ* neoplastic lesion. Since no animal model is available at present, GB and ITGNU in patients with DSD represent currently the earliest accessible stages of germ cell tumor development.

The following questions need to be answered:

- To what extent is maturation delay of germ cells a preceding common mechanism in various conditions (DSD and others) that are characterized by an increased risk for germ cell tumor development?
- What are the histopathologic findings characterizing maturation delay of germ cells? What are the diagnostic tools that allow to distinguish this condition from normal germ cell development on the one hand and early neoplastic changes on the other?
- Why do some patients with DSD develop a GB lesion and others an ITGNU?
- What exactly are the sequential steps preceding the formation of ITGNU and GB?
- Since not all patients with DSD will develop a germ cell tumor, are there additional risk factors that allow the early identification of high risk patients?

A detailed insight in the mechanisms preceding and determining germ cell tumor formation in patients with DSD might in the future lead to the elaboration of safe protocols for the follow-up of dysgenetic gonads left in place, in an attempt to progress towards a more conservative approach regarding the practice of systematic gonadectomy in this specific patient population.

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2

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

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Abstract

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, beta-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, beta-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 the 22nd to 24th week 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 do no longer proliferate.

Introduction

In humans and rodents, amongst other species, the embryonic precursors of the gametes are known as primordial germ cells (PGCs) (1), 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 (2). Subsequently, the gonads and sexual differentiation occur in the 6th and 7th week (3). Between the 7th and 9th week during ovarian development (referred to as prefollicular stage) the germ cells (oogonia) and granulosa cells characteristically arrange in cords and sheets without specific organization (4). 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 to 12 weeks of gestation and extends into the second trimester (5). Contradictory data exists 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 (6, 7). 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 (8). Whereas at 5 weeks of gestational age an estimated 700-1300 germ cells are present, germ cell number peaks between weeks 16 to 20, reaching an estimated $6x10^6$ cells per ovary (9). At birth, the total number of germ cells has been estimated to be around $1x10^6$ cells, and the number of follicles has been reported to be in the range of 1,3 to $3,8x10^5$, with 95% being represented by primordial follicles (9, 10). Maturation is a gradual process, as is demonstrated by the initiation of 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 (5).

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. Our results describe the maturation process seen in normal development of the ovary and can serve as a reference for future identification of pathological processes of maturation.

Material 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 hematoxylin/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 (11). For immunohistochemistry, sections were incubated with the primary antibody overnight at 4°C (PLAP, c-KIT, VASA, ß-catenin, E-cadherin) or 2 hours at room temperature (OCT3/4, Ki-67). The primary and secondary antibodies used are indicated in Table 2-1. All slides were counterstained with hematoxylin. For PLAP, ß-catenin, and E-cadherin, positive staining of the fallopian tube, and for c-KIT,

the presence of mast cells was used as an internal positive control. Negative controls were performed by omitting the primary antibody, resulting in complete absence of signal.

Table 2-1. Antibodies (source) and detection method used for immunohistochemistry.

Antibody	Company	Code	Pretreatment	Dilutions	Secondary antibody (1:200) (biotinylated)	Visualization
PLAP	Cell Marque, Hot Springs, AR, USA	CMC203	HIAR*	1:200	Rabbit-anti-mouse (Dako-Cytomation E0413)	ABCplx-ap[1]
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[2]
VASA	provided by D. Castrillon#		HIAR	1:2000	Swine-anti-rabbit	ABCplx-ap
ß-catenin	Zymed Laboratories, South San Francisco, USA	CAT-5H10	HIAR	1:2000	Rabbit-anti-mouse	ABCplx-hrp
E-cadherin	Zymed Laboratories, South San Francisco, CA, USA	HECD-1	HIAR	1:300	Rabbit-anti-mouse	ABCplx-hrp
Ki-67	Dako-Cytomation, Glostrup, Denmark	A047	HIAR	1:50	Swine-anti-rabbit	ABCplx-hrp

* Heat induced antigen retrieval (40)
# (23)
[1] ABC complex, alkaline phosphatase, DakoCytomation, Code: K0391
[2] ABC complex, horse radish peroxidase, DakoCytomation, Code: K0377

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, Steinheim, Germany) for a blue staining and 3-amino-9-ethyl-carbazole (A.5754 and D4254, Sigma, Steinheim, Germany)/H_2O_2 for a red staining, without counterstaining. Endogenous peroxidase activity and/or endogenous biotin was blocked using 3% H_2O_2 (for 5 minutes) and/or a blocking kit for endogenous biotin (Vector Laboratories, Burlingame CA, USA) to prevent background staining.

To semi-quantitatively assess expression of the markers investigated, cells showing a positive signal were counted in representative visual fields (magnification x 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 x 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 to 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 x 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 2-1 summarizes the results of a semi-quantitative assessment of the different markers. Each black spot represents one case. Table 2-2 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 2-3 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 2-2 and 2-3. Results of single and double-stainings are described below. The data are depicted graphically in Figure 2-4 (grey bars) and were compared to findings published so far (black bars). Figure 2-5 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 (see Figure 2-1). 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 2-2 and 2-3). In the medulla, where the majority of maturing germ cells is found during ovarian development, positive cells were much less frequent.

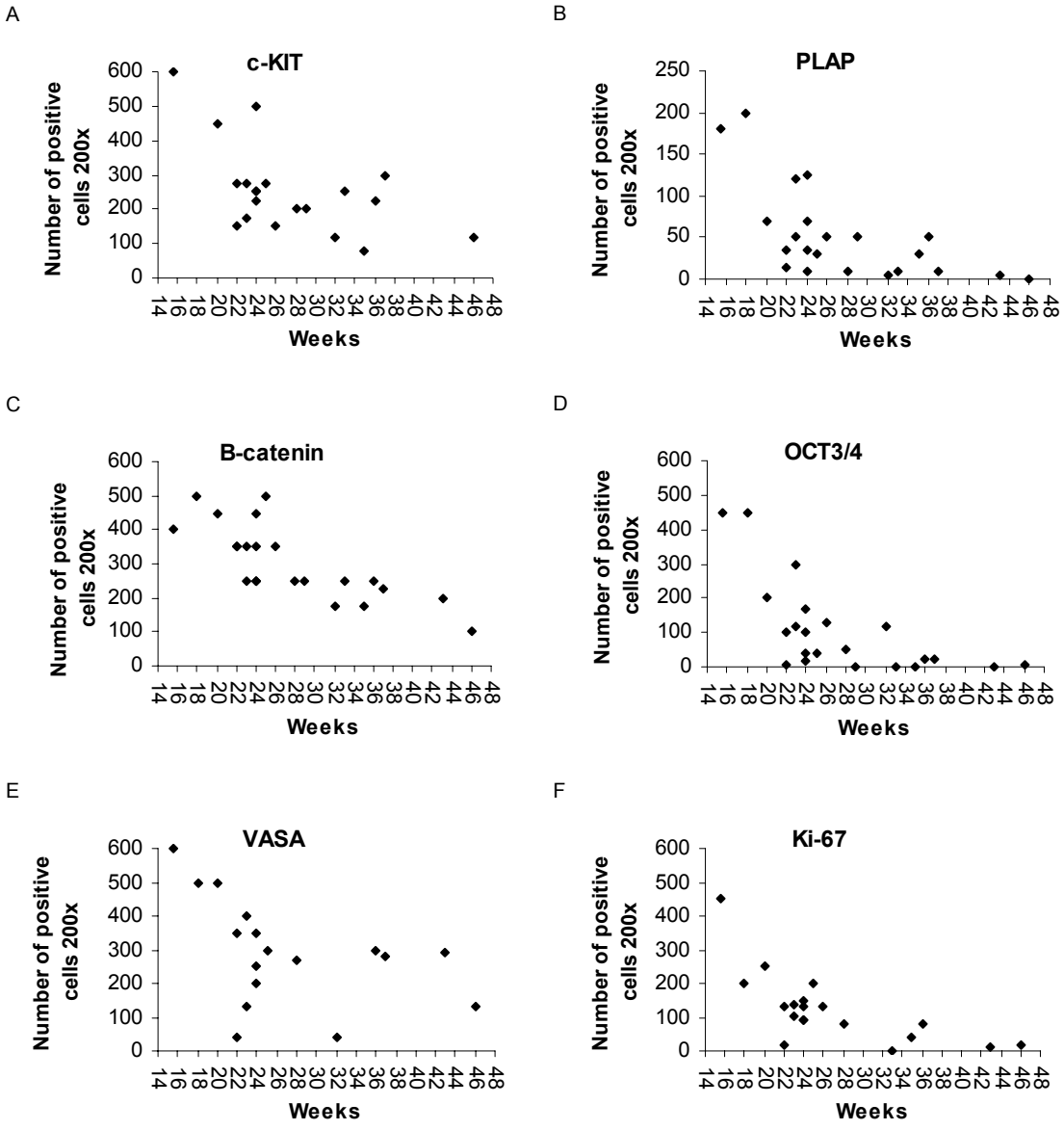

Figure 2-1. Absolute numbers of cells per representative visual field (magnification factor x 200) showing a positive staining with immunohistochemistry for the markers at different gestational ages (weeks). The earliest case studied in this series was 15.5 weeks, the oldest was the ovary of a neonate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

Table 2-2. Differences in the expression of PLAP, OCT3/4, and Ki-67 between the cortical and the medullary region of fetal ovaries aged 15.5 to 36 weeks. The average and the range of cell numbers found positive for the indicated markers in three independent high power magnification fields (x 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 interindividual variation during the third trimester.

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)

Table 2-3. Absolute numbers compared to total present, and percentage (between brackets) of cells positive for OCT3/4 in three ovaries of 15.5, 28, and 36 weeks gestational age. 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 x 200) in both the cortical and the medullary region of three fetal ovaries. In brackets, the percentage of germ cells expressing OCT3/4 is given, together with the range (in percentage) of three different countings 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.

Gestational age (weeks)		15.5	28	36
OCT3/4	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 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 2-2A). 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 (see Figure

2-1). PLAP was detected in early germ cells predominantly located in the cortical region (see Figure 2-2B, and Table 2-2). After birth, PLAP positive germ cells were hardly ever seen, with maximally 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 (see Figure 2-1 and Table 2-2). In addition to oogonia, OCT3/4 was occasionally seen in early oocytes, but was never detectable in cells involved in folliculogenesis (Figure 2-2C). Similar to the expression pattern of Ki-67 and PLAP, a decline in the expression of OCT3/4 in fetal ovaries was seen around 24 weeks of gestation. At term and in ovaries of neonates, hardly any positive germ cells were detectable.

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

A comparable staining pattern was seen for c-KIT, ß-catenin, and E-cadherin (see Figure 2-3, A-F). Both in early, immature germ cells (oogonia) and at all later stages of folicogenesis, these factors were predominantly localized at the membrane, but sometimes also showed a cytoplasmic localization (see also Figure 2-1). In primordial follicles, all three factors showed strong signal intensity at sites of cell-cell interaction (see Figure 2-3, A-F). As ß-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 (see Figure 2-1G 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 (see Figure 2-3G). The total number of germ cells, determined by VASA, decreased with gestational age: whereas at 15.5 weeks around 600 germ cells, mostly oogonia and early

oocytes, were seen per visual field, the average number of germ cells at term was around 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 two 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 ß-catenin, and PLAP and Ki-67 (Figures 2-2, D-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 throughout all ages investigated (see Figure 2-2D). 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 (see Figure 2-2E). 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 have already lost OCT3/4 expression (Figure 2-2F). Similar staining patterns were observed for the combinations OCT3/4 and VASA, and OCT3/4 and ß-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 did no longer show OCT3/4 (see Figure 2-2G). 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 2-2H). A fraction 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 2-4 (grey bars) and compared to findings in humans published so far in the literature (black bars). Figure 2-5 gives a scheme of marker progression during maturation from oogonia to primordial follicles.

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 G0-phase cells (12). 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 2-2). 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, around 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 to 40% of the few early germ cells found at weeks 28 gestation and 3 weeks after birth, respectively. This indicates that immature germ cells at early stages show strong

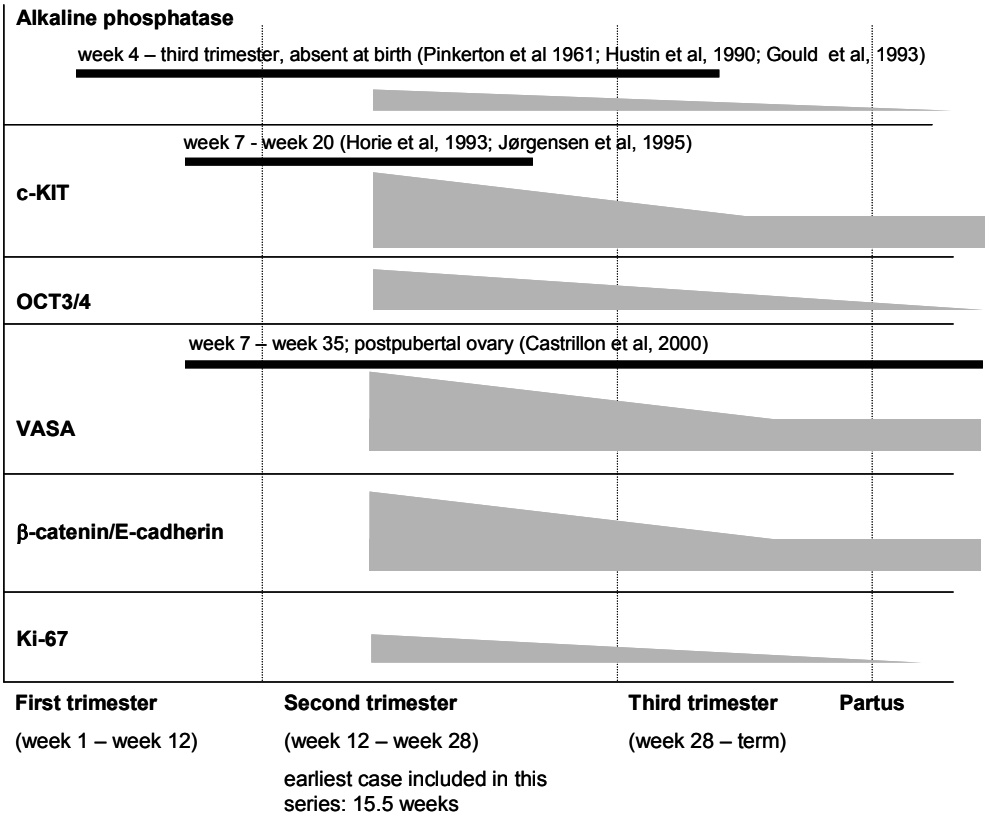

Figure 2-4. Overview of the data currently available from the literature on the expression of different antigens in germ cells during female intrauterine development (black bars), compared with findings of this study (grey bars). Bar sizes of grey bars schematically represent numbers of germ cells expressing the individual factor at different developmental ages.

	Oogonia	Oocyte	Primordial follicle
c-KIT	+	+	++
β-catenin/	+	+	++
E-cadherin	+	+	++
VASA	+	+	++
PLAP	+	+/-	-
OCT3/4	+	+	-
Ki-67	+/-	-	-

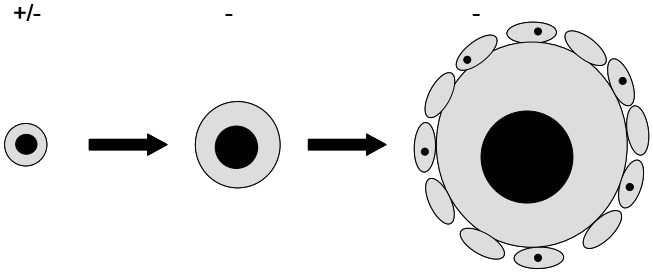

Figure 2-5. Progression of markers during germ cell maturation from oogonia to primordial follicles during the pre- and perinatal period.

proliferation around weeks 15.5 to 20, whereas at later ages both the number and the fraction of proliferating germ cells decrease. 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 (5). 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 (13, 14). The number of positive meiotic cells was that small, that it did not influence the overall conclusion, and did not allow conclusions regarding possible physiological meaning.

Alkaline phosphatases are regarded as archetypical onco-fetal proteins. They are detectable at the mammalian blastocyst stage and have been described in germ cells of human embryos of less than 4 weeks gestational age (15). PLAP expression has been described in primordial germ cells at 8 to 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 (16, 17). 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 PGCs (18, 19). In addition, presence of OCT3/4 has been described in germ cell tumor cells with pluripotent potential such as embryonal carcinoma and seminomas (20). In fetal human testes, OCT3/4 has been found to be highly expressed in PGCs between weeks 17 and 24 and to a lesser extend at later stages (20). Expression of OCT3/4 in normal and dysgenetic human ovaries has been reported recently (46). 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 2-4) (42). 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 (21). The finding that these tumors 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 (22). 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 pathologic conditions, e.g. in dysgenetic gonads, as has been described recently (46).

VASA is a member of the DEAD box family of RNA helicases and is specifically expressed in the germ cell lineage (23). Furthermore, VASA expression has been detected by immunohistochemistry in migratory PGCs at week 7 gestational age and in germ cells both before and after birth (23). 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 tumors retaining germ cell characteristics (24). Interestingly, in murine embryonic stem cells expression of Mvh, the mouse VASA homologue, has been described as an early event in the commitment of stem cells to the germ cell lineage (25, 26). In our study, expression of VASA has been found both in germ cells at all gestational ages. In accordance with previous reports (23), 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, ß-catenin, and E-cadherin are all involved in cell signaling 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 (27), for review). The c-KIT/SCF system has been found to be involved in survival and proliferation of migrating germ cells in mice (28). In human intrauterine gonadogenesis, *c-KIT* expression has been described in female PGCs at the period of arrival of PGCs at the gonadal ridges at week 7 and later between 13

and 21 weeks of gestational age (29-31). 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 (32).

Whereas some data is available on the cadherin-catenin complex in mouse PGC development and oocyte maturation (33, 34), 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 (35). Here we show that c-KIT, ß-catenin, and E-cadherin are all present in germ cells throughout all stages of female intrauterine development. 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 like OCT3/4 and PLAP with c-KIT and ß-catenin. As oogonia are often found in clusters, these factors seem to be involved in signaling 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 PGCs (36), and in oocyte growth and acquisition of meiotic competence in mice (37). The functional importance of E-cadherin has further been demonstrated by dissociation/re-association 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 (38).

The total numbers of germ cells seen in one visual field, as assessed by either c-KIT, ß-catenin, or VASA at different gestational ages (see Figure 2-1), are in accordance with previous reports, where a steady decrease of germ cells from around week 18-20 gestational age resulted in numbers that were only one sixth of peak values at term (9). We therefore conclude that assessment of cell numbers in one representative visual field at magnification x 200 and including comparable surface areas of cortical and medullary areas is a reliable method for semi-quantitative analyses. Our results allow some interesting comparisons with data on mouse germ cell maturation (see Table 2-4).

Table 2-4. Comparison of gene expression of female human and mouse germ cells during maturation. 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.

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	++	++[#,1]	+/-∗	-[1,2]	?∗	+[2]
c-KIT	+	+[#,1]	++	-[#,1]	++	+[3]
VASA	+	+[#,1]	++	++[#,1]	++[4]	++[5]

References: 1= 41; 2 =42; 3 = 43; 4 = 23; 5 = 25.
∗ Note that the exact time point of down-regulation of OCT3/4 with regards to onset of meiosis has not been established in human oocytes yet. We could not detect re-expression of OCT3/4 in primary and secondary follicles of a 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 (44, 45) warrants investigation at what stage this protein is re-expressed in mature/ovulating oocytes or during fertilization.
[#] Results are based on RT-PCR (expression of mRNA); all other results are based on immunohistochemistry.

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 to 20), the number of immature germ cells is high, and the pool of these cells decreases significantly after week 22 to 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 compartimentalization, where the cortex is the area where expansion of immature germ cells takes place, whereas the medulla provides 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 get into close contact with nurturing Sertoli cells (47). 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, ß-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 (39), Cools et al, manuscript in preparation).

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3

Maturation delay of germ cells in trisomy 21 fetuses results in increased risk for the development of testicular germ cell tumors

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Abstract

Trisomy 21 is associated with an increased risk for the occurrence of germ cell tumors in males. The development of these tumors is thought to be related to events in fetal life. A delay in the maturation of germ cells is one of the mechanisms that have been proposed for the development of these tumors in high-risk groups such as intersex patients. To investigate whether a delay in germ cell development also occurs in trisomy 21, we examined the gonads of 30 trisomy 21 fetuses, neonates and infants (19 males and 11 females) for the expression of several immunohistochemical germ cell markers throughout pregnancy, and compared them to a series of 46 age-matched controls. The results of our study reveal a significant delay in germ cell development in trisomy 21 fetuses, especially in males. Prolonged expression of OCT3/4, in combination with an increased expression of TSPY might have pathogenetic relevance for the development of testicular germ cell tumors in this population.

Introduction

In addition to a high incidence of leukemias, trisomy 21 (Down syndrome) is associated with an increased risk for gonadal and extragonadal germ cell tumors (1). The incidence of testicular germ cell tumors (TGCT) in trisomy 21 individuals is estimated at 0.5%, compared to an expected incidence of 0.087% in the general population (2). Some studies even mention a 50-fold increase in incidence (3, 4). Seminomas, non-seminomas and 2 fetal carcinoma *in situ* (CIS) lesions (the precursor lesion of these tumors, also called intra tubular germ cell neoplasia unclassified (ITGCNU) have been reported, the first occurring most frequently (4-6). In view of the young age at which these tumors are diagnosed, a genetic background is suspected. Cryptorchidism, which occurs frequently in Down syndrome is also a risk factor, though TGCT are mainly found in normally descended testes. Additional contributing factors are excessive FSH stimulation due to hypergonadotrohpic hypogonadism, a possible gene dosage effect of putative oncogenes on chromosome 21, increased sensitivity of trisomic cells to carcinogenic agents and increased maternal age (2, 4, 7). Recently, maturation delay of germ cells and prolonged expression of the stem cell factor receptor c-KIT during intrauterine development have been related to the development of CIS in various intersex conditions and chromosomal anomalies, by increasing the survival and proliferative chances of the primordial germ cells (PGC) (8, 9).

To investigate if and to what extent intrauterine maturation delay of germ cells is present in trisomy 21, we studied the expression of several immunohistochemical markers for germ cells in the gonads of 30 trisomy 21 fetuses, neonates and infants, and compared their expression profiles throughout pregnancy and early infancy with a series of 46 normal controls of comparable gestational age (GA) published previously (10, 11).

Immunohistochemistry was performed with the antibodies OCT3/4, c-KIT, PLAP, TSPY, VASA and Caspase 3. OCT3/4 (Octamer binding transcription factor 3/4)/POU5F1 (POU domain class 5 transcription factor 1) is a transcription factor, staining pluripotent stem cells and early germ cells. OCT3/4, c-KIT (the stem cell factor receptor) and PLAP (Placental-Like Alkaline Phosphatase) are well-established markers for the diagnosis of CIS and invasive TGCT (12, 13, 14) and are normally expressed during early fetal life in primordial germ cells/gonocytes, the putative cells of origin of TGCT (10, 11). TSPY (Testis Specific Protein - Y

encoded), encoded by the TSPY gene, is thought to regulate the mitotic proliferation of spermatogonia, just before the onset of meiosis (15). However, this is most likely not its only function, because TSPY is also expressed, mainly in prespermatogonia, during embryonic life, where no meiosis in males occurs (10). VASA, the human homologue of the mouse *vasa* gene (*mvh*) is a general marker for germ cells (16). As such, it can be used to estimate the total number of germ cells in gonadal tissue samples. In fetal gonadal tissues, VASA was found to be expressed at a constant level from the second trimester of pregnancy onwards. VASA stains maturing germ cells (oocytes and prespermatogonia) with a higher intensity than immature germ cells. Therefore, a reverse correlation exists between the staining intensity of VASA and the expression of OCT3/4, the latter staining the immature, pluripotent germ cells (oogonia and gonocytes) (10, 11). Caspase 3 is an early product and further executioner in the apoptotic cascade. Therefore, immunohistochemical detection of activated Caspase 3 is a new and reliable method to specifically stain early apoptotic, but not necrotic or autolytic cells (17). Caspase 3 expression in fetal gonads has not been studied previously.

Materials and Methods

Tissue samples

Gonadal tissue of trisomy 21 fetuses, neonates and infants was obtained after induced abortion or sudden death (2 due to heart failure, 1 due to meningococcal septicemia and 1 due to necrotising enterocolitis). Eleven trisomy 21 females, 18-36 weeks GA, and 19 trisomy 21 males, 11-19 weeks GA, and 1 week-15 months old, were included. Unsatisfactory preservation of tissue samples, as evaluated by hematoxylin-eosin (HE) staining, led to the exclusion of 17 samples. Gonads from 21 females (15-46 weeks GA) and 25 males (15-37 weeks GA and 5-17 months old) without developmental anomalies (referred to as controls) were obtained after spontaneous or induced abortion or cot death. The diagnosis of trisomy 21 was confirmed by amniocentesis and karyotyping in all cases, trisomy 21 mosaicism led to exclusion of one additional case. GA of patients and controls was calculated in relation to the mother's last menstrual cycle and was in accordance with the crown-heel length measurements at autopsy.

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).

Immunohistochemical staining

After fixation in 10% formalin, tissue sections of 5µm thickness were prepared. The antibodies used for immunohistochemistry and a schematic representation of the different protocols for the various antibodies are represented in Table 3-1. Slides were incubated with the primary and appropriate secondary antibodies in the indicated dilutions. Between the different incubation steps, slides were washed in a PBS-Tween 0.01% solution. Staining was performed using DAB/H_2O_2 or New Fuchsin/Naphtol ASMX phosphate. Sections were lightly counterstained with hematoxylin. The following positive controls were included: normal adult male gonadal tissue for VASA and Caspase 3 and a seminoma sample for PLAP, c-KIT, TSPY and OCT3/4.

Table 3-1: Schematic representation of origin and protocols used for the different antibodies.

Primary antibody	Origin	Dilution	Pre-treatment	HIAR	Incubation	Secondary antibody	AB-complex	Chromogen
VASA	Kindly provided by Dr DH Castrillon*	1/2000	no	yes	overnight, 4° C	SWAR-bio	ABC-AP	New Fuchsine
PLAP	Cell Marque, Hot Springs, AR, USA	1/200	no	yes	overnight, 4° C	RAM-bio	ABC-AP	New Fuchsine
c-KIT	Dako-Cytomation, Glostrup, Denmark	1/500	no	yes	overnight, 4° C	SWAR-bio	ABC-AP	New Fuchsine
TSPY	Kindly provided by Prof C Lau#	1/3000	no	no	overnight, 4° C	SWAR-bio	ABC-AP	New Fuchsine
OCT3/4	Santa Cruz Bio-technology, Santa Cruz, CA, USA	1/1000	H_2O_2 for 5'	yes	2 h, RT	HAG-bio	ABC-HRP	DAB
Caspase 3	R&D systems, Minneapolis, MN, USA	1/8000	H_2O_2 for 5'	yes	overnight, 4° C	SWAR-bio	ABC-HRP	DAB

Secondary antibody	Origin	Dilution
SWAR	Dako-cytomation	1/200
RAM	Dako-cytomation	1/200
HAG	Vector Laboratories, Burlingame, CA, USA	1/200

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# Department of Medicine, VA Medical Center, University of California, San Francisco, CA, USA
HIAR: heat induced antigen retrieval (30); SWAR-bio: swine anti rabbit antibody, biotin labeled; RAM-bio: rabbit anti mouse antibody, biotin labeled; HAG-bio: horse anti goat antibody, biotin labeled; AB-complex: streptavidin-biotin complex; ABC-AP: streptavidin-biotin-alkaline phosphatase complex; ABC-HRP: streptavidin-biotin-horseradish peroxidase complex, RT: room temperature.

Quantification of results

In male gonads, the expression of a marker was assessed by counting the number of positive germ cells in 10 tubular cross-sections. Additionally, the number of TSPY positive gonocytes (defined as immature luminal germ cells) per 50 TSPY positive germ cells (gonocytes + prespermatogonia, defined as maturing germ cells on the basal membrane) was determined.
In females, all germ cells positive for a given marker were counted in one representative visual field (magnification x 200) including comparable surface areas of cortical and medullary tissue of the ovaries. Caspase 3 activity was evaluated separately in oogonia (defined as germ cells not yet engaged in follicle formation) and oocytes (germ cells included in primordial follicles) as follows: Caspase 3 positive oogonia were counted in one visual field (magnification x 200) including comparable surface areas of cortical and medullary tissue. Apoptotic oocytes were counted in one high power visual field (magnification x 400) including only medullary ovarian tissue and expressed as a percentage of the total number of oocytes.
All counts were performed by the same observer (MC), who was blinded for GA and origin of the tissue material (patient or control).
Statistical analysis was performed using the SPSS statistical program (SPSS 11.5 for Windows).

Results

Staining results for OCT3/4, PLAP and c-KIT in trisomy 21 males and in male controls

The expression profiles throughout the second and third trimester of pregnancy were similar for the three markers in trisomy 21 and in controls: the expression was highest during the first half of the second trimester, but decreased sharply thereafter. However, in trisomy 21, the number of positive cells was higher at all gestational ages and the decrease in the number of positive cells was slower: In controls, around birth, only a few positive cells were encountered and at five months these markers had virtually disappeared, whereas in trisomy 21, at 5 months a considerable number of positive cells were still found (Figure 3-1 and Figure 3-2). The prolonged expression of markers in trisomy 21 was more pronounced for OCT3/4 and PLAP as compared to c-KIT. The difference in mean number of positive germ

cells per 10 tubule cross-sections was statistically significant for the three markers (OCT3/4: p= 0.004; PLAP: p = 0.003; c-KIT: p = 0.03).

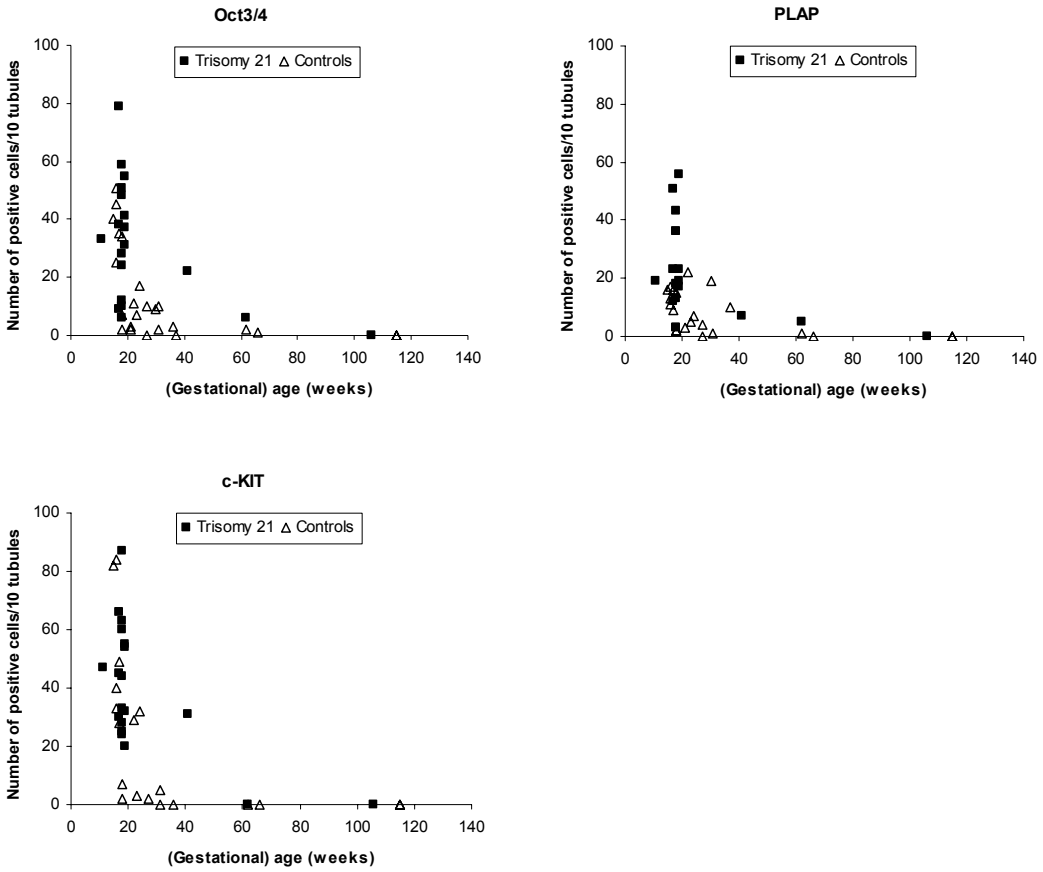

Figure 3-1. Staining results for OCT3/4 (top left), PLAP (top right) and c-KIT (bottom) in male individuals with trisomy 21 (black squares) and controls (triangles). The expression patterns for the three markers are similar in trisomy 21 and in controls: the expression is high in the second trimester but decreases sharply thereafter. The number of positive cells is higher in trisomy 21 than in controls at all ages and the decline in the number of positive cells is slower.

Staining results for OCT3/4, PLAP and c-KIT in trisomy 21 females and in female controls

The expression profiles throughout the second and third trimester of pregnancy were similar in trisomy 21 females and in controls for OCT3/4 and PLAP. Positive cells were mainly found in the cortical region, where immature oogonia reside, whereas in the medulla, which is rich in maturing oocytes included in primordial follicles, very few positive cells were seen. In both patients and controls, expression of OCT3/4 and PLAP was high early in the second trimester, and decreased around 25 weeks GA. Around birth, only a few positive cells were seen. The number of positive cells in trisomy 21 was higher as compared to controls during

early pregnancy, but since the decline was sharper in this group than in controls, no maturation delay of germ cells in trisomy 21 was observed anymore around 35 weeks GA (Figure 3-3 and Figure 3-4). The differences in mean number of positive cells per visual field was statistically significant for both OCT3/4 and PLAP (Oct 3/4: $p = 0.005$; PLAP: $p = 0.003$). In contrast to OCT3/4 and PLAP, c-KIT was not only expressed in oogonia, but also in oocytes. c-KIT expression in females (trisomy 21 cases and controls) was highest early in the second trimester, and decreased afterwards, but in contrast to the situation in males, it did not disappear but remained stable at a certain level around birth and during the first weeks thereafter (Figure 3-3). The number of c-KIT positive cells did not differ between trisomy 21 females and controls ($p = 0.28$).

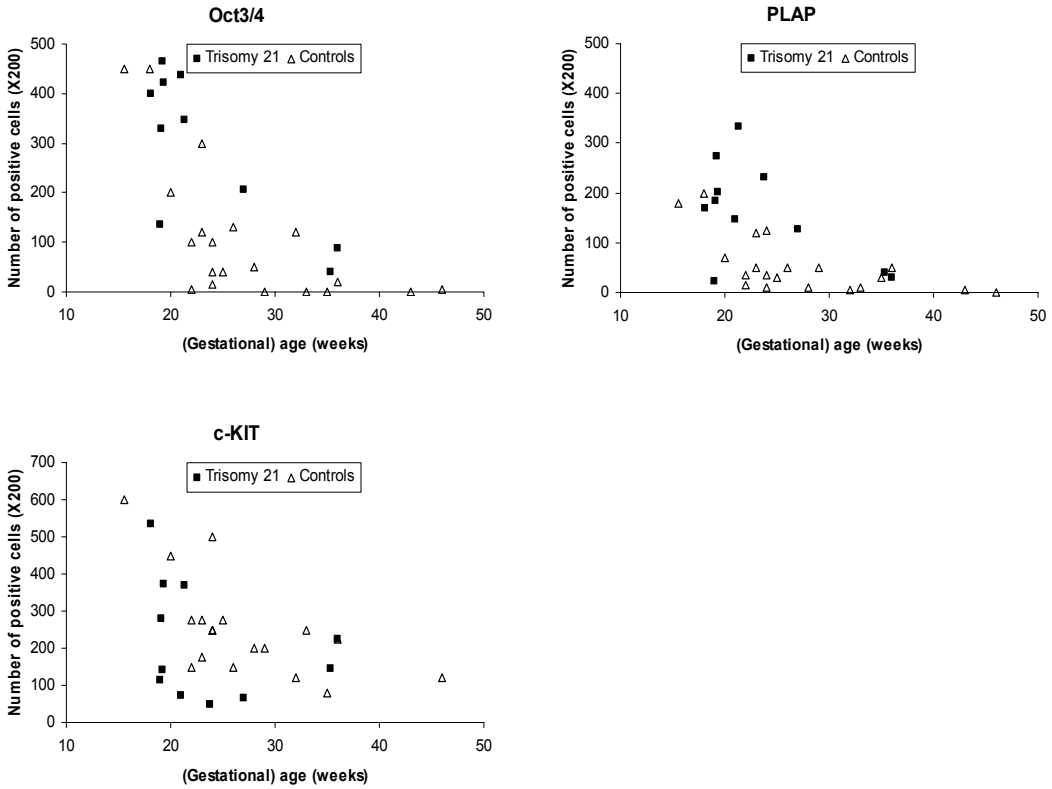

Figure 3-3. Staining results for OCT3/4, PLAP and c-KIT in female individuals with trisomy 21 (black squares) and controls (triangles). The expression patterns are similar for OCT3/4 (top left) and PLAP (top right) in trisomy 21 and in controls: high expression early in the second trimester and a sharp decrease thereafter. The number of positive cells is higher in trisomy 21 than in controls at all ages but the decline is sharper, so that around birth, very few positive cells are found in patients as well as in controls. c-KIT expression (bottom) is highest early in the second trimester and decreasees afterwards, to remain stable at a certain level around birth and during the first weeks thereafter. The number of c-KIT positive cells does not differ between patients and controls.

Staining results for VASA and TSPY in trisomy 21 males and in controls

VASA expression in trisomy 21 males and controls was mostly seen in maturing germ cells on the basal membrane (prespermatogonia), whereas staining in luminal germ cells (the immature gonocytes) was less frequent and showed lower intensity. The expression profiles were similar in trisomy 21 and in controls: VASA was highly expressed around 20 weeks, and decreased afterwards to reach a stable level around birth and the first months thereafter. The number of positive cells throughout pregnancy was higher in controls, suggesting a higher number of maturing germ cells in this group, compared to the population with trisomy 21 (Figure 3-5). However, this difference did not reach statistical significance ($p = 0.07$).

The expression pattern of TSPY throughout pregnancy was similar to the expression pattern of VASA. However, in spite of the lower total germ cell number in trisomy 21, as evaluated by the VASA staining, more TSPY positive cells were found in this group than in controls (Figure 3-5). This difference was statistically significant ($p = 0.02$). In the control group, TSPY expression was mainly confined to prespermatogonia, confirming results from our previous study (10). Yet, in individuals with trisomy 21, TSPY was frequently seen in gonocytes as well as in prespermatogonia. The number of TSPY positive gonocytes per 50 TSPY positive germ cells was calculated for trisomy 21 males and controls (Figure 3-5). The difference between the two groups was statistically significant ($p = 0.002$). Moreover, the staining intensity appeared to be stronger in trisomy 21 than in controls (Figure 3-6).

Staining results for VASA in trisomy 21 females and controls

The expression profile of VASA throughout pregnancy in trisomy 21 females and controls was comparable to the profile in males (high expression early in the second trimester and decreasing thereafter to reach a stable level around birth) (Figure 3-7). No difference in VASA staining was seen between cortical and medullary regions of the ovary. In contrast to the males, the total number of VASA positive cells did not differ between trisomy 21 females and controls ($p = 0.77$).

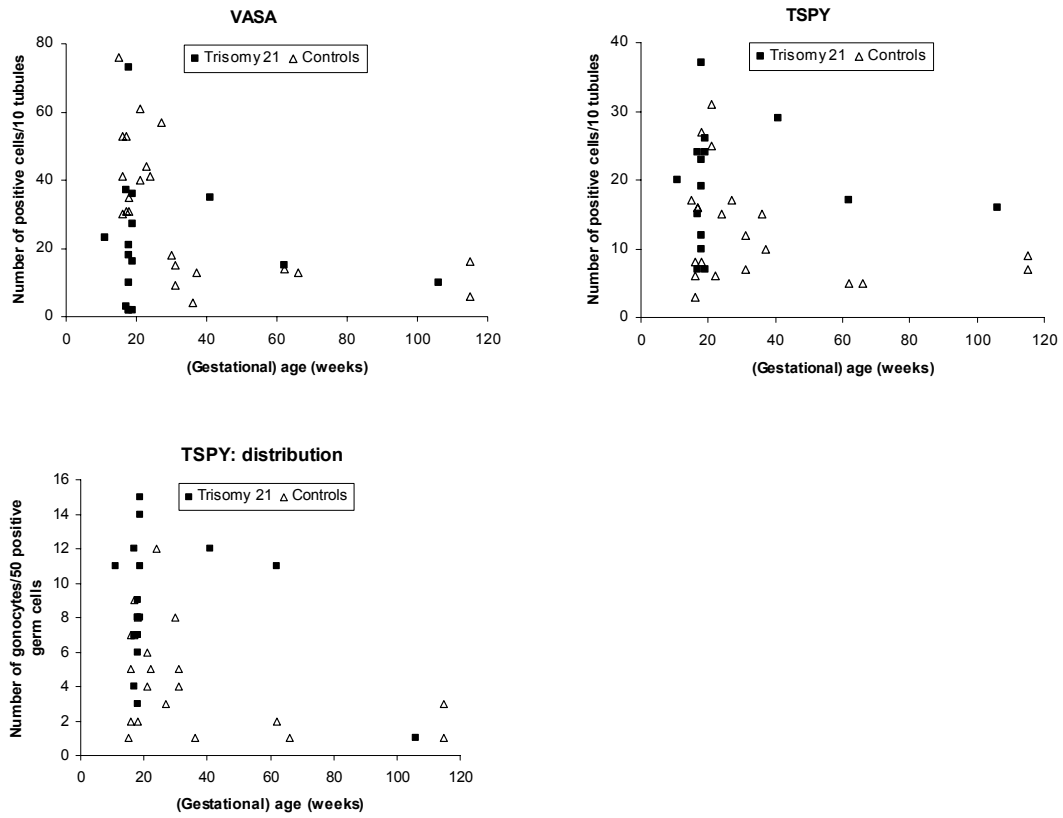

Figure 3-5. Staining results for VASA and TSPY in male individuals with trisomy 21 (black squares) and controls (triangles). Top left: VASA expression is high around 20 weeks GA, decreases thereafter and remains at a constant level from birth onwards. VASA expression is slightly higher in controls than in trisomy 21 males. Top right: The pattern of TSPY expression is similar to VASA expression but the number of TSPY positive cells is higher in trisomy 21 than in controls. Bottom: This difference is due to the more frequent positivity for TSPY in gonocytes of trisomy 21 patients as compared to the control group where TSPY expression is predominantly seen in prespermatogonia.

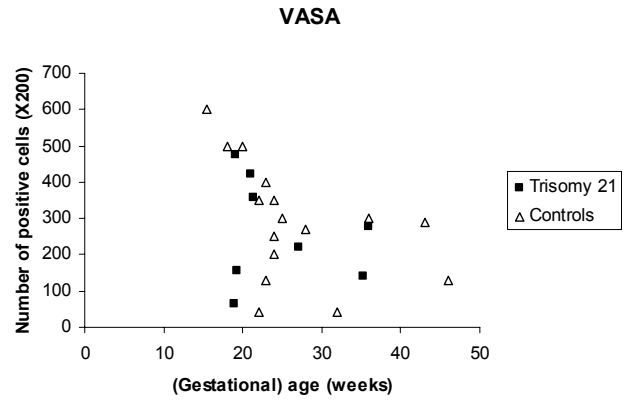

Figure 3-7. Staining results for VASA in female individuals with trisomy 21 (black squares) and controls (triangles). VASA expression is high in early pregnancy and starts to decline from the 20th week onwards to remain at a stable level around birth. There is no difference in the number of VASA positive cells between females with trisomy 21 and controls.

Staining results for Caspase 3 in male and female individuals with trisomy 21 and controls

In males, apoptotic activity, as indicated by a positive staining for Caspase 3, was mainly seen in gonocytes and peaked around 20 weeks GA. Around birth, hardly any apoptotic germ cells could be detected (Figure 3-8). No difference was found between trisomy 21 males and controls ($p = 0.17$). In females, some apoptotic oogonia were observed before 20 weeks GA. The rate of apoptosis in oogonia remained constant throughout pregnancy, without difference between trisomy 21 females and controls ($p = 0.40$). Apoptotic follicles appeared around 20 weeks GA, their number showed a slight increase towards the end of pregnancy (Figure 3-8). Again, no difference could be detected between trisomy 21 females and controls ($p = 0.55$).

Discussion

Boys with trisomy 21 (Down syndrome) have an increased risk of developing a TGCT (1-3). The earliest steps of tumor formation are believed to occur in fetal life. The current hypothesis is that disturbed migration and maturation of germ cells can result in testicular dysgenesis, and finally leads to an increased risk for CIS and invasive neoplasia (8, 18). Prolonged expression of immunohistochemical markers such as OCT3/4, PLAP and c-KIT in various intersex conditions has been interpreted as a result of maturation delay of germ cells, and has been linked to the high incidence of germ cell neoplasia seen in these patients (9, 19, 20). In order to investigate whether germ cell development in trisomy 21 is characterized by maturation delay, we examined the expression profiles of an established set of immunohistochemical markers (see Table 3-2 for an overview of expression profiles and references) during fetal life and early childhood in trisomy 21 individuals and compared them to age-matched controls.

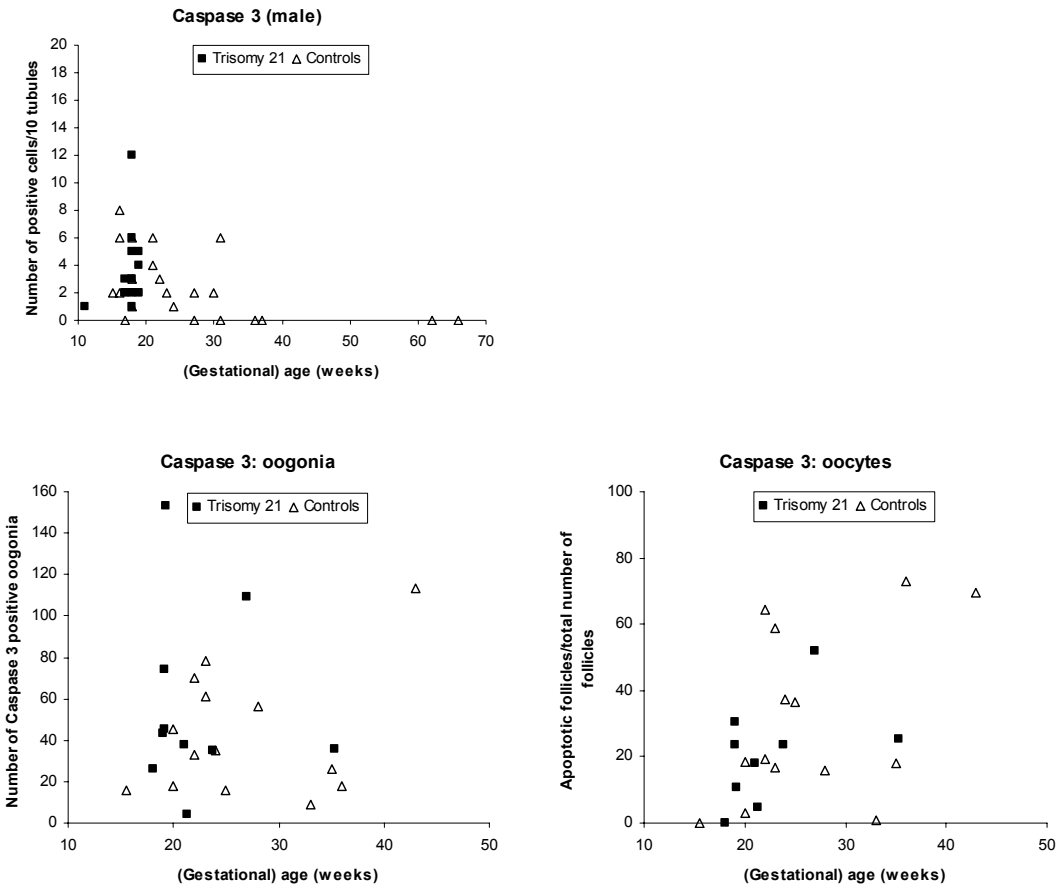

Figure 3-8. Staining results for Caspase 3 in male and female individuals with trisomy 21 (black squares) and controls (triangles). Top: In males, apoptotic activity is highest in early pregnancy but decreases afterwards and is almost undetectable around birth. There is no difference in apoptotic activity between trisomy 21 and control males. Bottom left: Apoptosis is frequently observed in oogonia throughout pregnancy. There is no difference in the number of apoptotic oogonia in trisomy 21 females and controls. Botom right: Apoptotic oocytes appear at 20 weeks GA and their number increases towards the end of pregnancy. There is no difference in the number of apoptotic oocytes in trisomy 21 females and controls.

Table 3-2: Immunohistochemical markers used for the diagnosis of CIS and invasive germ cell tumors.

	CIS	Seminoma	Non- seminoma	Reference
VASA	+	+	-	(16)
TSPY	++	variable	-	(15, 26)
OCT3/4	++	+	EC	(14)
c-KIT	++	variable	-	(12,31)
PLAP	++	++	EC	(13)

EC: embryonal carcinoma; CIS: carcinoma *in situ*

The expression profiles of OCT3/4, c-KIT, PLAP, TSPY and VASA during fetal life have recently been described in detail by our group (10, 11). The same series of fetal gonads served as controls for the present study. In male trisomy 21 fetuses, OCT3/4, PLAP and c-KIT are expressed longer and at higher levels throughout pregnancy than in controls. For OCT3/4

and PLAP, this differential expression extends well beyond birth. In female trisomy 21 fetuses, OCT3/4 and PLAP are also expressed at a higher level during the first half of the second trimester, but thereafter, their expression decreases more sharply than in controls, so that no differences between female trisomy 21 fetuses and controls are seen around 35 weeks GA. c-KIT expression did not differ between female individuals with trisomy 21 and controls. Based on these results, it is concluded that germ cell development is delayed in males and females with trisomy 21, but that this delay is more pronounced in the male group.

According to the results for VASA, no difference in germ cell numbers between female patients and controls was observed. However, in males with trisomy 21, lower numbers of VASA positive cells as compared to controls point at a disturbed development of maturing germ cells.

TSPY is expressed at a higher level and the staining intensity is more pronounced in the germ cells of the trisomy 21 males than in controls. Interestingly, in trisomy 21, TSPY expression is not only seen in prespermatogonia, but is aberrantly expressed at a high frequency in gonocytes. In controls, TSPY expression is mainly confined to prespermatogonia, TSPY positive gonocytes are only rarely encountered in this group. A summary of the expression profiles throughout pregnancy for the different markers in trisomy 21 and controls is presented in Figure 3-9.

Prolonged expression of OCT3/4 and aberrant expression of TSPY as it is found in PGC/gonocytes of trisomy 21 males may be related to tumorigenesis in these cells. OCT3/4 is normally expressed in embryonic stem cells, regulating pluripotency and differentiation of these cells (21). However, OCT3/4 expression in PGC is related to the survival of these cells, preventing them from premature apoptosis (22). Moreover, OCT3/4 is consistently expressed in various germ cell tumors, including CIS and gonadoblastoma (14, 20, 21, 23). In an in vitro model investigating mouse embryonic stem cell-derived tumors, modulating the level of OCT3/4 expression changed the malignant phenotype of the tumor cells, thereby suggesting a pathogenetic relevance of OCT3/4 expression in these tumors (24).

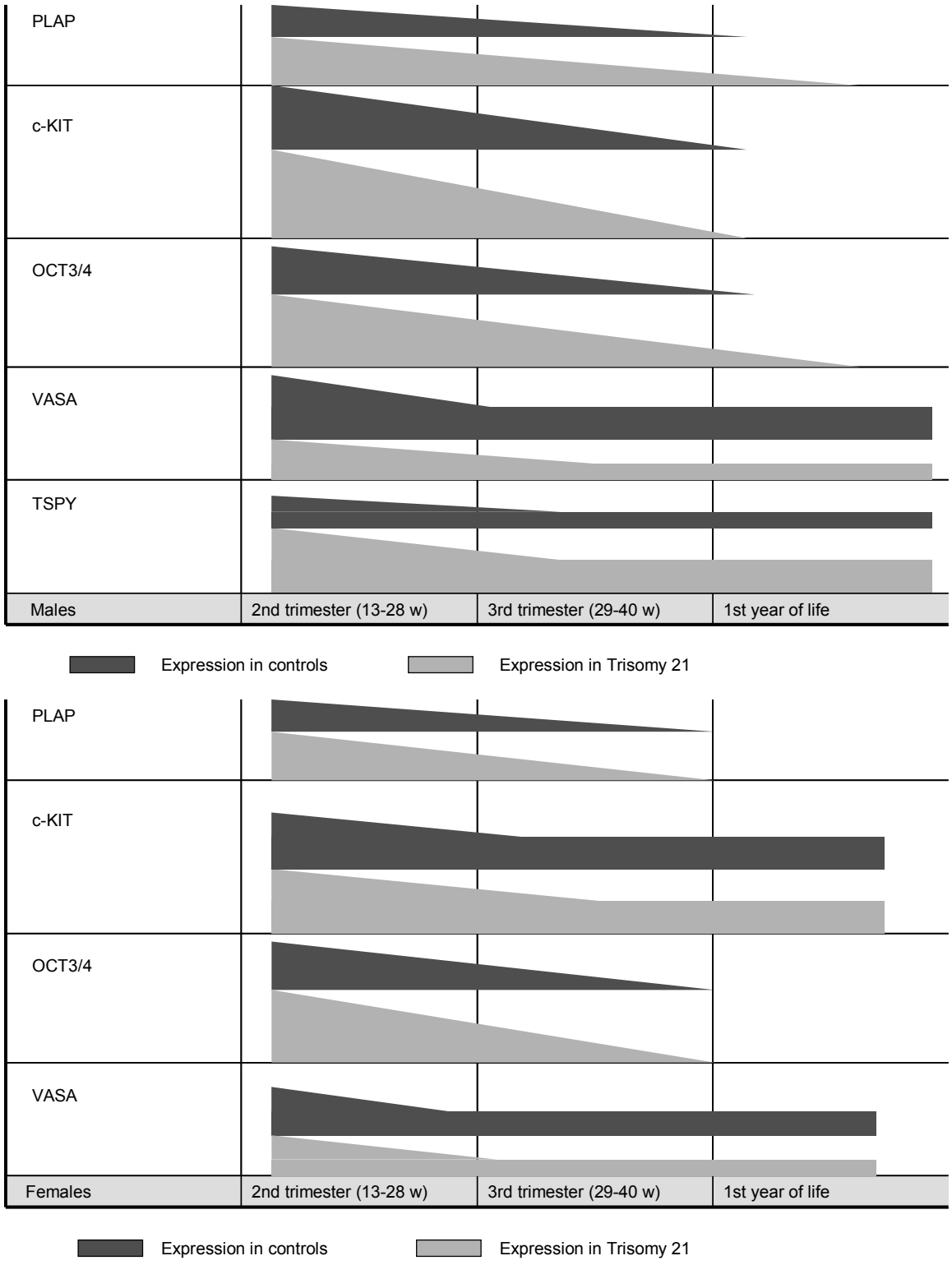

Figure 3-9. Summary of the expression profiles for the different markers in male (top) and female (bottom) trisomy 21 gonads (grey bars) and controls (dark grey). The PGC markers OCT3/4, PLAP and c-KIT are expressed at a higher level throughout pregnancy, and the expression of OCT3/4 and PLAP is prolonged in trisomy 21 as compared to controls, pointing at a delay in the maturation of germ cells in trisomy 21. The delay is more pronounced in males than in females with trisomy 21. In males, VASA is expressed at a higher level in the control group, indicating that in this group, more maturing germ cells can be found as compared to trisomy 21 males. Aberrant TSPY expression is observed in gonocytes of trisomy 21 patients, resulting in a higher total number of TSPY positive cells in this group as compared to controls.

The *TSPY* gene maps to the short arm of the Y chromosome, close to the centromeric region (the so-called GBY region or gonadoblastoma-susceptible region on the Y chromosome), where it is highly repeated (25). Although the function of TSPY is not fully clarified, it is thought to be involved in pre-meiotic proliferation of male germ cells (15). Aberrant (increased) expression of TSPY in patients with undervirilization syndromes and gonadal dysgenesis has been repeatedly related to the development of CIS and gonadoblastoma in these patients (9, 15, 23, 26 and unpublished observations).

Increased apoptosis of germ cells has been described in sex chromosome aneuploid fetal human gonads (27). Apparently, aneuploidy of autosomes does not provoke a similar apoptotic wave during fetal life, since in our study, no difference in apoptotic activity between patients and controls was observed. Yet, the mechanisms by which germ cell numbers in fetal human ovaries are regulated remain to be elucidated (28).

Taken together, our results suggest that trisomy 21 leads to delayed and possibly disturbed maturation of fetal germ cells. An effect of trisomy 21 on maturation of germ cells has been reported previously (8). With this analysis, we present a detailed and differential expression profile in male and female individuals with trisomy 21 throughout pregnancy for the first time. According to our results, the maturation delay of germ cells is more pronounced in males than in females. An increased incidence in germ cell tumors is only seen in males with Down syndrome. We hypothesize that the prolonged expression of OCT3/4, as is predominantly observed in male individuals with trisomy 21, in combination with an increased TSPY expression, could provide these PGC/gonocytes with an important advantage to survive and proliferate. Eventually, this might, after additional, still unknown pathogenetic hits, lead to clonal expansion of these cells and the development of CIS in the minority of cases. In contrast, earlier downregulation of OCT3/4, possibly due to entry into meiosis, and the absence of TSPY expression, might prevent female individuals with trisomy 21 from malignant transformation.

Moreover, trisomy 21 is characterized by male infertility, while female reproductive capacity seems to be preserved (29). Whether the more pronounced maturation delay in males eventually accounts for the observed difference in gonadal function remains to be elucidated.

Finally, according to our data, identification of male germ cells with aberrant expression of markers such as PLAP or OCT3/4 in individuals with trisomy 21 during embryonic development or in the early postnatal period points at delayed germ cell development rather than plain CIS. Clearly, other pathogenetic hits must be involved, as even though an increased risk exists, only a minority of male individuals with trisomy 21 will eventually progress to CIS and the development of invasive germ cell tumors.

Conclusion

By comparing the expression of several immunohistochemical markers in normal fetal gonads with the presence of these markers in gonads of trisomy 21 fetuses, we were able to demonstrate that germ cell maturation is seriously delayed in the latter group. In contrast to conditions affecting the sex chromosomes, trisomy 21 does not lead to an increase in apoptosis during fetal germ cell maturation. For reasons that still need to be elucidated, the observed maturation delay is more pronounced in male than in female individuals with trisomy 21. The observed delay in maturation could result in a prolonged retention of germ cells in a phase vulnerable to further hits, thereby increasing the risk for the development of CIS.

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4

Morphological and immunohistochemical differences between gonadal maturation delay and early germ cell neoplasia in patients with undervirilization syndromes

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Abstract

The pathogenetic process from delayed maturation towards malignant transformation in the development of germ cell tumors in patients with gonadal dysgenesis is not well understood. The morphology of a developmentally delayed germ cell resembles a carcinoma *in situ* (CIS) cell. Maturation delay of germ cells causes prolonged expression of well-established immunohistochemical markers for CIS such as OCT3/4, PLAP and c-KIT, that normally disappear shortly after birth. This questions their use for the detection of CIS in young children. Additional criteria are needed in order to clearly distinguish the expression of these markers due to maturation delay from the presence of a CIS lesion. This is essential before a biopsy can eventually be proposed as a (temporary) alternative for gonadectomy in intersex patients.

We studied 58 gonads of 30 patients with undervirilization syndromes (androgen insensitivity syndrome (AIS), 17 β-HSD deficiency and Leydig Cell Hypoplasia) with immunohistochemical staining techniques and ploidy determination. These patients have an increased risk for the development of germ cell tumors. OCT3/4 was the most reliable marker for the detection of maturation delay and CIS. Patient age, distribution of OCT3/4 positive cells throughout the gonad and their position within the seminiferous tubule allow to distinguish between maturation delay and CIS. Based on these findings, we detected CIS in 3/30 patients (10%) and in 4/58 gonads (6.9%). Prolonged OCT3/4 expression and excessive TSPY expression appear to be of pathogenetic relevance in the development of these lesions.

Introduction

Undervirilization syndromes can be caused by errors in testosterone biosynthesis, by testicular unresponsiveness to HCG and LH or by defects in androgen-dependent target tissues (1). Patients have an increased risk for the development of germ cell tumors. In the complete androgen insensitivity syndrome (CAIS), this risk is estimated at 2 - 5% (2). In other subgroups, due to low prevalence, incomplete diagnostic information and confusing nomenclature, the incidence is actually unknown. Moreover, criteria for the correct diagnosis of early neoplasia in young intersex patients are lacking (see below). Gonadectomy is the therapy of choice, to exclude tumor development, and to avoid virilization (1).The optimal timing for gonadectomy is controversial, especially in CAIS patients, where peripheral conversion of testosterone into estradiol allows spontaneous development of secondary sexual characteristics. Patients often underscore the unphysiological bioactivity of hormonal replacement therapy as compared to their endogenous hormone production. Therefore, and since the risk of malignancy seems to increase with increasing age, some authors advise to postpone gonadectomy (2-5). However, since germ cell tumors have been described in (pre)pubertal AIS patients, others advocate to remove the gonads or at least to perform a biopsy at the time of diagnosis (6-8).

The diagnosis of carcinoma *in situ* (CIS) in children with intersex conditions is difficult. Morphologically atypical germ cells are commonly seen in their gonads (8-11), and seem to correspond with delayed maturation rather than with malignant transformation (12, and personal observations). Immunohistochemical markers, such as OCT3/4, PLAP and c-KIT, that are normally expressed in embryonic germ cells are well-established markers to detect CIS and some invasive germ cell tumors in adult patients (13-16). These markers are also used to demonstrate CIS in children with intersex conditions (17, 18) and even in fetal gonads (19). However, many of these markers show prolonged expression in dysgenetic gonads (20, 21) and are even normally expressed in young children shortly after birth (22). Therefore, these markers cannot be used as such to detect CIS in young patients with gonadal dysgenesis.

Germ cells in gonadal dysgenesis exhibit a developmental delay and are prone to malignant transformation if they are able to survive in their inappropriate environment (23). The aim of

our study was to describe the process from maturation delay towards germ cell death or development of CIS in a series of patients with undervirilization syndromes and to identify additional tools to distinguish morphological and immunohistochemical features of developmental arrest of the germ cells from a developing CIS. Ploidy determination in the CIS samples was used to gain further insight into the chronological steps leading from maturation delay towards malignancy. The ability to distinguish between these different conditions is essential before a biopsy can be proposed as a safe and temporary alternative for gonadectomy in those patients where preservation of proper hormone production is relevant (see above).

Materials and methods

Tissue samples

Gonadal tissue samples of 30 patients with undervirilization syndromes, aged 1 month to 23 years, were obtained after biopsy (5.4%) or gonadectomy (94.6%), performed in the Sophia Children's Hospital or collaborating centers. All patients had 46,XY karyotypes. The individual diagnoses were established on clinical grounds and hormonal profiles and where possible, confirmed by characterization of the underlying genetic defect (Table 4-1). In all patients, gonads were removed as a prophylactic measure and no cases gave rise to clinical suspicion of a tumor. Bilateral specimens were available from 28 patients and a unilateral specimen from 2 patients.

The gonads of 8 control patients, aged 3 months - 13 years, who suffered from SIDS or sudden death after trauma, were examined to compare staining and counting results.

Table 4-1. Diagnosis of patients with undervirilization syndromes, sex of rearing and localization of the gonads at gonadectomy. CIS according to diagnosis, age and gonadal localization

Diagnosis	Gene defect identified	Sex of rearing	Localization		CIS		
			Abdominal	Inguinal	Age	Abdominal	Inguinal
CAIS	13/15	female	5	10	18	1/5	0/10
PAIS	3/5	female	1	4	13	0/1	1/4
17β-HSD deficiency	5/6	female	0	6	4	0/0	1/6
Leydig Cell Hypopl	1/2	female	0	2		0/0	0/2
unknown	0/2	1 female, 1 male	0	2		0/0	0/2
Total	22/30	29 female, 1 male	6	24		1/6	2/24

CIS: carcinoma *in situ;* hypopl: hypoplasia

Table 4-2. Schematic representation of origin and protocols used for the different antibodies.

Primary antibody	Origin	Dilution	Pre-treatment	HIAR	Incubation	Secondary antibody	AB-complex	Chromogen
VASA	Kindly provided by Dr DH Castrillon*	1/2000	no	yes	overnight, 4°C	SWAR-bio	ABC-AP	New Fuchsine
PLAP	Cell Marque, Hot Springs, AR, USA	1/200	no	yes	overnight, 4°C	RAM-bio	ABC-AP	New Fuchsine
c-KIT	Dako-Cytomation, Glostrup, Denmark	1/500	no	yes	overnight, 4°C	SWAR-bio	ABC-AP	New Fuchsine
TSPY	Kindly provided by Prof C Lau#	1/3000	no	no	overnight, 4°C	SWAR-bio	ABC-AP	New Fuchsine
OCT3/4	Santa Cruz Bio-technology, Santa Cruz, CA, USA	1/1000	H2O2 for 5'	yes	2 h, RT	HAG-bio	ABC-HRP	DAB
2nd AB	**Origin**	**Dilution**						
SWAR	Dako-cytomation	1/200						
RAM	Dako-cytomation	1/200						
HAG	Vector Laboratories, Burlingame, CA, USA	1/200						

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AB: antibody; HIAR: heat induced antigen retrieval (38°; SWAR-bio: swine anti rabbit antibody, biotin labeled; AB-complex: streptavidin-biotin complex; ABC-AP: streptavidin-biotin-alkaline phosphatase complex; ABC-HRP: streptavidin-biotin-horseradish peroxidase complex, RT: room temperature.

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).

Immunohistochemical staining

After routine fixation in 10% formalin, 5µm thickness slices were prepared. Poorly preserved samples were excluded from the study. The antibodies used for immunohistochemistry and a schematic representation of the applied protocols are represented in Table 4-2. Slides were incubated with the primary antibodies in an appropriate dilution. In between the incubation steps, they were washed in a PBS-Tween 0.01% solution. Staining was performed using DAB/H_2O_2 or New Fuchsin/Naphtol ASMX phosphate and counterstaining with hematoxylin. As positive controls, normal adult male gonadal tissue for VASA and seminoma for PLAP, c-KIT, TSPY and OCT3/4 were used.

Double-staining: After pre-treatment with H_2O_2 and pressure-cooking, sections were incubated with VASA (overnight) and OCT3/4 (2 hours). OCT3/4 was detected using the avidin-biotin-alkaline phosphatase complex and Fast Blue/Naphtol ASMX phosphate for a blue staining. After this, free biotin was blocked using a blocking KIT (Vector Laboratories,

Burlingame CA, USA). VASA was detected, using the avidin biotin-horseradish peroxidase complex with 3-amino-9-ethyl-carbazole (Sigma, Steinheim, Germany)/H_2O_2 resulting in a red staining. A male gonad containing normal tissue and CIS served as a positive control.

Quantification of results

General morphology of tissue samples was assessed by a pathologist experienced in germ cell pathology (JWO). Eventual artefacts, due to the fixation procedure were not taken into account in the final analysis.

Results were quantified as follows: For each tissue sample, 500 seminiferous tubules were assessed for the staining pattern of OCT3/4, VASA and TSPY. One tubule was considered positive for a marker if at least one germ cell in the tubule stained clearly positive. All counts were performed by the same observer (MC), blinded for age and origin (patient or control) of the sample. The obtained data were graphically depicted using the SPSS statistical system.

Determination of ploidy

Ploidy of the CIS samples was determined by fluorescent *in situ* hybridization (FISH). Therefore, tissue sections of 5 μm were digested with 0.0005% pepsin (Sigma-Aldrich, Zwijndrecht, The Netherlands) in 0.01 mol/L HCl for 1 minute at 37°C, rinsed in demi water and dehydrated. The protocol was used according to Hopman et al (24). The following centromeric probes were used: chromosome 1 (pUC1.77), 12 (pα12h8) (25, 26), X (BamH1) and Y (DYZ3). Probes were labelled with biotin-16-dUTP (Roche, Mannheim, Germany) using a nick-translation kit (Gibco BRL, Paisley, UK). After denaturation (73°C for 5 min in hybmix) they were pre-annealed with an excess of Cot-1 DNA (Life Technologies/ Gibco BRL, Paisley, UK), added to denatured slides (3 min in 70% formamide/2*SSC pH 7.0, 5 min 70% ethanol at -20°C and dehydrated) and hybridized for 48 hours. Slides were washed in 50% formamide/2xSSC. The hybrids were visualized with Cy3 conjugated avidin antibody (1:50, Jackson ImmunoResearch, West Grove, PA, USA).

Table 4-3. WHO criteria for the diagnosis of CIS (World Health Organization Classification of Tumors (international agency for research on cancer) (39)

CARCINOMA *IN SITU*
Larger than normal spermatogonia
Clear or vacuolated cytoplasm, rich in glycogen
Nuclei: large, irregular, hyperchromatic
(Abnormal) mitoses
Basally located cells
Spermatogenesis commonly absent
Segmental involvement of tubules

Results

General morphology of gonads and germ cells

The gonads of patients exhibited an immature aspect of tubules and cells. Dissociation between epididymis and testis was observed in 4 samples. Seminiferous tubules were small as compared to controls, branching tubules were encountered in 4 patients. The degree of peritubular and interstitial fibrosis and thickening of the basal membrane was variable. Apparent Leydig cell hyperplasia was found after puberty (6 samples). Sertoli cell only nodules and nodular hyperplasia of Sertoli cells were encountered in older patients (8 samples).

Germ cells were found in 28/30 patients and 50/58 gonads. They were very large and irregular of shape, with an abundant pale cytoplasm and a hyperchromatic nucleus. Multinucleated spermatogonia were often encountered. No maturation of germ cells, expected based on age, was observed, except for two pubertal PAIS patients in whom spermatocytes and spermatids were seen. Spermatozoa were never found. The number of germ cells was high in the youngest patients but a progressive loss of germ cells was noted with advancing age. This loss presented itself as patchy: certain areas maintained an adequate number of germ cells while in other areas all germ cells had disappeared. Except for the PAIS patients, the end result as could be appreciated in post pubertal patients was a Sertoli-cell only pattern in atrophic tubules throughout the gonad. On pure morphological grounds, an adult CIS pattern was discovered bilaterally in one 18 year old CAIS patient. In many other patients, the aberrant appearance of the germ cells raised suspicion about the presence of CIS since their morphology largely met the WHO criteria (Table 4-3).

Immunohistochemical staining for OCT3/4, c-KIT, PLAP, TSPY and VASA, and double staining for the combination OCT3/4-VASA was performed.

Staining results for OCT3/4, c-KIT and PLAP (Figure 4-1)

OCT3/4 positive germ cells were found in all the gonads of patients below 9 months of age. OCT3/4 was also widely present in a control of 3 months old, whereas at 5 and 6 months, only 3/500 and 2/500 tubules respectively contained an OCT3/4 positive germ cell. Thereafter, no OCT3/4 positive cells were identified in controls. In all but 3 patients OCT3/4 expression had disappeared at 9 months: one 4 year old patient with 17 β-HSD deficiency, one 13 year old PAIS patient and the CAIS patient of 18 years in whom CIS was discovered in the HE staining. In this patient, OCT3/4 positive cells corresponded with the CIS cells on the HE staining. In the former 2 patients, the morphology of the germ cells was judged as clearly aberrant (see above). The distribution of the OCT3/4 positive cells throughout the gonad in these 3 patients was different from the distribution pattern in younger patients and controls: in the former, OCT3/4 positive tubules were confined to a specific area of the gonad, separated from the unstained areas by interstitial fibrous septa, whereas in young patients and controls, OCT3/4 positive cells were scattered throughout the gonad, without any preference for a certain area (Figure 4-2). Also within the tubules, the distribution of OCT3/4 positive cells was different: In young patients and controls, they were found almost exclusively centrally in the tubule, separated from the basal lamina by at least one layer of Sertoli cells. In the 13 year old PAIS patient and the 18 year old CAIS patient, OCT3/4 positive cells were confined to the basal lamina, whereas in the 4 year old patient with 17 β-HSD deficiency, OCT3/4 positive cells were equally present centrally and along the basal lamina. This was nicely demonstrated in the OCT3/4-VASA double staining (see below).

Staining patterns for c-KIT and PLAP were comparable to the OCT3/4 staining, both for timing of disappearance of the marker as for localization of positive cells within the tubules but the color intensity was generally weaker than the OCT3/4 staining.

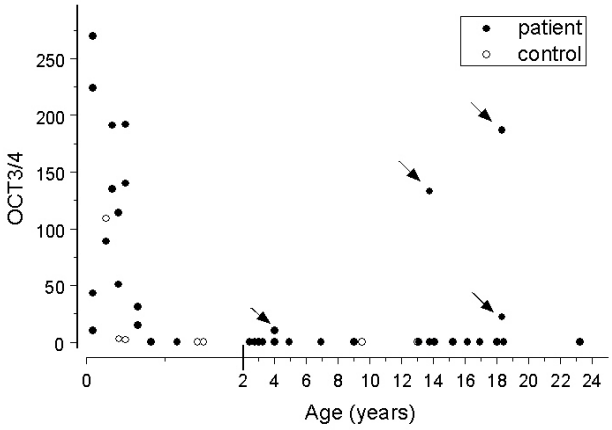

Figure 4-1. Staining results for OCT3/4: Number of tubules containing at least one OCT3/4 positive germ cell (Y-axis) according to age (X-axis). Remark the high and prolonged OCT3/4 expression in patients as compared to controls during the first year of life. No OCT3/4 positive tubules are found anymore in the control patients above the age of 6 months and in the patient population above the age of 8 months, except for the 4 year old patient with 17 β-HSD deficiency (10/500 positive tubules in one gonad), the 14 year old PAIS patient (133/500 positive tubules in one gonad) and the 18 year old CAIS patient (187/500 positive tubules in one gonad and 22/500 positive tubules in the other gonad) (arrows).

Staining results for VASA and TSPY (Figures 4-3 and 4-4)

VASA is a general marker for germ cells (13), and thus allowed us to estimate the total number of germ cells throughout time in the study population relative to the controls. In the latter, VASA positive germ cells were seen in nearly all the tubules, at every age. In the study population, the number of tubules expressing VASA was comparable to controls only shortly after birth. From the 10th month onwards, a rapid and progressive decline in VASA expression was observed, suggesting a massive loss of germ cells. This loss was manifest in all diagnostic groups, except in the PAIS patients, who maintained about 2/3 of their germ cell population as compared to controls at the age of 15.

TSPY stains spermatogonia of adult men (27) and was also observed in prespermatogonia of fetal testes during the second and third trimester of gestation (22). No data exist on the expression of TSPY in prepubertal children. In controls, TSPY expression clearly differed from VASA expression: while VASA stained all the germ cells, irrespective of age, TSPY expression was gradually upregulated, and reached the level of VASA expression only around puberty. In patients however, staining of adjacent slides for TSPY and VASA demonstrated that, as for VASA, TSPY stained all the (remaining) germ cells at every age. Thus, in controls, VASA was expressed at a constant level throughout time, pointing at a continuous presence of germ

cells in almost every tubular cross-section, while TSPY expression increased gradually. In patients, both TSPY and VASA were highly expressed during the first year of life and showed a parallel decrease thereafter. Interestingly, the intensity of the TSPY staining in the patient population was extremely high as compared to controls, a normal adult male testis and a series of fetal testis published elsewhere (22) (Figure 4-5).

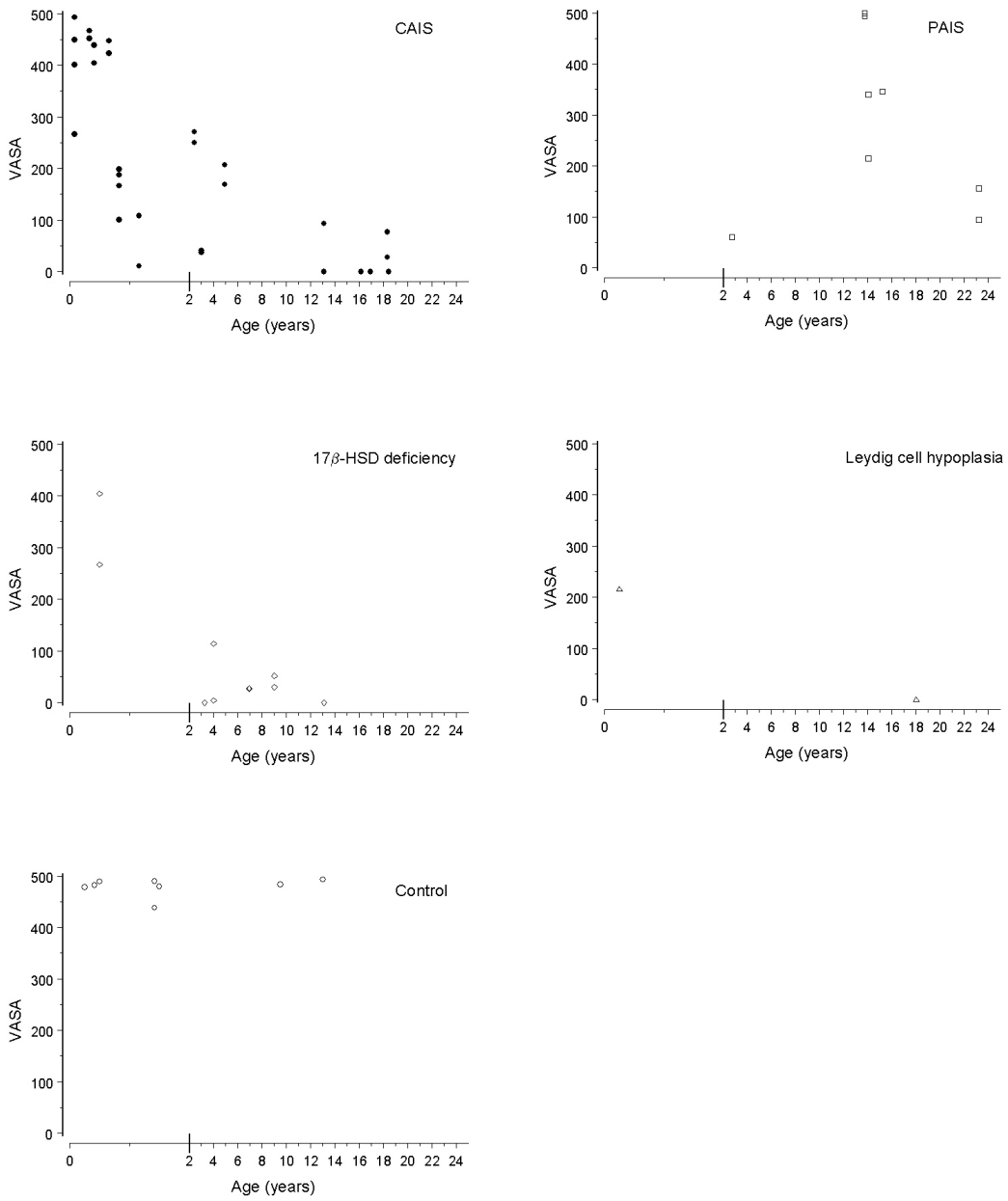

Figure 4-3. Staining results for VASA: Remark the loss of germ cells around puberty in all the patient groups, except for the PAIS patients and the 18 years old CAIS patient with bilateral OCT3/4 positive cells.

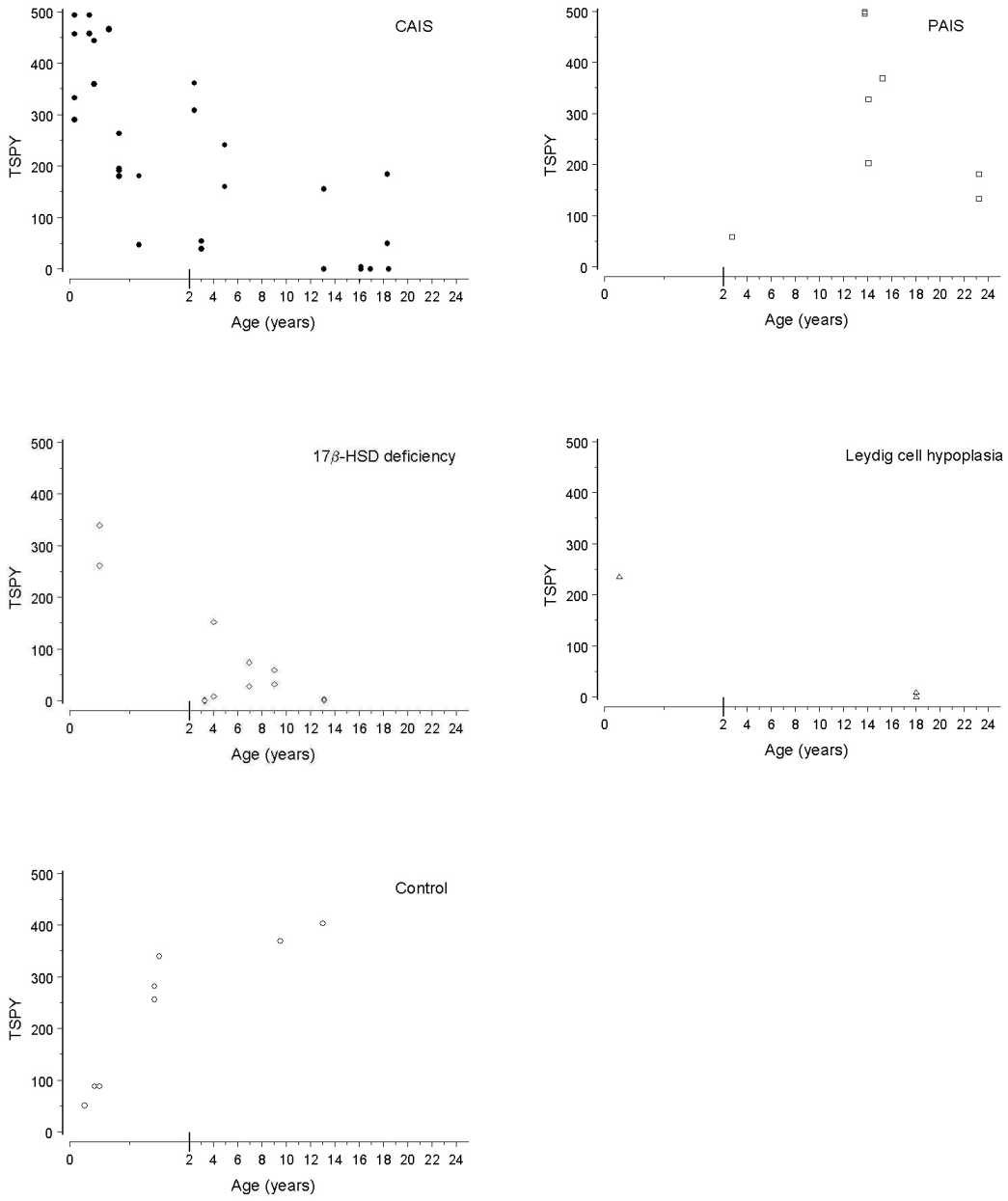

Figure 4-4. Staining results for TSPY: Remark the analogous pattern of germ cell loss for the different patient groups as compared to VASA, but the increase in TSPY expression in the controls, which is different from the constant VASA expression in this group.

OCT3/4-VASA double staining (Figure 4-6)

This staining was performed to appreciate the position of OCT3/4 positive germ cells relative to the overall germ cell population and to the basal lamina within the tubule.

The 3 month old control and the patients below 9 months of age showed the same staining patterns: OCT3/4 positive cells were almost exclusively located centrally in the tubule, whereas VASA positive cells lined up along the basal lamina. OCT3/4 positivity excluded

VASA positivity in the same germ cell. The 13 year old PAIS patient and the 18 year old CAIS patient showed a different distribution pattern with OCT3/4 positive cells lying almost exclusively on the basal lamina. VASA expression was lost in tubules expressing OCT3/4. The 4 years old patient with 17 β-HSD deficiency showed an intermediate pattern: OCT3/4 positive cells were found equally in the tubular center and on the basal lamina. Some of these germ cells co-expressed VASA, and VASA positive-OCT3/4 negative cells were also seen within the same tubule.

Results of ploidy determination

The precursor CIS lesions of seminomas and non-seminomas are aneuploid (28, 29). To evaluate the ploidy of OCT3/4 positive cells in the 13 year old PAIS patient and the 18 year old CAIS patient, FISH analysis with centromeric probes for chromosomes 1, 12, X and Y was performed. In these patients, the OCT3/4 positive regions were found to be diploid.

Discussion

We studied 58 gonadectomy and gonadal biopsy samples in 30 patients with undervirilization syndromes to investigate the relationship between maturation delay and progression to CIS. To our knowledge, this represents the largest series ever published. In spite of a certain heterogeneity in clinical diagnoses, we consider the group of undervirilization syndromes as homogenous, taken into account the normal embryonic gonadal development and the common end result (a defective or absent action of androgens on the end organs) that characterizes these syndromes and opposes them to the gonadal dysgenesis syndromes. An important variable that we were not able to correct for is the localization of the gonads, which was abdominal in 6/30 patients (20%) and inguinal in 24/30 patients (80%). However, the finding that 2 of 3 CIS lesions were detected in inguinal gonads underscores the malignant potential of partially descended testes (Table 4-1).

The general morphology of gonads and germ cells in our patient series did not differ from previous findings (8, 9, 18), but the timing and pattern of germ cell loss are described systematically for the first time. The cryptorchid position of the gonads in our study population certainly accounts for this loss of germ cells, alone or in combination with other pathogenetic mechanisms relative to the underlying disease.

OCT3/4, PLAP and c-KIT are well-established markers for the diagnosis of CIS and invasive germ cell tumors in adults (14-16). They are also used to detect CIS in dysgenetic gonads of young children (17, 18) and in fetal gonads (19). However, the expression of these markers is not limited to malignant conditions. They have been demonstrated in the developing fetal testes, only to disappear well beyond birth (22), while TSPY and VASA are expressed throughout life (13, 27). High and prolonged expression of c-KIT, OCT3/4 and PLAP has been observed in dysgenetic gonads and in gonads of patients with chromosomal anomalies and has been related to a degree of maturation delay (20, 21, 30 and Cools et al, submitted). This condition itself predisposes germ cells to malignancy (23).

OCT3/4 is a transcription factor, normally expressed in embryonic stem cells and embryonic germ cells, and is essential in regulating pluripotency and differentiation (14). Recently, a specific role of OCT3/4 for the survival of migratory primordial germ cells has been demonstrated, preventing them from premature apoptosis (31). Moreover, OCT3/4 has been shown to be consistently present in various germ cell tumors, including CIS and gonadoblastoma (14, 32). By modulating the level of OCT3/4 expression in vitro in mice embryonic stem cell-derived tumors, the malignant phenotype of the tumor cells could be changed, suggesting that OCT3/4 is of pathogenetic relevance in the development of these tumors (33).

In our series, patients below 9 months of age showed a prolonged and higher OCT3/4 expression level compared to controls. This is in line with previously published data where OCT3/4 was detected in the gonads of a 9 months old AIS patient, but disappeared in older patients (20). OCT3/4 expression was never seen in patients older than 9 months, except for three cases. In the 2 oldest, in contrast to younger patients and controls, OCT3/4 positive cells were limited to one specific region of the gonad, where the germ cells homogenously stained positive for this marker and did not express VASA. Within the tubule, OCT3/4 positive cells stuck to the basal lamina, a pattern that is generally found in CIS. The normal central position of OCT3/4 positive cells in the developing tubules, as it was encountered in young patients and controls, was described previously and it was suggested that by reaching the basal lamina, early germ cells loose their pluripotency and start to differentiate (22). An intermediate pattern was found in one of six patients with 17 β-HSD deficiency, 4 years of

age: OCT3/4 positive germ cells were confined to a specific region of the gonad, but within the tubule, they were found both in a peripheral and a central position. Tubules with OCT3/4 positive cells also expressed VASA, sometimes showing co-expression of these two markers in the same cell. The remaining 5 patients with 17 β-HSD deficiency did not differ from other patients in our series, suggesting that this group does not represent a separate entity.

Thus a possible pathogenetic mechanism for the development of germ cell neoplasia emerges: The central OCT3/4 positive germ cells, scattered throughout the gonad are delayed in maturation. By moving towards the basal lamina, they will loose their pluripotency and eventually start to differentiate. If this process does not occur in one OCT3/4 positive cell, and the cell is not removed by an apoptotic or other mechanism, clonal expansion of this pluripotent cell may lead to the CIS pattern as it was encountered in the 4 year old patient, and at a later age to the typical CIS pattern as it is seen in adults. The expression of PLAP and c-KIT was similar to OCT3/4, but showed less consistency. Therefore, we conclude that OCT3/4 is the best marker to describe maturation delay and CIS in patients with undervirilization syndromes. Age of the patient, distribution of OCT3/4 positive cells throughout the gonad and staining pattern within the tubule seem to be useful additional tools to differentiate between maturation delay and CIS in the young patient. Our results suggest that the presence of germ cells positive for OCT3/4, PLAP or c-KIT in patients younger than 1 year is in accordance with the expected maturation delay in this patient group and is thereby insufficient for the diagnosis of CIS. However, the relevance and applicability of these tools in larger patient series and other diagnostic groups must be tested before they can be widely accepted as essential diagnostic criteria.

An intriguing finding in our patient population, and similar tot the findings of Schnieders and colleagues (27) was the abnormal and abundant expression pattern of TSPY. The TSPY gene maps to the short arm of the Y chromosome, close to the centromeric region, where it is highly repeated. Although its function is not fully clarified, it is thought to regulate the mitotic proliferation of spermatogonia (27). The expression of TSPY in dysgenetic gonads has been related to the development of gonadoblastoma (32, 34). It is tempting to speculate that TSPY expression is upregulated in germ cells residing in an unfavorable environment in an attempt to survive and proliferate. The combination of maturation delay, prolonged

expression of OCT3/4 and abundant TSPY expression can provide the surviving germ cell with an important proliferative advantage, rapidly leading to clonal expansion and the development of CIS.

The CIS cells in the 18 years old CAIS patient and in the 13 years old PAIS patient were diploid. However, polyploidisation is known to be an early event in the development of CIS in the adult testis (28, 29, 35). On the other hand, gonadoblastomas arising in dysgenetic gonads are often found to be diploid (28, 36), although aneuploidy was also described (37). It is unclear so far whether polyploidisation did not take place yet in our patients, thereby representing a very early CIS stage, or alternatively, whether polyploidisation is not essential in the development of CIS in the gonads of undervirilized patients.

In conclusion, we discovered CIS lesions in 3/30 patients (10%) and in 4/58 gonadectomy or biopsy specimens (6.9%). Morphological criteria for the detection of CIS in adult patients and the classic use of immunohistochemical markers were insufficient to describe CIS in our patients. Based on our results, patient age, distribution pattern of OCT3/4 positive cells throughout the gonad and their position within the seminiferous tubule can additionally be used to distinguish maturation delay from CIS in young patients with undervirilization syndromes. However, larger patient series and extension of these tools to other situations that are characterized by gonadal maturation delay and increased risk for germ cell neoplasia are essential before they can be accepted as essential criteria for the diagnosis of CIS in young patients.

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5

Gonadoblastoma arising in undifferentiated gonadal tissue within dysgenetic gonads

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Abstract

Purpose: To define the histological origin of gonadoblastomas, allowing the identification of high-risk patients.

Experimental design: Sixty paraffin embedded gonadectomy or gonadal biopsy samples of 43 patients with gonadal dysgenesis were selected from our archives. We studied the morphology and immunohistochemical properties of the germ cells in 40 samples without neoplastic transformation and compared these findings to the morphological and immunohistochemical characteristics of 20 samples containing gonadoblastoma/dysgerminoma.

Results: The overall incidence of germ cell tumors in our patient series was 35%. In dysgenetic gonads without germ cell neoplasia, besides the presence of areas with testicular and/or ovarian differentiation, areas of undifferentiated gonadal tissue were identified in 13/40 (32.5%) samples. A subpopulation of germ cells within these undifferentiated areas stained positive for OCT3/4, c-KIT, PLAP and TSPY. Gonadoblastoma germ cells display identical staining results. Moreover, in gonads containing gonadoblastoma, adjacent to this lesion, areas of undifferentiated gonadal tissue with identical immunohistochemical characteristics were identified in 10/20 (50%) samples. No adjacent tissue was available in 5 cases, while in the 5 remaining cases it consisted of streak tissue. In 3 cases, an accumulation of OCT3/4 positive germ cells in the proximity of the malignant lesions was found, suggesting clonal expansion and final organisation into gonadoblastoma nests.

Conclusions: Based on these observations, we hypothesize that gonadoblastomas originate from surviving OCT3/4 positive germ cells in areas of undifferentiated gonadal tissue within the dysgenetic gonad. Supportive evidence was obtained that carcinoma in situ arises in regions with testicular differentiation.

Introduction

Gonadoblastoma is histologically defined as "a tumor composed of two principal cell types: large germ cells similar to those of seminoma and small cells resembling immature Sertoli and granulosa cells; elements resembling Leydig or lutein-like cells may also be present" (1). This premalignant lesion of the dysgenetic gonad is the counterpart of the more frequent carcinoma in situ (CIS) lesion, which is found in well-differentiated testicular tissue (2). Gonadal dysgenesis is defined as an incomplete or defective formation of the gonads, resulting from a disturbed process of migration of the germ cells and/or their correct organization in the fetal gonadal ridge. It is caused by structural or numerical anomalies of the sex chromosomes or mutations in one of the genes involved in the formation of the urogenital ridge and in sex determination of the bipotential gonad. Neoplastic transformation of germ cells in dysgenetic gonads (the formation of gonadoblastoma and/or an invasive germ cell tumor) occurs, according to literature data in 20-30% of cases and is associated with the presence of (part of) the Y chromosome in the patients' karyotype (3, 4). It is usually diagnosed at a young age (3, 5, 6). Therefore, early gonadectomy, often combined with gender reassignment and genital surgery, is mostly advocated (3, 6, 7). This safe but radical approach results definitely in infertility and life-long dependence of hormonal replacement therapy in all patients. However, genital surgery and early gender assignment procedures have become controversial (8-11). Hormonal substitutes are sometimes considered as unphysiological as compared to endogenous hormone production. Advances in surgical techniques allow now rearing an individual born with ambiguous genitals as a male, preferably with his gonads positioned into the scrotum (9, 12). The incidence of malignancy in true hermaphroditism is estimated at 2-10% (4, 13), and is thus considerably lower than in other diagnostic groups. Interestingly, preservation of gonadal function has been described mainly in this specific group (13, 14) but also in some other patients with gonadal dysgenesis (15, 16). These observations led the French group of Josso and co-workers to propose a more conservative approach regarding gonadectomy in true hermaphroditism (14).

Dysgenetic gonads containing a germ cell tumor have been examined in more detail in several patient series (5-7, 15, 17). These combined data reveal that the gonad of origin is considered as a dysgenetic testis in 19.8% of cases, a streak in 26.1% and that it could not be

determined in 54.1% of cases. The impossibility to predict (e.g. from a gonadal biopsy) which gonad is prone to neoplastic transformation hampers the application of a more conservative approach relative to gonadectomy on a wider scale.

To gain insight into the nature of the gonads in which gonadoblastoma and invasive tumors may arise, we studied the histological and immunohistochemical properties of 40 dysgenetic gonads removed as a prophylactic measure and in which no malignancy was detected on routine pathological examination, and compared them to the histological and immunohistochemical characteristics of 20 gonads containing gonadoblastoma and/or dysgerminoma lesions. Immunohistochemistry was performed with the antibodies OCT3/4 (Octamer binding transcription factor 3/4), c-KIT (the stem cell factor receptor) and PLAP (Placental Like Alkaline Phosphatase), which are normally expressed in primordial germ cells/gonocytes and are well-established markers for the diagnosis of CIS and gonadoblastoma (18-22). Furthermore, the expression of TSPY (Testis Specific Protein-Y encoded), encoded by the TSPY gene and the main candidate gene responsible for the development of gonadoblastoma (19-21, 23, 24) and VASA (the human homologue of the mouse vasa gene), a general marker for germ cells (20, 25) was examined (Table 5-1). Sertoli/granulosa cells were examined for their expression of AMH (Anti Müllerian Hormone).

Materials and Methods

Tissue samples

Forty-five patients with gonadal dysgenesis were retrieved from our archives. Samples of 20 dysgenetic gonads containing gonadoblastoma and/or dysgerminoma in 16 different patients and 40 dysgenetic gonads without apparent malignancy in 27 different patients were selected; three samples in two patients were excluded due to bad preservation of material. Patient and tissue characteristics are summarized in Table 5-2. Two fetal samples (Table 5-2, patients 1 and 2), obtained after induced abortion were included. Patient 5 underwent a bilateral gonadal biopsy at four months and a right gonadectomy two months later. A left gonadectomy was performed at 14 years. Patient 26 underwent a right gonadectomy at 8 years and a left gonadectomy at 25 years.

Use of tissues for scientific reasons was approved by an institutional review board. 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 (version 2002) (26). Samples originating from collaborating centers in Belgium, Poland and Indonesia were treated in accordance with the above mentioned as well as local medical ethical guidelines.

Immunohistochemical staining

Tissue material was fixed in 10% formalin or Bouin's fixative, according to local fixation procedures. Slices of 3-5 µm thickness were prepared.

The antibodies used for immunohistochemistry and a schematic representation of the applied protocols are represented in Table 5-1. Slides were incubated with the primary antibodies at appropriate dilutions, staining was performed using DAB/H2O2 or New Fuchsin/Naphtol ASMX phosphate, and counterstaining with hematoxylin. As positive controls, a normal adult male gonad for VASA, a seminoma sample for PLAP, c-KIT, TSPY and OCT3/4 and a male fetus, 8w GA for AMH were included.

Table 5-1: Schematic representation of origin and protocols used for the different antibodies

Primary antibody	Origin	Code	Dilution	Pre-treatment	HIAR	Incubation	Secondary antibody	AB-complex	Chromogen
VASA	Kindly provided by Dr DH Castrillon*		1/1000	no	yes	overnight, 4°C	SWAR-bio	ABC-AP	New Fuchsine
PLAP	Cell Marque, Hot Springs, AR, USA	CMC203	1/200	no	yes	overnight, 4°C	RAM-bio	ABC-AP	New Fuchsine
c-KIT	Dako-Cytomation, Glostrup, Denmark	A4502	1/500	no	yes	overnight, 4°C	SWAR-bio	ABC-AP	New Fuchsine
TSPY	Kindly provided by Prof C Lau#		1/3000	no	no	overnight, 4°C	SWAR-bio	ABC-AP	New Fuchsine
OCT3/4	Santa Cruz Bio-technology, Santa Cruz, CA, USA	SC8629 and SC5279	1/1000	H_2O_2 for 5' + biotin blocking	yes	2 h, RT	HAG-bio	ABC-HRP	DAB
AMH	Kindly provided by Prof A Themmen$		1/200	H_2O_2 for 5' + biotin blocking	yes	overnight, 4°C	RAM-bio	ABC-HRP	DAB

Secondary antibody	Origin	Dilution
SWAR	Dako-cytomation	1/200
RAM	Dako-cytomation	1/200
HAG	Vector Laboratories, Burlingame, CA, USA	1/200

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HIAR: heat induced antigen retrieval (44); SWAR-bio: swine anti rabbit antibody, biotin labeled; RAM-bio: rabbit anti mouse antibody, biotin labeled; HAG-bio: horse anti goat antibody, biotin labeled; AB-complex: streptavidin-biotin complex; ABC-AP: streptavidin-biotin-alkaline phosphatase complex; ABC-HRP: streptavidin-biotin-horseradish peroxidase complex, RT: room temperature.

Double-staining experiments were performed using the same detection methods but with different substrates: Fast Blue/Naphtol ASMX phosphate (F3378 and N500, Sigma, Steinheim, Germany) for a blue staining and 3-amino-9-ethyl-carbazole (A.5754 and D4254, Sigma, Steinheim, Germany)/H2O2 for a red staining, without counterstaining. To reduce background signal, endogenous peroxidase activity and endogenous biotin were blocked using 3% H2O2 (5 minutes) and a blocking kit for endogenous biotin (Vector Laboratories, Burlingame CA, USA). For a correct interpretation of histology, HE staining was performed on parallel slides.

Interpretation of results

General morphology and interpretation of the staining results were assessed by 2 observers with experience in germ cell pathology (MC and JWO). Part of the results obtained in five gonadoblastoma samples were reported previously (21).

Statistical analysis

Results were analyzed using the SPSS (SPSS 11.0 for Windows) statistical program (Fisher Exact test for 2 x 2 tables).

Results

Morphology and staining results in dysgenetic gonads without neoplastic transformation

Four gonadal differentiation patterns were recognized in various combinations (Tables 5-2 and 5-3 and Figure 5-1):

Tissue containing seminiferous tubules was encountered in 24/40 gonads (60%) and was considered as a testicular differentiation pattern. The tubules often displayed abnormalities such as a thin basal lamina, shape irregularity, increased intertubular spaces and branching structures, reflecting their dysgenetic nature (Figure 5-1A). Germ cells were found in 20/24 samples (83%) and were easily recognized by positive staining for VASA and TSPY. The TSPY staining was consistently very intense as compared to normal adult and age-matched controls as well as normal fetal testicular samples (personal observations). Staining of germ cells for OCT3/4, c-KIT and PLAP was performed in 18/24 samples, in the remaining six it

could not be performed due to limited material. A subpopulation of germ cells stained positive for the three markers in 14/18 samples (78%). In all cases, positive cells were found centrally in the tubule, pointing at a delay in maturation, but 7/14 patients (50%) also displayed positive germ cells on the basal lamina (Figure 5-1B). OCT3/4-VASA double staining experiments confirmed these findings. In general, OCT3/4 expression was lost as cells became positive for VASA, yet sometimes OCT3/4-VASA co-expression was observed within a single cell (Figure 5-1C). Regardless of their dysgenetic aspect, in all samples the majority of the tubules displayed positive staining for AMH in Sertoli cells.

An ovarian differentiation pattern was defined as gonadal tissue containing germ cells enclosed in primordial and eventually growing follicles, comparable to the ovaries of female neonates (Figure 5-1D). It was encountered in 10/40 gonads (25%). Ova homogeneously expressed VASA and c-KIT, whereas OCT3/4, PLAP or TSPY positive ova were never found. A unique situation was found in patient 3l: follicles, containing VASA positive and TSPY negative ova were enclosed in seminiferous tubules, containing VASA positive, TSPY positive spermatogonia (see Figure 5-2).

A third differentiation pattern, further referred to as undifferentiated gonadal tissue (UGT), consisted of gonadal tissue containing germ cells not enclosed in seminiferous tubules or follicles, but either organized together with Sertoli/granulosa cells in cord-like structures, or residing without apparent organization in a background of fibrous stroma (Figure 5-1E), or a combination of these two. This pattern, though present in 13/40 gonads (32.5%) was mostly not mentioned in the original pathology reports, or alternatively, it was referred to as a streak. Expression of markers in the germ cells within UGT was similar to that found in testicular tissue. Germ cells could easily be identified by their expression of VASA and abundant expression of TSPY. Due to limited material, OCT3/4, PLAP and c-KIT staining could be performed in 11/13 samples. In 9 of them (82%), a subpopulation of germ cells within UGT stained positive for these three markers. OCT3/4-VASA double staining confirmed these results (Figure 5-1F). Sertoli/granulosa cells within UGT differed from their apparently more differentiated counterparts in testicular tubules: in most samples, AMH expression was totally absent, although sporadic weakly positive Sertoli/granulosa cells were found in two samples.

Table 5-2: Overview of patient and tissue characteristics

Patient	Age	Karyotype	Phenotype	Histology					
				T	O	UGT	S	Tumor	Remarks
1	17 w	45,X/46,XY	male	+	-	+	-	-	
2	19 w	45,X (7/20) /46,XY (13/20)	male	+	+	-	-	-	
3l	1 m	46,XX	female	+	+	-	-	-	
3r	1 m			-	+	+	-	-	
4l	4 m	46,XX	female	-	-	-	-	calcified GB	
4r	4 m			-	+	-	+	GB	
5r	4 m	46,XY	female	+	-	-	-	-	r biopsy
5l	4 m			-	-	+	-	-	l biopsy
5r*	6 m			+	-	+	-	-	* r gonadectomy
5l**	14 y			-	-	+	-	GB+DG	** l gonadectomy
6l	6 m	45,X (22/50) /46,X der(Y) (28/50)	male	-	-	+	-	-	
6r	6 m			+	-	-	-	-	biopsy
7l	6 m	45,X/46,X iso(Y)	male	-	-	-	+	-	
8	11 m	46,XYder(9) t(7;9) (q13.2;p24.2)	male	+	-	-	-	-	
9l	1y	45,X/46,XY	female	+	-	+	-	GB	
9r	1 y			-	-	-	+	-	
10l	1 y	46,XY	male	+	-	+	-	-	
11	1 y	46,XX/46,XY	female	+	+	+	-	-	
12	1 y	45,X (10/100) /46,XY (90/100)	male	+	-	-	-	-	biopsy
13l	1 y	45,X (12/50)/46,XY (38/50)	male	-	-	+	+	-	
14l	2 y	45,X (53/74) /46,Xi(Yp) (21/74)	male	+	-	+	-	-	
15	2 y	46,XY	female	-	-	+	-	-	
16l	3 y	45,X/46,XY	female	+	-	-	+	-	
17r	3 y	46,XX (30/100) /46,XY (70/100)	male	-	+	-	-	-	
18l	4 y	45,X, inv(5) (q22q33.2); 46,X	female	+	-	+	-	-	
18r	4 y	i(Y9), inv(5) (q22q33.2)		+	-	+	-	-	
19l	4 y	46,XX	male, later	-	+	-	-	-	
19r	4 y		female	+	+	-	-	-	T: no germ cells
20	4 y	45,X (11/25) /46,XY (14/25)	male	+	-	-	-	-	
21	5 y	46,XY	male	+	-	-	+	-	
22l	6 y	46,XX	male	+	+	-	+	-	T: no germ cells
23	7 y	46,XY	female	-	-	-	-	GB	
24r	7 y	46,XX/46,XY	male	-	+	-	+	-	
24l	7 y			+	-	+	-	-	
25r	7 y	45,X/46,XY	male	+	-	-	-	-	biopsy, T: no germ cells
25l	8 y			+	-	-	-	-	biopsy, T: no germ cells
26r	8 y	45,X/46,XY	male	+	-	-	+	-	
26l	25 y			-	-	-	-	GB	
27r	9 y	46,XY	female	-	-	+	-	calcified GB + MT + IT	
28r	10 y	45,X (7/12)/46,XY (5/12) - 2nd count: 45,X (40/40)	male	+	-	-	-	-	
29r	14 y	46,XY	female	-	-	-	+	GB	
29l	14 y			-	-	-	+	-	
30r	14 y	46,XX	female	+	+	-	-	-	
31r	14 y	45,X/46,XY	female	-	-	-	+	-	
31l	14 y			-	-	-	+	-	
32r	16 y	46,XY	female	-	-	+	-	GB	
32l	16 y			-	-	-	+	GB	
33	16 y	46,XY	female	-	-	-	+	GB	
34l	17 y	45,X/46,XY	female	-	-	+	-	calcified GB	
35r	17 y	45,X (12/30) /46,XY (18/30)	female	-	-	-	+	-	
36l	17 y	45,X (5/50)/46,XY (45/50)	female	-	-	-	+	-	
37r	18 y	46,XY	female	-	-	+	-	GB+DG	
37l	18 y			-	-	+	-	GB	
38	18 y	46,XY	female	-	-	+	-	GB	
39r	19 y	46,XY	female	-	-	+	-	GB+DG	
39l	19 y			-	-	+	-	GB	
40	19 y	?	female	-	-	-	+	-	
41	20 y	45,X/46,XY	male	-	-	-	-	GB+DG	
42l	21 y	46,XY	male	-	-	-	-	GB+CIS	
43	25 y	46,XY	male	-	-	-	+	DG	

Laterality of the gonad and karyotype are indicated if known.
age = age at gonadectomy or biopsy; l = left; r = right; GB = gonadoblastoma; DG = dysgerminoma; CIS = carcinoma *in situ*; UGT = undifferentiated gonadal tissue; MT = mature teratoma; IT = immature teratoma; T = testis; O = ovary; S = streak; y = years; w = weeks gestational age; m = months

Table 5-3: Summary of staining results in the encountered gonadal differentiation patterns and in gonadoblastoma/dysgerminoma

	Testis	UGT	Ovary	Streak	GB	DG
OCT3/4	+	+	-	-	+	+
PLAP	+	+	-	-	+	+
c-KIT	+	+	+	-	+	+
TSPY	+++	+++	-	-	+++	+/-
VASA	+	+	+	-	+	+/-

UGT: undifferentiated gonadal tissue; GB: gonadoblastoma; DG: dysgerminoma

The fourth pattern, consisting of fibrous stroma devoid of germ cells was referred to as streak. It was found in 14/40 dysgenetic gonads (35%) in an age-related manner: 4/25 cases until 4 years of age contained streak tissue (15%), whereas it was found in 10/15 samples older than 4 years (66%) ($p = 0.002$). Cord-like structures were often recognizable and might represent UGT that has lost its germ cells and has undergone a fibromatous involution (Figure 5-1G). AMH expression was never observed in this tissue type.

Morphology and staining results in dysgenetic gonads containing gonadoblastoma and/or dysgerminoma (Table 5-2 and 5-3 and Figure 5-3)

Typical gonadoblastoma nests were found in 16/20 samples, four of them also containing an invasive dysgerminoma component and one combined with CIS. Three samples consisted of large calcifications (the so-called burnt out gonadoblastoma), one of them in combination with mature and immature teratoma. In one sample only dysgerminoma was identified. Germ cells within gonadoblastoma stained positive for OCT3/4, c-KIT, PLAP and TSPY. TSPY was abundantly expressed in every gonadoblastoma sample, but the expression decreased in the invasive tumor components. The expression of OCT3/4, c-KIT and PLAP was variable: in some gonadoblastoma samples, all the germ cells stained positive, whereas in others, only a subpopulation of germ cells expressed these markers; however, in the dysgerminoma samples, these markers were homogenously present. VASA expression within the gonadoblastoma samples was variable: in 12/15 examined samples germ cells were positive, the remaining three samples were negative for VASA.

Non-neoplastic gonadal tissue adjacent to the neoplastic lesions was available in 15/20 samples (75%). The four differentiation patterns described above were encountered: a streak was found in 5/15 cases (33%), one in combination with ovarian tissue, and UGT was present in 10/15 cases (67%), one in combination with testicular tissue (Figures 5-3A – 5-3C). Again,

in UGT, germ cells stained positive for TSPY with a remarkably strong intensity. In all samples containing UGT, OCT3/4, c-KIT and PLAP positive germ cells were found (Figure 5-3D). In patient 23 and 39 (bilaterally), in the proximity of the malignant lesions, an accumulation of OCT 3/4 positive germ cells was found, suggesting clonal expansion and final organisation into gonadoblastoma nests (Figure 5-3E).

These findings were confirmed in the OCT3/4-TSPY double staining results (Figures 5-3F and 5-3G).

Discussion

The overall incidence of germ cell tumors in 45 patients with gonadal dysgenesis, retrieved from our database was 35% (16/45 patients), with four bilateral cases. Invasive germ cell tumors were found in 6/45 patients (13%).

We studied the histological and immunohistochemical characteristics in dysgenetic gonads that have (not yet) undergone neoplastic transformation and compared these findings to the properties of the gonadal tissue adjacent to and within gonadoblastoma samples. Given the rarity of the syndrome, a relatively large patient series could be collected. However, due to limited material, some experiments were performed in selected samples.

In dysgenetic gonads, basically four patterns of gonadal differentiation (with each pattern displaying a broad spectrum of abnormalities in morphology, number and organization of the germ cells) were found: testicular and ovarian tissue, streak, and UGT. The frequent finding of UGT was unexpected since it had not been mentioned in the original pathology reports, nor is it routinely described in literature, although some histological descriptions might suggest the presence of UGT (17, 27, 28). UGT clearly differed from the other patterns: in contrast to streak tissue, it does contain germ cells but these are neither organized in seminiferous tubules nor in follicles. In contrast, these germ cells reside apparently randomly distributed in a background of stromal cells or align in clusters, in close contact with Sertoli/granulosa cells. Without close observation and the use of specific markers, the germ cells within UGT are easily overlooked, hence its frequent classification as a streak. Alternatively, due to their presence in ovarian-type stroma, and although they are not organized in follicles, the germ cells are sometimes misinterpreted as residing in ovarian tissue. However, their correct identification is of crucial importance, as is illustrated in patient

5. In this girl, a bilateral biopsy was performed at 4 months, followed by a gonadectomy of the right testis, because of its discordance with the sex of rearing. The left gonad, which was considered as ovarian tissue, was left in place. At the age of 14, she developed a gonadoblastoma, already having progressed to a dysgerminoma. A re-evaluation of the available biopsy material of the left gonad unequivocally demonstrated germ cells and cord-like structures (Figures 5-4A - 5-4C). A similar case is found in the literature (6).

A significantly increased incidence of streak tissue was found in patients older than four years as compared to younger patients ($p = 0.002$). Cord-like structures were often recognized, suggesting a loss of germ cells in UGT, analogous to the germ cell loss that is observed in dysgenetic testicular or ovarian tissue (e.g. the testicular tissue in hermaphrodites (13) or the testes of patients with undervirilization syndromes (19). This finding is in accordance with previous reports (7).

The fate of the germ cells during normal human gonadal development was described by Gondos (29), and is largely analogous to the gonadal development and differentiation process analyzed in detail in mice (30). At their arrival in the genital ridge around 5 weeks GA, the germ cells and pre-Sertoli/granulosa cells lay intermingled without specific organization in the undifferentiated gonad. The first sign of sexual differentiation (the formation of primitive cords) coincides with SRY expression, around week 6 GA. In the absence of SRY, no changes occur in the undifferentiated gonad until the 12th week, when the germ cells enter meiosis. As stated above, germ cells within UGT lay either randomly in fibrous stroma or line up together with Sertoli/granulosa cells in cord-like structures. It is conceivable that the first pattern represents the undifferentiated state of the gonad, where no accurate SRY expression has taken place, but where under the influence of unknown (male) characteristics, meiosis and progression along the default pathway are inhibited. The second pattern might represent early sex cords, blocked in their progression towards seminiferous tubules (Figure 5-5). The differential staining results for AMH in testicular tubules and cord-like structures support this hypothesis.

Germ cells were stained with the markers TSPY, OCT3/4, c-KIT, PLAP and VASA. TSPY expression was never encountered in ova enclosed in follicles. However, it was abundantly expressed in germ cells within dysgenetic testes and UGT, as compared to the intensity of

the TSPY staining in fetal and age-matched normal gonads (19, 20, 31) (and personal observations), thereby suggesting an upregulation of TSPY when germ cells reside in an unfavorable environment. This is in line with previous observations in gonadoblastoma (21). TSPY is thought to be related to the pre-meiotic proliferation of spermatogonia, although its function is not fully clarified (23). Evidence is growing that TSPY is the main candidate for the hypothetical gene in the GBY region leading to the development of gonadoblastoma (21, 24, 32). Abnormal TSPY expression has also been related to the development of CIS in undervirilized patients (19).

OCT3/4, c-KIT and PLAP are well-established markers for the diagnosis of various germ cell tumors and are normally expressed during fetal gonadal development (20, 33). Maturation delay of germ cells, which has been described in intersex conditions and in patients with chromosomal anomalies, is characterized by a prolonged expression of these markers and is considered to be a risk factor for malignant transformation (2, 19, 31, 34, 35). In the present series, these three markers revealed similar staining patterns, however, OCT3/4, resulting in a well-circumscribed and intense nuclear staining was the most stable marker, and was easiest for interpretation. OCT3/4, a transcription factor regulating pluripotency of embryonal stem cells and essential for the survival of migratory primordial germ cells (36) is consistently expressed in specific germ cell tumors and might play a pathogenetic role in their development (37, 38). In line with previously reported results (33), OCT3/4 expression was never found in ovarian follicles. In contrast, within testicular tissue and UGT of dysgenetic gonads without gonadoblastoma and of tissue adjacent to gonadoblastoma, a subpopulation of germ cells expressed OCT3/4. Gonadoblastoma and dysgerminoma also consistently expressed this marker (Figures 5-1 and 5-3). In the proximity of a gonadoblastoma lesion, an accumulation of OCT3/4 positive cells was observed, suggesting clonal expansion within UGT towards gonadoblastoma formation (Figure 5-3E). Previously we demonstrated that OCT3/4 positive prespermatogonia located centrally in the seminiferous tubule reflect a state of maturation delay, whereas OCT3/4 positive cells on the basal lamina are prone to malignant transformation (19). In the present study, the latter pattern was encountered in 7 samples. It is tempting to speculate that these patients were at high risk for

developing CIS, eventually progressing to invasiveness within the dysgenetic testis if a gonadectomy had not been performed.

Based on our results, a model for the development of gonadoblastoma and CIS in the dysgenetic gonad containing Y chromosome material (i.e. the TSPY gene) emerges: A dysgenetic gonad may consist of different parts with various degrees of differentiation: ovarian tissue, in which the germ cells evolve along the default pathway of meiosis. These germ cells have lost OCT3/4 expression and do not express TSPY, therefore, they cannot give rise to a malignant germ cell tumor. Testicular tissue, showing variable degrees of dysgenesis, contains germ cells that abundantly express TSPY, possibly in an attempt to survive and proliferate in an unfavorable environment. Some of these germ cells maintain OCT3/4 expression even after having reached the spermatogonial niche, due to a block in their maturation. Over time, most of these germ cells will die; however, some, possibly due to abundant TSPY levels and prolonged OCT3/4 expression will survive and proliferate, eventually leading to clonal expansion and CIS formation. In UGT, a similar process takes place, ending either in a streak in case all the remaining germ cells die, or alternatively in the development of gonadoblastoma (Figure 5-5). If the latter evolution takes place more rapidly than the progression towards CIS, this would explain the more frequent finding of gonadoblastoma rather than CIS in dysgenetic gonads containing both UGT and testicular tissue. Alternatively, it is conceivable that immature germ cells, blocked in their maturation but residing in UGT, which is their natural environment during early embryonic life, have increased survival chances as compared to immature germ cells residing in more differentiated testicular tissue. Our findings are not disconcordant with the hypothesis that gonadoblastomas originate from germ cells developing along the female pathway but failing to complete the meiotic prophase and to organize in primordial follicles (39).

In conclusion, extrapolation to a clinical situation would suggest that a gonadal biopsy revealing the presence of UGT or testicular tissue with OCT3/4 positive cells on the basal lamina contains a high risk for germ cell tumors and should imperatively lead to gonadectomy. Ovarian tissue can safely be left in place, testicular tissue displaying maturation delay of germ cells can be left in situ given that its localisation allows adequate follow-up. A streak is not functional, making its preservation controversial. Evidently, the

decision with regard to gonadectomy is not only based on a pathological analysis of biopsy material but must be inspired by the combined interpretation of the patients' karyotype, internal genitalia, phenotype, gender and psychological well-being. The value, safety and applicability of this model should be thoroughly tested in large patient series and multicenter studies before it may contribute to a more conservative approach regarding gonadectomy in selected patients with gonadal dysgenesis.

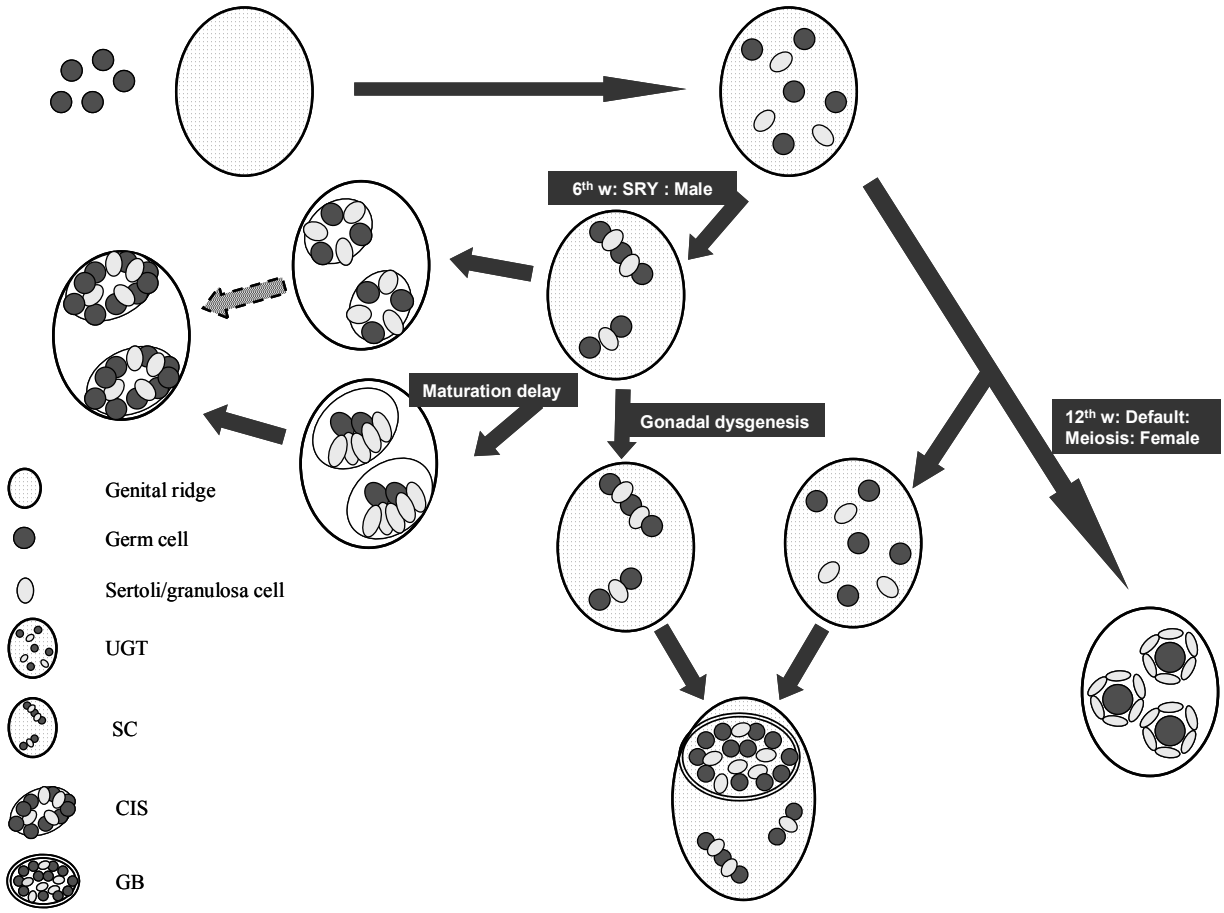

Figure 5-5: Model for the development of UGT, gonadoblastoma and CIS within the dysgenetic gonad: Upper panel: In the developing embryo, germ cells migrate from the yolk sac into the bipotential gonad and intermingle with pre-Sertoli/granulosa cells. Middle panel, from the right to the left: In the male, SRY expression in the 6th week GA induces organization of pre-Sertoli cells and germ cells in primitive sex cords. Under the influence of other male sex-determining genes downstream of SRY, these sex cords differentiate into seminiferous tubules. Pathological conditions can cause a block or delay in the normal germ cell development, thereby increasing the risk for ITGNU formation. Lower panel: In gonadal dysgenesis, inaccurate or absent SRY expression or a disturbed expression of other male-determining genes prohibit sex cord formation or further differentiation of sex cords whereas progression along the meiosis-default pathway is also blocked (lower panel, right), resulting in the persistence of UGT. Surviving germ cells residing in UGT (including immature sex cords) contain a high risk for the development of GB (lower panel, middle). UGT: undifferentiated gonadal tissue; GB: gonadoblastoma; ITGNU: intra tubular germ cell neoplasia unclassified

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6

Identification of germ cells at risk for neoplastic transformation in gonadoblastoma

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Abstract

Carcinoma *in situ* (CIS) is the precursor of malignant testicular germ cell tumors of adolescents and young adults (TGCTs), being the neoplastic counterpart of primordial germ cells/gonocytes. CIS cells will develop into invasive seminoma/nonseminoma. Gonadoblastoma is the precursor of invasive GCTs in dysgenetic gonads, predominantly dysgerminoma. 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 PLAP, c-KIT and OCT3/4, as well as TSPY on a series of gonadoblastomas, and adjacent invasive dysgerminomas. All five patients were XY individuals (four females and one male). In contrast to c-KIT, PLAP was positive in all cases. The immature germ cells of gonadoblastomas were positive for OCT3/4, while the mature germ cells were negative for this marker, but positive for TSPY. In every gonadoblastoma, a minor population of germ cells positive for both markers could be identified, similar to the majority of CIS cells and early invasive dysgerminoma. On progression to an invasive tumor, TSPY can be lost, a process that is also detectable in invasive TGCTs compared to CIS.

These results indicate that gonadoblastoma 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 germ cell tumor. In addition, the data support the model that gonadoblastoma represents the earliest accessible developmental stage of malignant GCTs.

Introduction

Human germ cell tumors (GCTs) represent a unique and complex group of neoplasms. Recently, we proposed a classification system based on various parameters, including age at clinical presentation, anatomical site of the tumor, sex of the patient, histology and chromosomal constitution (1, 2 for review). This classification has been acknowledged by the WHO (3). The group of tumors referred to as type II GCTs are the seminomas and 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, 5, 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 (4). Like CIS, it shows the potential to progress to an invasive GCT, mainly dysgerminoma, and less frequently to other tumor components, as embryonal carcinoma, teratoma, yolk sac tumor, and choriocarcinoma. Being a rare condition, gonadoblastoma has not been studied extensively, even though it might increase our understanding of the different pathogenetic steps involved in the development of malignant GCTs. Both the comparatively young age at presentation (4), and the genomic constitution (8) suggest that gonadoblastoma could be the earliest accessible stage in the development of a malignant germ cell tumor.

There are strong indications that both gonadoblastoma 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 proto-oncogene receptor c-KIT in CIS and gonadoblastoma (9-11). Besides c-KIT and PLAP, gonadoblastomas 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 gonadoblastomas has been linked to the presence of a specific part of the Y chromosome, namely the GBY (GonadoBlastoma 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 embryonal carcinoma (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 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 gonadoblastoma 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 gonadoblastomas. To further investigate the pathogenesis of gonadoblastoma, and to shed light on its pathogenetic relationship with testicular CIS, we performed a detailed investigation of these markers in a series of six gonadoblastomas and adjacent invasive tumor components. The findings were compared to those obtained in testicular CIS and adjacent invasive germ cell tumors. Our results confirm that gonadoblastoma 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 germ cell tumors. 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, while OCT3/4 remains positive. These observations are in line with the model that gonadoblastoma is an intermediate between normal, immature germ cells and CIS of the testis.

Materials and Methods

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 gonadoblastoma with or without an invasive tumor component were collected in the southwestern part of the Netherlands in collaboration with pediatric surgeons, urologists, and pathologists (Table 6-1). Furthermore, 21 seminomas and 38 nonseminomas of different, sometimes mixed histologies (18 embryonal carcinoma, seven yolk sac tumors, 9 teratomas, 4 choriocarcinomas) of the adult testis were included, as well as 31 cases of CIS adjacent to invasive 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 post mortem examinations were carried out in our department. Tissues were routinely formalin-fixed and paraffin-embedded. Diagnosis of gonadoblastoma and invasive tumor parts was made according to the WHO classification by a pathologist experienced in GCT pathology (JWO).

Based on morphology, the germ cells present in gonadoblastomas 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 a lower nuclear to cytoplasmic ratio, are similar to oocytes/prespermatogonia.

Table 6-1. Clinical data and summary of immunohistochemical results of PLAP, c-KIT, OCT3/4 and TSPY staining experiments of germ cells in gonadoblastoma, dysgerminoma and testicular germ cell tumors of adolescents and adults. Immunohistochemistry for c-KIT was not always positive in gonadoblastoma, all cases consistently showed expression of TSPY, OCT3/4 and PLAP. Expression of the latter two was mainly found in immature germ cells (indicated by +-). Staining results were the same on both sides (data not shown).

Organ	Sex	Case	Age (y)	Histology	PLAP	c-KIT	OCT3/4	TSPY
Dysgenetic gonad	F	1	18	GB	+	-	++	+
				Early invasive DG	+	-	++	+
	F	2	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	++	+/-	++	+-
				Nonseminoma:				
		n=18	>16	EC			+	-
		n=9	>16	TE			-	-
		n=7	>16	YST			-	-
		n=4	>16	CH			-	-

#Case 2 presented with bilateral gonadoblastoma.
F: female; M: male; y: years; GB: gonadoblastoma; DG dysgerminoma; EC: embryonal carcinoma; TE: teratoma; YST: yolk sac tumor; CH: choriocarcinoma; - : negative; +- : heterogeneity of staining; + : moderate; ++ : strong intensity of a homogenous staining

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, AR, USA), c-KIT (1:500, Dako-Cytomation, Glostrup, Denmark), and TSPY (1:3000, provided by C. Lau). Ki-67 (1:100, Dako-Cytomation, Glostrup, Denmark) were incubated for one hour, and OCT3/4 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for two hours at room temperature. All slides of the single staining experiments were counterstained with hematoxylin. Alkaline phosphatase staining with new fuchsine 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 (DAB) was used as chromogene 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/Naphtol ASMX phosphate (F3378 and N500, Sigma, Steinheim, Germany) for a blue staining (TSPY) and 3-amino-9-ethyl-

carbazole (A.5754 and D4254, Sigma, Steinheim, Germany)/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 CA, USA).

Fluorescent *in situ* hybridization (FISH)

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 two different tumor areas, both in regions containing gonadoblastoma and dysgerminoma, if present. The mean number of spots per nucleus is indicated; minimum number of cells assessed was 117.

Results

c-KIT and PLAP are established markers suitable for the immunohistochemical detection of CIS cells of the adult testis (for review, see 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 gonadoblastomas. In total, six gonadoblastomas were included, of five independent patients with a 46,XY karyotype (one was bilateral). In four cases also an adjacent invasive dysgerminoma component was available for investigation. Three of the invasive tumors showed an early stage of invasiveness in close proximity to the gonadoblastoma areas (termed early invasive dysgerminoma, Table 6-1), and one case also contained large invasive tumor areas at a distance from the precursor lesion (termed progressed dysgerminoma, Table 6-1). The results of the staining experiments are summarized in Table 6-1, and representative examples of staining results are shown in Figure 6-1. Double-staining experiments were performed for TSPY and OCT3/4, and TSPY and Ki-67, of which representative examples are shown in Figures 6-1G and 6-2. To assess the presence of chromosome X and Y, FISH analysis was performed using centromer probes for these chromosomes.

Morphologically, gonadoblastoma lesions contain various cell types, including somatic cells and germ cells (Figure 6-1A). 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.

Results of single staining experiments

c-KIT was the least consistent marker investigated in the gonadoblastomas in this study. It showed no staining in three gonadoblastomas, and the two matched dysgerminomas. In contrast, PLAP was detected in all gonadoblastomas and adjacent invasive components, with a heterogeneous pattern in the most advanced dysgerminoma. However, not all germ cells present in gonadoblastomas were positive for PLAP (Figure 6-1B). While the majority of mature germ cell was negative, the immature germ cells were mainly positive, although also stromal cells could show staining. Most of the tumor cells in invasive dysgerminoma showed a weaker signal intensity, or were negative for PLAP (Table 6-1). This indicates that PLAP is present in gonadoblastoma, but weaker to negative in invasive dysgerminoma. A similar pattern has been observed in testicular CIS and seminoma, in which PLAP is downregulated upon tumor progression (Table 6-1).

OCT3/4 was readily detectable as a nuclear staining in all gonadoblastomas (Figure 6-1C), in which the protein was restricted to the immature germ cells, while the more differentiated germ cells were negative. In contrast to the heterogeneity seen in gonadoblastoma, all tumor cells of dysgerminomas, both adjacent to and at distance from gonadoblastoma areas showed a homogenous staining for OCT3/4 (see also Figure 6-2). Therefore, a selection of OCT3/4 positive cells takes place during development of an invasive tumor from gonadoblastoma. These data are in accordance to observations in CIS and testicular germ cell tumors (see below, and reference 17 for discussion).

A heterogeneous staining pattern was found in gonadoblastoma for TSPY. In contrast to OCT3/4, TSPY was detected in both a nuclear localization and in the cytoplasm (Figure 6-1D). The protein was not detectable in somatic cells resembling granulosa/Sertoli cells. Tumor cells of early invasive dysgerminoma closely adjacent to the gonadoblastoma areas consistently showed a strong and homogenous staining for TSPY, similar to the pattern seen

for OCT3/4 (see Figure 6-2 for examples). TSPY expression could be lost in invasive dysgerminoma areas at distance from the pre-invasive 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 6-1 and Figure 6-1, E-G). Intratubular seminoma was consistently positive, whereas invasive seminoma, like dysgerminoma, can lose expression of TSPY. In contrast, all nonseminomas lack TSPY. Interestingly, even the intratubular embryonal carcinoma component is already negative (data not shown). As 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 Figure 6-1H, right panel). However, the staining intensity of TSPY in CIS was stronger than in spermatogonia (Figure 6-1H left panel).

Results of double-staining experiments

To further investigate the observed heterogeneity in expression of OCT3/4 and TSPY in gonadoblastoma, 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 dysgerminoma components showed a homogenous staining for both OCT3/4 and TSPY, in which the majority of tumor cells were positive for both markers (Figure 6-2A). In gonadoblastoma, 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 (Figure 6-2B). Only a subpopulation of germ cells in gonadoblastoma showed co-expression of both markers. To investigate whether co-expression of OCT3/4 and TSPY is a physiological event during normal intra-uterine male germ cell development, we stained two normal testes of fetuses of 16 and 21 weeks of gestational age. The majority 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 (Figure 6-2C). Interestingly, these cells were located on the basal membrane of the seminiferous tubule. Whereas the overall number of OCT3/4 positive cells decreased from week 16 to week 21 of gestational age, the fraction of germ cells showing expression of both markers remained unchanged.

These results were compared to 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 (Figure 6-2D, 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 (Figure 6-2D right panel, and Figure 6-1G). 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 (Figure 6-2E). In two cases of CIS without invasive tumor, all CIS cells expressed TSPY (Figure 6-2F). These data indicate that TSPY is lost upon invasiveness of germ cell tumor cells. This step can already occur in the *in situ* stage (Figure 6-2E).

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 gonadoblastoma, also the two normal fetal testes were investigated. The majority (around 90%) of malignant germ cells 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 gonadoblastoma without invasive tumor was much more heterogeneous (case 5, Figure 6-2G). 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 rarely co-expression of both markers was seen. This was similar to the pattern seen in fetal testes, where the two factors were not frequently co-expressed. 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 Figure 6-2H).

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 germ cell tumors (GCTs). The invasive tumor components, referred to as dysgerminomas and nondysgerminomas, resemble those of the adult testis (TGCTs), seminoma, and nonseminoma of various histologies. The precursor lesion is known as gonadoblastoma (4). Histologically, gonadoblastomas 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 gonadoblastoma 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 (i.e., 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 gonadoblastoma (8). We undertook the present study to further understand the biology of this condition, and to compare similarities and differences between gonadoblastoma and CIS.

Immunohistochemical analysis of both known (PLAP, c-KIT) and recent markers of early male germ cells (OCT3/4, TSPY) was performed in gonadoblastomas and adjacent dysgerminomas. 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 gonadoblastomas, 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 gonadoblastoma. A heterogeneous staining pattern was found for both OCT3/4 and TSPY in all gonadoblastomas investigated. Early invasive dysgerminoma cells closely adjacent to the gonadoblastoma component were positive for both OCT3/4 and TSPY. A similar pattern was found in the majority of CIS cells and could be confirmed by double-staining experiments. TSPY has previously been reported in a few cases of gonadoblastoma (12, 13), while, to our knowledge, the presence of TSPY in early invasive dysgerminomas and the loss of expression on tumor progression is novel. These findings support a model in which progression from early gonadoblastoma to invasive growth is associated with expression of both TSPY and OCT3/4 in immature germ cells (see Figure 6-3 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 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, co-expression was found in the vast majority of CIS and early invasive seminoma cells, as

well as in gonadoblastoma 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 gonadoblastoma. TSPY and Ki-67 were less frequently co-expressed in one gonadoblastoma case that had not yet developed into an invasive tumor (case 5, Table 6-1). In our view, this could indicate that this specific gonadoblastoma case had not yet progressed to the next pathogenetic step, i.e., 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 gonadoblastomas (17, 18). In the present series, OCT3/4 was readily detectable in all gonadoblastomas in the nuclei of cells showing morphologic features of immature germ cells. Furthermore, as OCT3/4 remained positive throughout all stages of tumor development from gonadoblastoma to dysgerminoma, it can serve as a marker to identify cells showing a high risk for malignant transformation. However, it is important to notice that the co-expression 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 co-express both TSPY and OCT3/4, although only in a minority of all cells.

Results of the double-staining experiments support the notion that gonadoblastoma 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 gonadoblastoma resemble more the male germ cell lineage than the female (33). Together with the higher incidence of these tumors in males compared to females this 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, following a process of selection and consecutive clonal expansion, immature germ cells in gonadoblastoma 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.

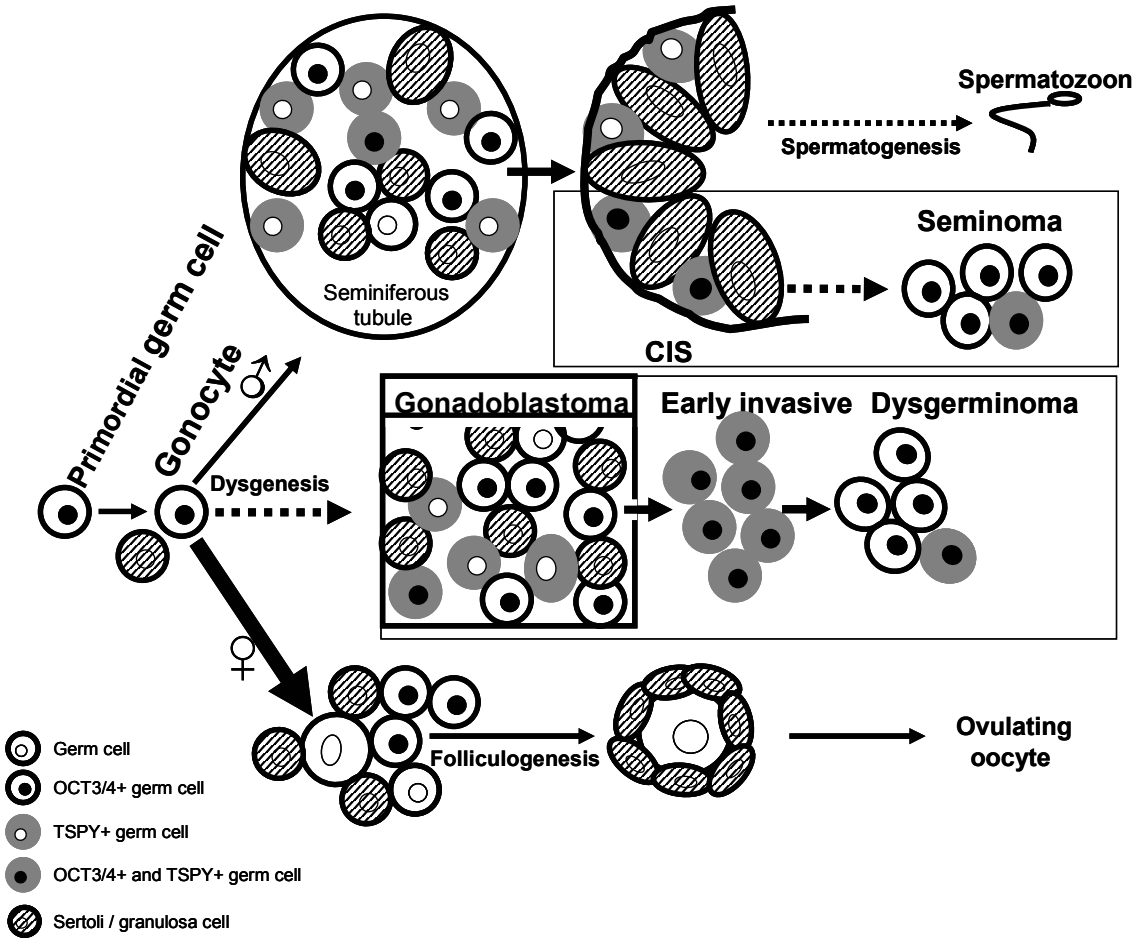

Figure 6-3: Schematic representation of normal and neoplastic germ cell development. The earliest stage of maturation (starting with a primordial germ cell) is indicated on the left side of the illustration, and the most mature (differentiated) stage at the right. Maturation of germ cells is graphically depicted by increasing size of cells and nuclei. Normal male development (top row) and female development (bottom row) leads 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). The different cells are represented as follows: black nuclei = germ cells positive for OCT3/4; cytoplasmatic grey cells= germ cells positive for TSPY. During normal male fetal germ cell development (and in gonadoblastoma), only a minority of cells show co-expression of OCT3/4 and TSPY (depicted by a black nucleus and a grey cytoplasm). A disturbed process of germ cell maturation can result in invasive germ cell tumors (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 dysgerminoma 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 gonadoblastoma is an earlier stage in the process of GCT formation than CIS

In conclusion, our results show that gonadoblastoma consists of a heterogeneous population of germ cells. Both in dysgerminoma 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, i.e. dysgerminoma or seminoma. Our analysis places gonadoblastoma 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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7

Analysis of Y containing cell lines in the gonads of patients with gonadal dysgenesis: impact on gonadal differentiation patterns and risk for gonadoblastoma formation

Submitted

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Abstract

Gonadal karyotyping is considered as a tool to increase our knowledge on the disturbed gonadal development in patients with gonadal dysgenesis and to better estimate the risk for gonadoblastoma formation in these gonads. To investigate the possible relations between peripheral blood karyotype, gonadal karyotype, morphological differentiation patterns of the dysgenetic gonad and tumor formation, we examined 22 gonadectomy or gonadal biopsy samples in 19 different patients with gonadal dysgenesis (14 patients with 45,X/46,XY (and variants) mosaicism, three patients with 46,XY and two patients with 46,XX karyotypes). The following analyses were performed: morphological examination and immunohistochemical staining for TSPY, and fluorescent and non-fluorescent *in situ* hybridization directly on gonadal tissue. No correlation was found between peripheral blood karyotype and gonadal karyotype and between gonadal karyotype and the corresponding differentiation pattern. A Y containing cell line was not more frequently encountered in Sertoli cells as compared to other cell types, including granulosa cells. We conclude that the distribution of the Y containing cell line in peripheral blood is not a suitable indicator to predict the histological differentiation pattern found in the gonads of patients with gonadal dysgenesis. The analysis of Y containing cell lines in the gonads of patients with gonadal dysgenesis could be informative with regard to the specific characteristics of gonadal development in humans as compared to chimeric mouse models. Moreover, it is essential to understand the mechanisms underlying disturbed gonadogenesis in these patients. However, in estimating the risk for gonadoblasotma formation in the individual patient, this analysis is not contributive.

Introduction

In spite of our increasing knowledge on genes involved in normal sex development, the disturbed gonadal differentiation processes that underlie gonadal dysgenesis (GD) syndromes are less understood (1-5). Little is known about the correlations between peripheral blood (PBL) karyotype and/or skin fibroblasts on the one hand, and the gonadal karyotype and morphological differentiation patterns of the gonad on the other (6-11). Traditionally, in GD, three differentiation patterns have been described: (dysgenetic) testicular tissue, ovarian tissue and streak gonads. Recently, a fourth pattern was recognized, the so-called undifferentiated gonadal tissue (UGT), which consists of germ cells lining up together with Sertoli/granulosa cells to form cord-like structures or residing without specific organization in a background of stromal cells (12). These four patterns do not appear strictly separated but may constitute a continuum of gonadal tissue areas, more or less differentiated into the male or female direction (Figure 7-1).

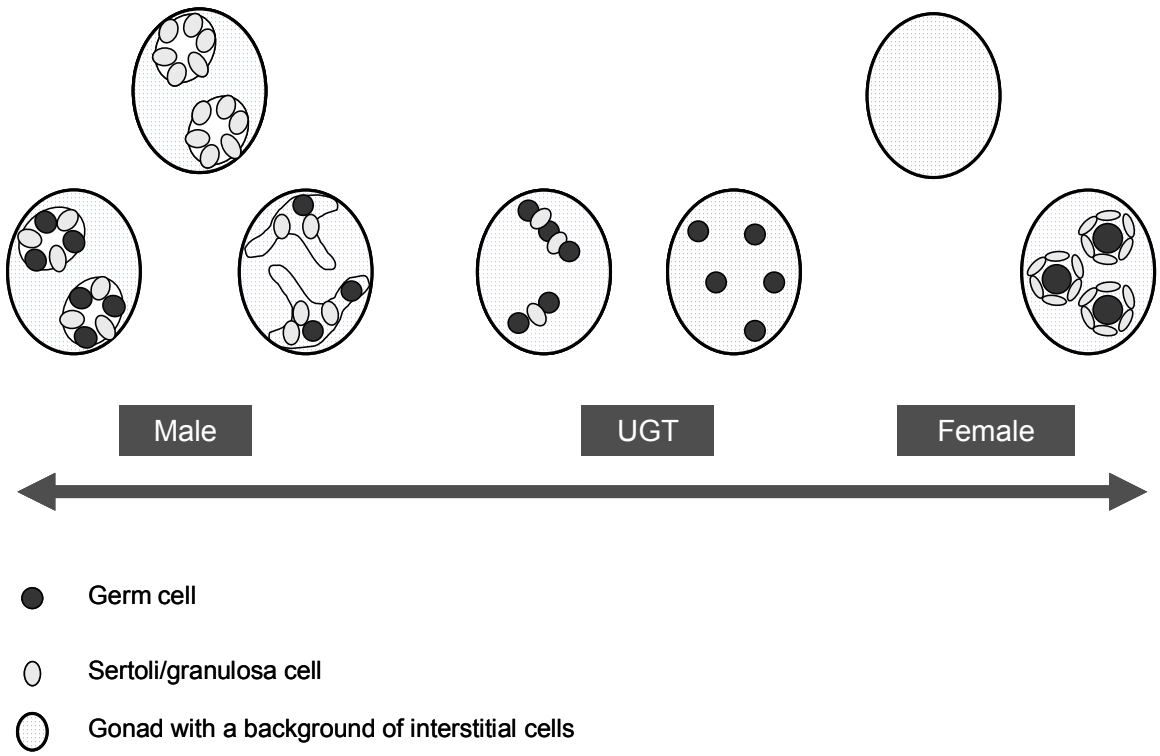

Figure 7-1: Schematic representation of gonadal differentiation patterns in patients with gonadal dysgenesis. Differentiated testis with well-formed tubules and ovaries in which all germ cells are enclosed in primordial and growing follicles are at the ends of the differentiation spectrum. In between, the patterns that can be found range from poorly formed testicular tubules with or without germ cells and germ cells enclosed in cord-like structures to germ cells lying without organization in a background of stromal cells and fibrous stroma without germ cells. UGT: undifferentiated gonadal tissue.

Patients with GD are at increased risk for the development of malignant germ cell tumors (13). In patient series selected for the presence of Y chromosome material, the incidence of germ cell neoplasia is estimated at 30% or more (14). Evidence is growing that the relevant gene in this context is the Testis Specific Protein - Y encoded (TSPY), located within the putative gonadoblastoma (GB) region on the Y chromosome (GBY) (12, 15-20). It was shown that in the presence of TSPY, areas of gonadal tissue with a low degree of differentiation (UGT) are at highest risk for GB development (12). The exclusion or confirmation of Y chromosomal material directly in the dysgenetic gonad is more relevant in this context than PBL karyotyping but is technically more difficult (7, 10). Due to the risk for germ cell tumors, a prophylactic gonadectomy has been advised in all GD patients (21-24). At present, this traditional policy is under debate and a tendency towards a more conservative approach regarding gonadectomy is observed (25-28). However, before safe protocols for the follow-up of patients with dysgenetic gonads left *in situ* can be established, research is mandatory to recognize additional risk factors for the development of germ cell tumors in these patients. The aim of this study was to examine the relationship between PBL karyotype, gonadal karyotype and the pattern and degree of gonadal differentiation within the dysgenetic gonad.

Materials and methods

Patients

Twenty-two gonadectomy or gonadal biopsy samples in 19 different patients with GD were studied. Patient and tissue characteristics are summarized in Table 7-1. Samples were available as formalin fixed, paraffin-embedded material. Ten additionnaly available samples were excluded due to bad preservation of material. Five normal testicular parenchyma samples of adult men were included as positive controls, all displayed normal spermatogenesis.

Use of tissues for scientific reasons was approved by an institutional review board. 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 (version 2002) (29).

Table 7-1: Patient and tissue characteristics

Code	Patient age*	Sex of rearing	PBL karyotype	Gonadal differentiation pattern					Remarks
				T	O	UGT	St	GB	
1	17w	m**	45,X/46,XY	+	-	+	-	-	
2	19.5w	m**	45,X[7]/46,XY[13]	+	+	-	-	-	
3l	6mo	m	45,X[22]/46,X der(Y)(pter-q11.2::q11.2-pter)[28]	-	-	+	-	-	
3r	6mo			+	-	-	-	-	
4	6mo	m	45,X/46,X isoY	-	-	-	+	-	
5l	1y	f	45,X/46,XY	+	-	+	-	+	very small GB lesion
5r	1y			-	-	-	+	-	
6	1y	m	45,X[12]/46,XY[38]	-	-	+	-	-	skin fibroblasts: 45,X[33]/46,XY[17]
7	1y	f	46,XX/46,XY	+	+	-	-	-	
8	2y	m	45,X[53]/46,X i(Yp)[21]	+	-	+	-	-	skin fibroblasts: 45,X[35]/46,X i(Yp)[15]
9	3y	m	46,XX[30]/46,XY[70]	-	+	-	-	-	
10	4y	f	45,X inv(5) (q22q33.2)[2]/46,X i(Y9) inv(5) (q22q33.2)[8]	+	-	+	-	-	
11	7y	m	46,XX/46,XY	-	-	-	+	-	
12	4y	m	45,X[11]/46,XY[14]	+	-	-	-	-	
13	10y	m	45,X[7]/46,XY[5]	+	-	-	-	-	
14	17y	f	45,X[5]/46,XY[45]	-	-	-	+	-	
15	6mo	f	46,XY	+	-	+	-	-	
16	1y	m	46,XY	+	-	+	-	-	
17	4y	m	46,XX	+	+	-	-	-	gender change into female; no germ cells in T
18	6y	m	46,XX	+	+	-	-	-	possibly Y containing cell line in genital skin, no germ cells in T
19r	14y	f	46,XY	-	-	+	-	+	GB completely calcified
19l	14y			-	-	-	+	-	

*patient age: age at gonadectomy or gonadal biopsy
** in these fetal samples, the phenotype was considered instead of sex of rearing
PBL: peripheral blood; T: testis; O: ovary; UGT: undifferentiated gonadal tissue; St: streak; GB: gonadoblastoma; m: male; f: female; w: weeks gestational age; mo: months; y: years; l: left; r: right.

Morphology and immunohistochemistry

Slides of 3 μm were prepared and stained with hematoxylin-eosin for morphological analysis. Per sample, the gonadal differentiation patterns (testis/ovary/UGT/streak) were determined. Parallel slides were incubated with a polyclonal TSPY antibody (15) in a 1:3000 dilution. After incubation overnight, application of a secondary antibody (Swine-anti-rabbit, biotin labeled; DAKO-cytomation, 1:200) and an amplification step (Streptavidin-biotin-alkaline phosphatase, 1:100), staining was performed using Naphtol ASMX Phosphate (Sigma-Aldrich,

Zwijndrecht, The Netherlands) and New Fuchsine (Fluka Chemica, Zwijndrecht, The Netherlands), and counterstaining with hematoxylin. Per differentiation type, germ cells were analyzed for their expression of TSPY. The morphological and immunohistochemical description of these samples has been published elsewhere (12).

Cytogenetic analysis

Metaphase chromosomes were obtained from phytohemagglutinin-stimulated PBL lymphocyte samples. The cultures were synchronized using an excessive amount of thymidine for 16 hrs. The block was released by a change of medium 6 hrs before harvesting. Standard karyotyping was performed using conventional Giemsa-trypsin-Giemsa banding.

Fluorescent *in situ* hybridization (FISH)

To avoid the generation of signals from overlapping nuclei, tissue sections of 5 μm were prepared. They were deparaffinized and pressure cooked analogous to standardized pre-treatment methods for immunohistochemistry (30). Afterwards, slides were digested with 0.01% pepsin (Sigma-Aldrich, Zwijndrecht, The Netherlands) in 0.01 mol/L HCl at 37oC. Due to variations in fixation techniques, the optimal digestion time per sample ranged from 0.5 min - 10 min. Slides were rinsed and dehydrated, and the probes, dissolved in hybridization mixture were applied. Probes for centromere X (BamH1) and Y (DYZ3) were used, in patients with an abnormal Y chromosome according to the PBL karyotype, additionally, the same FISH protocol was performed with a plasmid containing the TSPY gene in a 12.5-KB Y chromosome DNA fragment (31). Probes were labelled with biotin-16-dUTP or digoxigenin-11-dUTP (Roche, Mannheim, Germany), using a nick-translation kit (Gibco BRL, Paisley, UK). After denaturation on a heat plate (80° for 10 min), hybridization overnight, and washing steps, the hybrids were visualized with Cy3 conjugated avidin antibody (1:100, Jackson ImmunoResearch, West Grove, PA, USA) for centromere Y and TSPY and Sheep-anti-dig FITC (1:50, Roche Diagnostics, Mannheim, Germany) for centromere X. Results were analyzed using a fluorescent microscope (Leica Microsystems). Per slide, every gonadal differentiation pattern was photographed in two different areas at a 250X magnification and for each of the two photographs, the karyotype was determined in 100 cells. Afterwards, testicular tissue

was re-analyzed to determine the presence of the Y chromosome specifically in 100 intratubular cells as compared to 100 interstitial cells. Only non-overlapping cells with at least one signal were considered. In testicular tubules of controls, to avoid counting haploid, post-meiotic germ cells, only cells close to the basal lamina were considered.

In 46,XY controls, the mean percentage of Y positive cells in ten areas of testicular tissue analyzed (two per control) was 76% (71 - 81), indicating that false negative results due to the use of slides with a thickness of 5µm, occurred in 24% of cells. This number compares to what is found by other investigators: an independently performed study reveals 78% of Y positive cells in male controls, thus displaying 22% false negative cells (32). Therefore, we consider the results obtained by FISH analysis in the present study informative.

Non-fluorescent *in situ* hybridization

In 13 samples, non-fluorescent *in situ* hybridization (non-FISH) could be performed with good results. The other samples were excluded from this part of the study due to qualitatively insufficient preservation of tissue or poor signal intensity. For deparaffinization, pre-treatment and probe application, similar techniques were applied as for FISH on 5 µm slides. The optimal digestion time per sample ranged from 1 - 25 min. The same centromeric and TSPY probes were used. Hybrids were visualized using primary, secondary and tertiary antibodies and a final detection step (Table 2), resulting in a black signal for centromere X and a red signal for centromere Y/TSPY. All incubation steps were performed in a moist chamber at 37° for optimal binding results. Results were analyzed using a light microscope (Zeiss). In testicular tissue, 100 Sertoli cells and 20 germ cells were analyzed, in ovarian tissue, the karyotype of 100 granulosa cells, 100 stromal cells and 20 germ cells was determined, in UGT, 100 Sertoli/granulosa cells or interstitial cells (the two cell types could not be discriminated in this differentiation pattern) and 20 germ cells were analyzed, and in streak tissue, 100 fibroblasts were scored. Only non-overlapping cells with at least one signal were considered.

Table 7-2: Antibodies used for detection of hybridized probes by non fluorescent in situ hybridization

Probe	Step 1			Step 2			Step 3			Detection
	AB	Origin	Dil	AB	Origin	Dil	AB	Origin	Dil	
X-dig	Mouse-anti-dig	Boehringer-Mannheim, Germany 1333062	1/50	RAM-HRP	DAKO-cytomation P0260	1/50	SWAR-HRP	DAKO-cytomation P0217	1/40	DAB + cupper sulfate
Y-bio/ TSPY-bio	ABC-AP	DAKO-cytomation	1/100	goat-anti-avidin bio	VECTOR BA-0300	1/50	ABC-AP	DAKO-cytomation	1/100	New Fuchsin

AB: antibody; dil: dilution; dig: digoxigenin labeled; bio: biotin labeled, TSPY: testis-specific protein - Y encoded; ABC-AP: streptavidin-biotin-alkaline phosphatase complex; RAM: rabbit anti mouse antibody; HRP: horseradish peroxidase; SWAR: swine anti rabbit antibody; DAB: 3,3' – Diaminobenzidine – tetrahydrochloride dehydrate

Statistical analysis

Statistical analysis was performed using the SPSS (SPSS 11.0 for Windows) statistical program (Chi-square analysis with the number of Y containing cells as dependent variable and patients 15 and 16 as independent variables, these patients were compared to controls).

Results

Results for morphological, FISH and non-FISH analysis are summarized in Table 7-3. Representative examples are presented in Figures 7-2 (morphology), 7-3 (FISH) and 7-4 (non-FISH).

- Morphology and TSPY staining: In mosaic patients, testicular tissue was found most frequently (56%) as compared to UGT (37%), streak (25%) and ovarian tissue (19%) (see Table 4). The distribution of the different cell lines in PBL was available for 10 patients; 8/10 had more than 50% Y positive cells in PBL. The gonadal differentiation pattern in relation to the distribution of the Y containing cell line in PBL is presented in Table 7-4.

There were 5 non-mosaic patients (three 46,XY and two 46,XX). In two 46,XY patients (15 and 16), testicular tissue was found, both in combination with UGT. In patient 19, UGT with a small GB focus was present on the right side and a streak on the left. In 46,XX patients 17 and 18, testicular as well as ovarian tissue was present. However, in both samples, the testicular portion was devoid of germ cells.

In non-mosaic patients, most if not all germs cells in the testicular and UGT portions of the gonads expressed TSPY whereas in mosaic patients, TSPY negative germ cells were frequently found. Within UGT, there was no difference in TSPY expression between the germ cells organized in cord-like structures and those residing without specific organization in stroma.

In ovarian tissue of mosaic and non-mosaic patients, ova, all nicely enclosed in follicles, never expressed TSPY, although some of these ova were found to contain a Y bearing cell line (see below).

- FISH: Cells displayed the following signals: X, XX, XY or Y. In patients with an abnormal Y chromosome, as expected, results with the TSPY probe were similar to those obtained with the Y centromere probe. Per sample and per gonadal differentiation pattern, the percentage of Y bearing cells was calculated. Within one sample, counting results in the two photographed areas of the same differentiation pattern did not differ; therefore out of 200 counted cells, the mean percentage of Y bearing cells per pattern per sample is represented. As stated above, in 46,XY controls, false negative results occurred in 24% of cells. An estimation of the actual number of Y positive cells in a given gonadal area was calculated in patients for whom the distribution of cell lines in PBL was known (assuming that there is no differential growth of the cell lines cultured in vitro for cytogenetic analysis, see below): e.g. patient 2: 65% Y positive cells in PBL, testicular tissue contained 50.5/100 Y positive cells, the calculated % is (24 x 65/100) + 50.5= 66.1%.

For mosaic patients, the mean percentages of Y positive cells per differentiation pattern are summarized in Table 7-4. If the two fetal samples, containing relatively high amounts of Y positive cells are omitted from the calculations, these percentages, relative to each other, do not change considerably. In the two testicular samples without germ cells, a Y signal was scarce (18l, 3%) or absent (17r). Only few germ cells were found in the testis of patient 13, with only 5.5% Y positive cells.

In 4/9 testicular samples (44%), there were more Y positive intratubular cells (mainly Sertoli cells) than interstitial cells, in 4/9 samples, a predominance of Y positive interstitial cells was found, and in one sample, the Y signal was equally distributed.

In non-mosaic patient 15, the percentage of testicular Y positive cells was within the normal range as compared to controls ($\chi 2=0.13$, $p=0.72$), whereas in patient 16, it was significantly lower ($\chi 2=14.6$, $p<0.001$). The testicular and ovarian portions in 46,XX patient 17 revealed no Y positive cells. This was confirmed with quantitative PCR on DNA extracted from the gonad of this patient, with probes covering various parts of the Y chromosome (including SRY). In 46,XX patient 18, a Y containing cell line was first detected in genital skin fibroblasts

but this was not confirmed afterwards. However, the gonadal sample revealed a Y signal at a low degree in both the testicular and ovarian portions.

Table 7-3: Results of FISH and ISH analysis

Code	%Y in PBL	FISH					non-FISH			
		%Y in T	%Y in tc/%Y in ic	%Y in O	%Y in UGT	%Y in St	% Y in T	%Y in O	%Y in UGT	%Y in St
1	†	48.5	48/48	-	36	-	x	-	x	-
2	65	50.5 (66)	51/41	84 (100)	-	-	Sc: 76 gc: 65	80*	-	-
3l	56	-	-	-	24 (37)	-	-	-	x	-
3r	56	15.5 (29)	30/19	-	-	-	x	-	-	-
4l	†	-	-	-	-	4	-	-	-	4
5l***	†	27.5	13/16	-	20.5	-	x	-	x	-
5r	†	-	-	-	-	0	-	-	-	0
6l	76	-	-	-	44 (62)	-	-	-	-	30
7r	†	84	78/63	32	-	-	Sc: 82 gc: 70	x	-	-
8l	28	46.5 (53)	54/17	-	47 (54)	-	Sc: 12 gc: 40	-	S/grc: 12 gc: 80	-
9r	70	-	-	38.5 (55)	-	-	-	grc: 44 gc: 20 strc: 50	-	-
10l	80	14 (33)	13/30	-	43 (62)	-	x	-	x	-
11r	†	-	-	-	-	23	-	-	-	23
12	56	19.5 (33)	14/16	-	-	-	x	-	-	-
13r	42	5.5 (15.5)	0/13	-	-	-	x	-	-	-
14l	90	-	-	-	-	20 (42)	-	-	-	49
15r	100	77.5 (101.5)	74/68	-	x	-	Sc: 92 gc: 100	-	x	-
16l	100	58 (82)	57/36	-	61 (85)	-	Sc: 79 gc: 75	-	S/grc: 79 gc: 60	-
17r**	0	0** (0)	0/0**	0	-	-	Sc: 0**	grc: 0 gc: 0	-	-
18l**	0	3** (0)	0/1**	6.5	-	-	Sc: 6**	grc: 2 gc: 0	-	-
19r***	100	-	-	-	49 (73)	-	-	-	-	x
19l	100	-	-	-	-	60.5 (84.5)	-	-	-	x

patients 1 - 14: mosaicism in PBL, patients 15 - 19: no mosaicism in PBL
Calculated percentages of Y positive cells are given in parentheses.
† Information was unavailable
- This tissue type is not present in the sample
x Due to technical limitations, analysis was impossible for this sample
* Discrimination between the various cell types was not possible in this sample
** The testicular portion of the gonad contains no germ cells in these patients
*** Gonadoblastoma, apparently arising from the undifferentiated gonadal tissue present in the sample
PBL: peripheral blood; FISH: fluorescent *in situ* hybridization; non-FISH: non fluorescent *in situ* hybridization; T: testis; tc: tubular cells; ic: interstitial cells; O: ovary; UGT: undifferentiated gonadal tissue; St: streak; l: left; r: right; Sc: Sertoli cells; gc: germ cells; grc: granulosa cells; strc: stromal cells

Table 7-4: Results for morphological analyses and FISH in mosaic patients

	T	O	UGT	St	PBL	T	O	UGT	St	FISH	T	O	UGT	St
45,X/ 46,XY and variants (n=16)	9/16 (56%)	3/16 (19%)	6/16 (37%)	4/16 (25%)	>50% Y+ (n=8)	4/8	2/8	3/8	1/8	mean % Y+ cells	34.6% (5.5-84)	51.5% (32-84)	35.8% (20.5-47)	11.8% (0-23)
					<50% Y+ (n=2)	2/2	0/2	1/2	0/2	mean % Y+ cells*	30.4%	35.3%	35.7%	11.8%

Left: Frequency of the different gonadal differentiation patterns found in mosaic patients
Middle: The differentiation type of the gonad in relation to the distribution of the Y containing cell line in PBL
Right: Mean percentage of Y positive cells in the four gonadal differentiation patterns found in mosaic patients,
* fetal samples are omitted from these calculations
T: testis; O: ovary; UGT: undifferentiated gonadal tissue; St: streak

- Non-FISH: In mosaic patients, only a limited number of samples could be examined using this method. In testicular tissue, the mean percentage of Y positive Sertoli cells was 56% (12 - 82) and of Y positive germ cells 58% (40 - 65). In ovarian tissue, analysis of one sample (9r) revealed 44% Y positive granulosa cells, and 20% Y positive ova. In UGT, sample 8l revealed 12% Y positive Sertoli/granulosa cells or interstitial cells (the discrimination between these two cell types was not possible for UGT) and 80% Y positive germ cells. In streak tissue 21% (0 - 49) Y positive fibroblasts were found.

Testicular tissue in non-mosaic patient 15 revealed 92% Y positive Sertoli cells and 100% Y positive germ cells, as compared to 79% Y positive Sertoli cells and 75% positive germ cells in the testis of patient 16. In patient 18, 6% of Sertoli cells were positive for a Y signal, as compared to 2% of granulosa cells.

Discussion

To examine if the analysis of Y bearing cell lines contributes to a better estimation of the risk for germ cell tumor development in patients with GD, we investigated 22 dysgenetic gonads in fourteen 45,X/46,XY (and variants) mosaic patients, three 46,XY patients and two 46,XX patients. To our knowledge, this is the largest series ever described.

Previously, in small series of 45,X/46,XY mosaic patients, FISH analysis has been used to determine the presence of the Y chromosome in gonadal cells (7-10, 32). However, discriminating between germ cells, Sertoli/granulosa cells and stromal cells is extremely difficult, if not impossible with this technique. Therefore, we additionally examined the samples by non-FISH. However, this method reveals major limitations. The denaturation and digestion processes require optimal tissue preservation and slide preparation. Even in standardized conditions and after two amplification steps, the signal intensity can be too

weak to allow interpretation of results. The technique is only applicable to formalin-fixed material, with other methods of tissue preservation (such as Steve's fluid or Bouin's fixative), even extremely prolonged pepsin digestion does not result in binding of probes. Therefore, we currently consider non-FISH no suitable technique for routine gonadal karyotyping.

As in previous studies (7, 8, 10), we observed no correlation between the degree of mosaicism in PBL and in gonadal cells, nor was the PBL karyotype able to predict the differentiation patterns that had developed in the dysgenetic gonad. Differential growth of the 45,X and 46,XY cell lines in vitro and the selection of a subset of clones for PBL karyotyping cannot be excluded. Therefore, it is unknown if the PBL karyotype obtained from in vitro cultured lymphocytes reflects the true in vivo PBL karyotype, and by consequence, PBL karyotyping is insufficient, if not unreliable, to predict if and to what extent Y containing cell lines exist in the gonad. On the other hand, with the exception of sample 5r, the Y cell line retrieved in PBL was present in all gonadal samples, albeit at a low percentage in some of them (e.g. samples 3r, 10l, 13r). Thus, the finding of a Y containing cell line in PBL must warrant for the presence of gonadal mosaicism, and justifies gonadectomy.

In accordance with the PBL karyotype, no Y bearing cell line could be demonstrated in the gonad of patient 17. In contrast, in patient 18, a Y containing cell line was present at a low degree in both the testicular and ovarian portion. Since only two 46,XX patients were examined in this study, our data do not allow generalized conclusions regarding the appropriateness of gonadectomy in 46,XX GD.

A correlation between the gonadal differentiation pattern and the percentage of Y positive cells was suggested in XX ↔ XY chimeric mice models: it was found that if the gonad contains less than 30% Y positive cells, it develops as an ovary (33). First, this mouse model seemed to be confirmed in humans, based on an analysis of five 45,X/46,XY patients: testicular parts revealed a higher percentage of XY cells than ovarian parts (9, 10). However, two case reports describe testicular differentiation in the presence of low grade mosaicism for a Y bearing cell line (8, 11). Our results, obtained in a larger series of samples, reveal no correlation between the degree of gonadal mosaicism and its differentiation pattern.

Factors other than the mere quantitative influence of the Y signal seem to determine the fate of the bipotential gonad in X/XY mosaicism. Timing effects, the presence of a threshold level

and a sufficient density of the SRY signal to activate the downstream gene cascade have been mentioned (2, 10, 34). Near-threshold levels of SRY could give rise to opposite differentiation pathways (34) (as is demonstrated in patient 18). Minor variations in the expression of key genes (e.g. through allelic differences) may result in major outcome differences depending on whether or not the male determining cascade is activated (5). In mice, secondary feedback mechanisms firmly reinforce the chosen pathway, resulting in unambiguous testicular or ovarian tissue (5, 33, 35). In humans however, an undecided battle between testis-determining and ovarian promoting signals may end anywhere on the axis representing the gradual transition from dysgenetic testes to streak ovaries (Figure 7-1), meanwhile supporting the model that proper ovarian development is indeed an active process, requiring the activation of its own genes (2, 5).

In testicular samples, the mean percentage of Y positive Sertoli cells was 56%, whereas in ovarian samples, 44% Y positive granulosa cells were found. In XX ↔ XY chimeric mice, following SRY expression, mainly XY precursor cells develop into Sertoli cells, although some XX precursor cells also differentiate into this cell type, presumably following paracrine stimuli (5, 33). This is in contrast with the equal distribution of the different chromosomal lines in other gonadal cell types. By analogy, in X/XY and X/XY/XYY mosaic mice, follicle cells are predominantly Y negative. Our results on the individual karyotype of Sertoli and granulosa cells are in line with a previous study, where it is suggested that this recruitment process of XY positive precursor cells does not seem to take place at such a degree in humans or that the presumed paracrine actor is more effective (10).

In 46,XY patient 15, the percentage of Y positive cells in the testicular portion did not differ from controls, in contrast to patient 16. This suggests that gonadal mosaicism for a 45,X cell line could be the underlying mechanism for the disturbed gonadogenesis in the latter patient. In 46,XY patient 19, the complete absence of testicular differentiation rather points at a mutation in a major sex determining gene, which is currently under investigation.

In patients with GD as well as in undervirilization syndromes, a progressive loss of germ cells within testicular portions and UGT was demonstrated with increasing age (12, 36). The absence of germ cells in the testes of patients 17 and 18 (4 and 6 years old respectively), and

scarce germ cells in patient 13 (10 years old) suggest that very low numbers of surrounding Y positive cells may contribute to this process.

Previously we showed that the type and degree of gonadal differentiation is related to the risk of tumor formation (12): (dysgenetic) testicular portions may give rise to carcinoma *in situ* (CIS), germ cells residing in UGT are at high risk for GB development, follicles enclosing ova in well differentiated ovaries are not at risk, and streak gonads, devoid of germ cells cannot give rise to tumors (anymore). It was hypothesized that within testis and UGT, CIS and GB lesions result from a clonal expansion of TSPY positive germ cells that additionally have increased survival chances, immunohistochemically characterized by their abnormal OCT3/4 expression. Our results suggest that in 45,X/46,XY patients, both cell lines are represented in the residual germ cell populations in testis, UGT as well as in ovarian tissue. In testicular tissue and UGT, it is acceptable that the 46,XY germ cells coincide with the subpopulation of TSPY expressing cells. However, in ovaries, no TSPY positive ova were detected by immunohistochemistry, suggesting that the expression of TSPY is repressed in XY germ cells enclosed in follicles. These results are in line with the presumed function of TSPY, which is thought to be related to the pre-meiotic proliferation of male germ cells (16, 37) and suggest down regulation of TSPY at the beginning of meiosis. Further research is mandatory to confirm these findings and to elucidate the precise nature of the mechanisms that link TSPY expression to meiosis and to possible malignant transformation of germ cells.

In conclusion, this study demonstrates that the distribution of Y containing cell lines in PBL is unable to predict the gonadal differentiation pattern and gonadal karyotype in patients with gonadal dysgenesis. Gonadal karyotyping is essential to understand the specific characteristics of human gonadal development and is informative with regard to the mechanisms underlying disturbed gonadogenesis. Although in estimating the risk for GB formation in the individual patient, this analysis is not contributive, it confirms the need for prophylactic gonadectomy in 45,X/46,XY mosaicism.

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8

General discussion

Based on: Germ cell tumors in the intersex gonad: old paths, new directions, moving frontiers

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8.1 The incidence of germ cell tumors in patients with disorders of sex development

At present, our insight in the risk for germ cell tumor development in the various categories of patients with DSD is limited, and reported incidence numbers vary considerably. This is in our view caused by at least two major methodological problems: First, there are no well-established criteria for the diagnosis of early neoplastic changes (ITGNU) in young children, leading to a different interpretation of results by different research groups (1, 2). Second, the current terminology and classification systems are extremely confusing. Several synonyms and eponyms are used in literature. A definition for the used terminology in an individual article is often lacking. Moreover, the actual classification systems are based on phenotypic, genetic and pathological criteria at the same time and show several overlaps (for an example of an actual classification of patients with DSD and encountered synonyms in literature, see Table 8-1). This situation evidently leads to inconsistent classification of patients, making a correct insight in the incidence of malignancy in specific diagnostic groups illusory.

In the following paragraphs, an estimation of the incidence of germ cell tumors in patients with DSD is made, based on combined patient series from an updated review of the literature. For this purpose, patients with DSD were divided into three major groups, based on a common underlying pathophysiological mechanism per group.

8.1.1 Hypervirilization syndromes

These patients are not at risk for the development of germ cell tumors. The chromosomal constitution is 46,XX and the gonadal tissue always consists of well differentiated ovaries.

8.1.2 Undervirilization syndromes

For patients with "**male pseudohermaphroditism**" (the underlying defect not being further specified in the original articles), combined series reveal a tumor incidence of 2.3% (3/129 patients) (2-5).

Table 8-1: Traditional classification of patients with DSD, encountered synonyms in literature (adapted from (6))

Syndrome	Major group	Subgroups	Encountered Synonyms
Female PHP			Hypervirilized females
	CAH		
	CYP19 aromatase deficiency		
	Exogenous androgens		
Male PHP			Hypovirilized males
	End organ resistance for testosterone action	CAIS	Morris' syndrome, testicular feminization
		PAIS	Reifenstein's syndrome
	Disorders in testosterone and dihydrotestosterone biosynthesis	StAR deficiency	
		3β-HSDII deficiency	
		CYP17 deficiency	
		17β-HSD3 deficiency	
		5α-reductase II deficiency	
	Testicular unresponsiveness to LH and HCG		Leydig cell hypoplasia/agenesis
	Dysgenetic male PHP[1, **]		Incomplete GD[2], partial GD[2], mixed GD[3, **]
		XO/XY mosaicism*	
		structurally abnormal Y, Xp+, 9p-,10q-	
		Gene mutations (WT1, SOX9, SF1,…)	WT1: Denys-Drash, Frasier, WAGR (if WT1 deleted) SOX9: Campomelic dysplasia
	Defects in synthesis, secretion or action of AMH		Persistent Müllerian duct syndrome
	Exogenous estrogens/ progestagens		
GD			
	47,XXY		Klinefelter's syndrome
	45,X		Turner's syndrome
	45,X/46,XY and variants (+/- Turner stigmata)*		Turner's syndrome (if Turner stigmata)
	Pure (complete) GD[4]	complete 46,XY GD	Swyer's syndrome
		complete 46,XX GD	
	True hermaphroditism[5]		
	Incomplete forms of gonadal dysgenesis[2, **]		

[1] (Mostly ambiguous) phenotype resulting from the presence of bilateral dysgenetic testes
[2] (Mostly ambiguous) phenotype resulting from the presence of bilateral dysgenetic testes or one dysgenetic testis on one side and a streak on the other side
[3] (Mostly ambiguous) phenotype resulting from the presence of one dysgenetic testis on one side and a streak on the other side
[4] Normal female phenotype (without Turner stigmata) in the presence of bilateral streak gonads (devoid of germ cells) in a 46,XX or 46,XY individual
[5] (Mostly ambiguous) phenotype resulting from the presence of both testicular tissue consisting of seminiferous tubules, and ovarian tissue, containing germ cells that are all enclosed in primordial and eventually growing follicles in the same individual, either in a single gonad or in opposite gonads
*, ** Categories showing partial overlap
CAH: congenital adrenal hyperplasia; PHP: pseudohermaphroditism; CAIS: complete androgen insensitivity syndrome; PAIS: partial androgen insensitivity syndrome; HSD: hydroxy steroid dehydrogenase; GD: gonadal dysgenesis; AMH: Anti Müllerian Hormone; StAR: steroidogenic acute regulatory protein; WT-1: Wilms' tumor 1 gene; WAGR: Wilms' tumor aniridia genitourinary anomalies and mental retardation; SOX9: SRY-box - related gene 9; SF-1: steroidogenic factor 1

The first reported incidence of germ cell tumors in the AIS was 22% (7). Later this was corrected to 5 - 10% (8, 9). In combined more recent series of samples, mostly obtained after prophylactic gonadectomy, the calculated incidence is 5.5% (15/270 patients) (1-4, 10-16). Though data are limited, the risk seems to be markedly higher in the partial form (PAIS) (12/80 patients, 15%) (1, 13, 16) than in the complete variant (CAIS) (1/120 patients, 0.8%)

(1, 3, 13-16). In our view, this difference is explained by the fact that there is a rapid and total loss of germ cells in CAIS, starting from the age of 1 year, while PAIS patients have maintained their germ cell population at about 2/3 of the normal number at puberty (1). However, in this context it is important to note that at present, none of the existing *in vivo* or *in vitro* tests distinguish unambiguously between CAIS and PAIS (except in the presence of a mutation introducing a stop codon in the *AR* gene) (17-21).

Tumor incidence in AIS markedly increases after puberty and reaches 33% at the age of 50 years (3). However, no data exist on the estimated incidence in CAIS *versus* PAIS patients at this age.

For **other causes of undervirilization**, series are too small to draw conclusions: one tumor is reported in a series of 6 patients with 17β-Hydroxysteroid dehydrogenase (17β-HSD) deficiency (17%) (1) whereas no tumors are found in a series of 3 patients with 5α-reductase deficiency (14) and of 2 patients with Leydig cell hypoplasia (1).

With the exception of one case report of a GB, sufficiently supported by published histological findings in a CAIS patient (22), all the reported tumors in the group of patients with undervirilization syndromes are ITGNU lesions (81%) or seminomas (19%).

The incidence of pre-invasive tumor lesions found in prophylactic biopsy samples (usually performed at a very young age) in PAIS patients (15%) is unexpectedly high as compared to the total group of undervirilized patients (2.3%).

Due to the widespread policy of prophylactic gonadectomy, invasive tumors in these patients are rare at present but the hypothesis that all ITGNU lesions will finally result in an invasive tumor (23) explains the higher incidence in older series. However, in our view, the real incidence of ITGNU lesions in gonadectomy samples of young children might be significantly lower than the calculated 5.5%, since the benign condition of maturation delay can easily be misinterpreted as ITGNU and may cause a significant bias in some of the reported series (1, 2, 13, 14, 24) (see below). The recent tendency to leave the gonads in place, fixed in the scrotum in phenotypic males, and to include them in strict follow-up protocols will allow a better insight in the risk for malignant transformation of the testis in undervirilized patients in the future.

8.1.3 Gonadal dysgenesis

A clear insight in the incidence of germ cell tumors in patients with gonadal dysgenesis is hampered by the existing confusion regarding nomenclature, which is most pronounced for this patient category (see Table 8-I).

Four series report on the incidence of germ cell tumors in "**gonadal dysgenesis**" (not further specified): A germ cell neoplasia is found in 48/228 patients (21%) (2, 3, 25, 26). Most frequent are isolated GB (26/48, 54%) and DGs (DG) (11/48, 23%). Five (10%) consist of a combined GB and DG, two GB (4%) are associated with a non-seminoma, one is a combined GB and ITGNU lesion (2%) and three (6%) isolated ITGNU lesions are reported.

In two series of patients with "**46,XY gonadal dysgenesis**", the tumor incidence is 30 - 33% (27, 28). However, one series of 11 patients with "46,XY dysgenetic male pseudohermaphroditism" reports ITGNU in 100% of cases (29). In our view, most of the germ cell lesions described in this series reflect a state of maturation delay of the germ cells (see below). Therefore, this study will not be included in further calculations.

In series of patients with "**mixed gonadal dysgenesis**", or "**asymmetrical gonadal differentiation**", the overall incidence of germ cell tumors is 18/119 patients (15%) (3-5, 15, 30, 31), which is considerably lower than the previously reported incidence of 33% (3). The practice of performing an early prophylactic gonadectomy probably explains at least in part this difference. 38.5% of the tumors are GB, 23% are composed of GB and DG and 38.5% are isolated DG, no ITGNU lesions are found in these patient series. This distribution corresponds to older studies (3). However, in contrast with these data, one study describes 13 patients with "mixed gonadal dysgenesis" in which the incidence of malignant lesions is 77% (10/13), all of them being ITGNU, and 11 patients with "partial gonadal dysgenesis", in which the tumor incidence is 91% (10/11), all ITGNU, in 7 cases associated with GB (32). According to us, again, the ITGNU lesions described here correspond to a state of developmental delay of the germ cells (see below), and the data will not be used for further calculations.

In selected series of patients with "**true hermaphroditism**", the incidence of tumors is considerably lower. In 3 studies, 426 patients are described (2, 5, 33). In these patients, 11 tumors occurred (2.6%), the subtypes are 2/11 (18%) GB, 1/11 (9%) ITGNU, 6/11 (55%) DG and 2/11 (18%) embryonal carcinoma (EC).

According to the older literature, germ cell tumors occur in 15-20% of patients with proven **mosaicism 45,X/46,XY and variants**. Recent data may suggest a higher incidence: independent series describe 17 patients in whom 7 germ cell tumors occur (41%) (27, 34, 35): 4/7 tumors are GB (57%), 1/7 (14%) is a GB associated with a DG, 2/7 (28%) are DG. However, a bias due to selective reportage of positive gonadectomy samples cannot be excluded. In one series of four patients, ITGNU is found in all cases (100%) (36), but again, the criteria used for the diagnosis of what the authors call "an infantile CIS pattern" overlap with the characteristics of germ cells delayed in their maturation (see below). This study will not be used for further calculations.

Molecular genetic techniques (mainly polymerase chain reaction, PCR) allow a more reliable detection of a second (Y containing) cell line than classic cytogenetic analysis. Therefore, some studies examine the usefulness of routine PCR screening of all patients with Turner syndrome for the presence of Y chromosome material. We performed a meta-analysis of 11 studies dealing with this topic (37-47). This reveals that in 541 Turner syndrome patients without Y chromosome material on cytogenetic screening, 27 patients turned out to be mosaic for a Y containing cell line (5%). If a marker chromosome (mar+) was present in the original karyotype, the chance of detecting Y chromosomal derivatives approximates 100%. A total number of 557 patients were examined in these studies (in 16 patients Y chromosome derivatives were already detected on cytogenetic screening). Thus, the total number of patients bearing a Y chromosomal cell line was 43/557. In all, a gonadectomy was performed and GB was present in 5 of them (11.6%). From these data we conclude that routine PCR examination in patients with Turner syndrome in order to detect high-risk individuals for the development of GB is not indicated (the incidence of GB in the total group being 5/557 or 0.9%). In contrast, if a marker chromosome is found on cytogenetic analysis, the presence of Y chromosome material (and an elevated risk for the development of GB) must be suspected, and further examination by molecular genetic techniques is warranted.

In a review of 15 cases of **Frasier syndrome** (see Table 1-2), the incidence of germ cell tumors is as high as 60% (88% GB, 12% DG) (48). From these data, it is unclear whether this series reflects a reliable estimation of the tumor risk in Frasier syndrome or if an overrepresentation of positive cases has induced a selection bias. In other syndromes caused

by *WT-1* mutations (Denys-Drash, WAGR, see Table 1-2), the incidence of germ cell tumors may be equally high. Gonadal pathology is studied in one series of 10 patients with Denys-Drash syndrome: gonadoblastoma was found in 2/5 46,XY patients (40%), whereas 0/4 46,XX patients developed gonadal tumors (in 1 additional patient without GB, the karyotype was not determined) (49). No data are available concerning the incidence of germ cell tumors in cases of GD due to mutations in other newly discovered genes that are important for gonadal development (e.g. *SF-1, SOX9, DAX1*).

8.1.4 Conclusion

Combining of these data allows some important conclusions:

1. The overall incidence of germ cell tumors in the various patient series with GD is estimated at 97/817 (12%).
2. Within the group of undervirilization syndromes, germ cell tumors are extremely rare in CAIS and more frequent than expected in PAIS.
3. In contrast to patients with undervirilization syndromes, germ cell tumors in patients with GD are frequently found at a very young age (e.g. in the first year of life (26, 30, 50, 51)) or may even be present at birth (31).
4. Nearly all the *in situ* neoplastic lesions in patients with GD are GB, whether or not (partially) overgrown by seminoma/DG (56/61, 92%). The ITGNU lesion accounts for only 8% (5/61) of precursor lesions in patients with GD, and is probably only encountered in the presence of testicular tissue (personal observations).
5. The presence of the Y chromosome (and of the TSPY gene) was not an inclusion criteria in the large majority of the studies. Studies on patient series in which the presence of the TSPY gene or protein in the gonadectomy samples or at least in the patients' karyotype is confirmed are highly needed in order to get a better insight in the real malignant potential of the dysgenetic gonad in selected series of high-risk patients.
6. The use of an unambiguous classification system of clinical diagnoses and a clear definition of the used terminology is indispensable for a correct interpretation of data.

7. The routine search for mutations in genes involved in gonadal differentiation (e.g. WT1, SF1, DAX1, SOX9) in 46,XY individuals with gonadal dysgenesis is necessary to determine the tumor risk in specific patient series.

A summary of the estimated tumor incidences and the type of precursor lesion is given in Table 8-2.

Table 8-2: Summary of the estimated germ cell tumor incidence in patients with DSD and type of precursor lesion

	Estimated tumor incidence	**Type of precursor lesion**	
		ITGNU	**GB**
Undervirilization	**2.3% (probably higher if untreated)**	**~100%**	**~0%**
AIS	5.5% (20-30% if untreated)		
CAIS	0.8%		
PAIS	15%		
GD	**12% (probably >30% if untreated and/or selected for the presence of the Y chromosome)**	**8%**	**92%**
45,X/46,XY and variants	15 - 40%		
46,XY GD	30%		
mixed GD/asymmetrical gonadal differentiation	15% (>30% if untreated and/or selected for the presence of the Y chromosome)		
True hermaphroditism	2.6%		
Frasier Syndrome	60% (limited data)		
Denys-Drash syndrome with 46,XY karyotype	40% (limited data)		

ITGNU: intratubular germ cell neoplasia unclassified; GB: gonadoblastoma; AIS: androgen insensitivity syndrome; CAIS: complete androgen insensitivity syndrome; PAIS: partial androgen insensitivity syndrome; GD: gonadal dysgenesis

8.2 The use of immunohistochemical markers for the diagnosis of germ cell tumors in patients with disorders of sex development

The development of germ cell tumors in general is related to events during fetal gonadogenesis, the malignant germ cell being the neoplastic counterpart of a PGC or gonocyte (52). Therefore, immunohistochemical markers that are expressed by normal PGCs can be used for the diagnosis of malignant germ cell tumors. However, it is important to note that none of these markers is able to distinguish unambiguously between maturation delay of germ cells (a benign condition) and an *in situ* neoplasm. Since such a discriminative marker is not to be expected in the near future, additional criteria are indispensable to diagnose ITGNU in young children.

The use of these immunohistochemical markers was specifically examined in large series of gonads from intersex patients by our group (for results and references, see Table 8-3). The markers OCT3/4, c-KIT and PLAP show overlapping expression patterns, but the use of the

newer (both monoclonal and polyclonal forms of) OCT3/4 results in a well-circumscribed and intense nuclear staining, is easiest for interpretation, and is very robust, even if different methods of pretreatment and tissue storage are applied (53). Therefore, it is largely preferred to the other two markers.

The *in situ* neoplastic lesions of the intersex gonad - GB and ITGNU - are unambiguously characterized by a consistent expression of OCT3/4 and an abundant expression of TSPY (1, 26, 54). A careful analysis of the staining results summarized in Table 8-3 reveals that they can only arise from abnormal (developmentally delayed and/or dysgenetic) testicular tissue or from UGT, but not from normal testes or ovaries, since in these differentiation patterns, OCT3/4 and TSPY expression is always absent. The possible roles of OCT3/4 and TSPY in the development of germ cell tumors are discussed in the next paragraphs.

Table 8-3: Immunohistochemical markers in germ cells and germ cell tumors

	Testis			UGT	Ovary	ITGNU	GB	Seminoma	Non-Seminoma	References
	Normal	Delay	Dysgenetic							
OCT3/4	-	+	+	+	-	+	+	+	EC	(1, 25, 52-57)
PLAP	-	+	+	+	-	+	+	+	EC	(1, 25, 55-58)
c-KIT	-	+	+	+	+	+	+	+/-	-	(1, 25, 55-57, 59, 60)
TSPY	+	++	+++	+++	-	+++	+++	+/-	-	(1, 25, 53, 55, 56, 61, 62)
VASA	+	+	+	+	+	+/-	+/-	+/-	-	(1, 25, 55-57, 63)

UGT: undifferentiated gonadal tissue; ITGNU: intratubular germ cell neoplasia unclassified; GB: gonadoblastoma; EC: embryonal carcinoma

8.2.1 OCT3/4 (POU5F1)

OCT3/4 is consistently and specifically expressed in all germ cell tumors with pluripotent potential, and is therefore used as a reliable marker for the diagnosis of ITGNU, GB, seminoma/DG/germinoma and EC (53, 55). The diagnostic value of OCT3/4 has been confirmed in a series of independent studies (64-69). The expression of the recently proposed immunohistochemical marker AP2-γ (70) is similar to OCT3/4 expression but offers no additional information.

The Octamer binding transcription factor OCT3/4 is a member of the family of POU transcription factors, genes that regulate the expression of other target genes during mammalian development (71). It is highly expressed during the earliest stages of embryogenesis and in embryonic stem cells (ESC) (64, 72), and is essential for the

maintenance of pluripotency of these cells. Up- or down regulation of OCT3/4 in ESC as well as in EC cell lines induces differentiation (72, 73). In the early embryo, OCT3/4 is quickly repressed, and becomes exclusively confined to the germ cell lineage. However, loss of OCT3/4 expression in PGCs does not lead to differentiation of these cells but to apoptosis (74). Thus OCT3/4 is required for the surival of PGCs. In female gonadal development, OCT3/4 is expressed in oogonia and early oocytes, but never in germ cells included in follicles (58, 65), and, in accordance with this observation, it was suggested that downregulation of OCT3/4 is related to the entry of female germ cells into meiosis (71). As a result, in the female gonad, expression of OCT3/4 is high until 25 weeks GA, but decreases sharply thereafter. At birth, hardly any positive germ cell is detectable (58). In male embryonic development, OCT3/4 expression is confined to gonocytes, which are positioned in the centre of the tubule. During gonadal development, gonocytes migrate towards the periphery and once they make contact with the basal lamina (they are now referred to as prespermatogonia), they loose OCT3/4 expression. This results in a high expression pattern of OCT3/4 early in the second trimester and a constant decrease thereafter. In the perinatal period, few tubules display a single, centrally located positive germ cell. (56, 57). Subsequently it was shown that the expression of OCT3/4 is abnormally prolonged in situations where germ cells are delayed in their development (see below) (1, 26, 57, 65).

In embryonic stem cell derived tumors in mice, the level of expression of OCT3/4 was highly correlated with the formation and aggressive properties of the tumors (absent or low levels of OCT3/4 expression hampered tumor formation or resulted in poorly aggressive, well differentiated tumors, whereas high levels of OCT3/4 led to frequent and highly aggressive tumors, downregulation of OCT3/4 in these tumors resulted in their regression). Extrapolation of these data to the noticed OCT3/4 expression in germ cell tumors led to the suggestion that aberrant OCT3/4 expression might be of pathogenetic relevance in the development of these tumors and might determine their oncogenic potential (64).

8.2.2 The Testis Specific Protein - Y encoded (TSPY)

It was observed for many years that GB almost exclusively arise in the dysgenetic gonads of patients with DSD and the presence of (part of) the Y chromosome. Rare case reports of GB arising in Y negative patients date from an era before the use of molecular genetic

techniques to exclude the presence of Y chromosome material (3, 25, 28, 50, 75). This led Page in 1987 to postulate the hypothesis that a gene on the Y chromosome with a physiological function related to spermatogenesis in normal males may act as an oncogene in the context of a dysgenetic gonad. He referred to this hypothetical gene as *GBY* (gonadoblastoma locus on the Y chromosome) (76). By comparing the karyotypes of patients with GB and partially deleted Y chromosome material, the GBY susceptibility region was further sublocalized to a small region on the short arm (deletion interval 3E-3G and 4B) (77) or long arm (deletion interval 5E) of the Y chromosome (78, 79), both close to the Y centromere (Figure 1-5). The main candidate for *GBY* among the seven known genes of the interval on Yp is *TSPY*, which has also functional copies in the defined interval on Yq. In accordance with the original hypothesis of Page, the TSPY protein is normally expressed in spermatogonia of the adult male, and though its function is not fully understood, it is thought to be related to their mitotic proliferation (62, 80). Variations in the level of TSPY expression, resulting in fluctuations in the staining intensity in immunohistochemical experiments have been reported by different observers (1, 26, 57, 62, 80). In the fetal gonad, TSPY is expressed at a constant level throughout pregnancy (56). However, TSPY staining is more intense in fetal germ cells of trisomy 21 patients, which are delayed in their maturation (57), and becomes abundant in germ cells of patients with DSD (undervirilization syndromes as well as gonadal dysgenesis) (1, 26). Furthermore, the intensity of the TSPY staining is also abundant in ITGNU (whether or not arising in patients with DSD) (1, 62), GB (26, 54, 63), seminoma (63) and possibly in prostate cancer (81) and hepatocellular carcinoma (82). Whether or not the observed increase in staining intensity results from an up regulation of transcribed and translated TSPY copies has not been examined so far.

From this and the previous section it can be hypothesized that prolonged expression of OCT3/4 and increased expression of TSPY can provide the germ cell residing in an unfavourable environment with additional tools to survive and proliferate.

8.2.3 The use of serum tumor markers

Although β-hCG (choriocarcinoma) and AFP (yolk sac tumor) are well-established serum tumor markers for the diagnosis and follow-up of specific Type II germ cell tumors (83-85),

no studies exist at present on the value and applicability of these and other (PLAP, OCT3/4) serum markers for screening and early tumor detection in patients with DSD.

8.3 Maturation delay versus *in situ* neoplasia - Transitional changes of the germ cells

8.3.1 Maturation delay of germ cells

According to the "testicular dysgenesis syndrome" proposed by Skakkebæk and co-workers, germ cells that are not optimally nourished by Sertoli cells at the time of fetal gonadogenesis undergo a delay in their maturation (86), resulting in an increased risk for subfertility and germ cell tumor formation (see Chapter 1). This model suggests that developmental delay of germ cells is the common underlying mechanism in various apparently unrelated conditions, such as exposure to xenoestrogens and antiandrogens (the so-called "endocrine disruptors") of the male fetus, certain conditions caused by chromosomal aberrations (e.g. trisomy 21), and DSD.

To test this hypothesis, we quantitatively and qualitatively examined the expression of germ cell markers in male and female fetuses throughout pregnancy (56, 58) and compared the expression patterns to those obtained in trisomy 21 fetuses, undervirilization syndromes and GD (1, 26, 57). We found that in all these conditions, a subpopulation of germ cells is indeed affected by maturation delay. In the testes of males with trisomy 21 and of patients with undervirilization syndromes and GD, the normal migration of these early germs cell towards the basal lamina and their subsequent differentiation into prespermatogonia is interrupted. This becomes apparent by their morphological similarity with the primitive gonocyte, by their increased and prolonged expression of the tumor markers OCT3/4, c-KIT and PLAP, and by increased intensity of immunohistochemical staining for TSPY. The developmental delay present in the gonads of male trisomy 21 patients, who have a moderately elevated risk (estimated at 0.5%) for the development of germ cell tumors is rather mild, whereas a more pronounced maturation delay was demonstrated in the germ cells of patients with undervirilization syndromes and especially in the testicular parts of gonads from patients with GD (1, 26, 57). In UGT, characteristic for patients with GD, and at high risk for the development of GB, germ cells, extremely retarded (or blocked) in their maturation reside in a

background of supporting and stromal cells that failed themselves to differentiate properly and to organize in structures characteristic for normal male or female gonadal development (*i.e.* the formation of testes or ovaries) (26).

Thus, our data confirm the model that a delay or block in germ cell development is a common underlying mechanism in various conditions such as trisomy 21 and DSD. To what extent these data can be extrapolated to gonadal development in male fetuses exposed to endocrine disruptors remains to be examined at present.

8.3.2 Pitfalls in the diagnosis of early germ cell neoplasia

The prolonged expression of fetal germ cell markers/tumor markers in the gonads of young patients with DSD has led to a significant overdiagnosis of ITGNU in this patient population, since the mere presence of a tumor marker in a germ cell with aberrant morphology (the morphology of a ITGNU cell also closely resembles that of a primitive gonocyte) was considered as a hallmark for its malignant transformation, and was thought to represent a "prepubertal CIS lesion" (13, 14, 29, 32, 87). Therefore, we have proposed three additional criteria - patient age, position of OCT3/4 positive cells within the seminiferous tubule and distribution of OCT3/4 positive cells throughout the gonad - that allow distinguishing between maturation delay and ITGNU in the gonads of patients with undervirilization syndromes (1) (Table 8-4). These criteria have recently been validated in an independently performed study (88). The two last criteria are also applicable on the dysgenetic testes of patients with GD, however, the age criterion cannot be used in this population since the process of maturation delay is more pronounced in this condition and was found up to the age of 10 years (personal observations).

Table 8-4: Additional criteria for the diagnosis of maturation delay and CIS in patients with undervirilization syndromes

	Maturation delay	Transition	ITGNU
Patient age	< 1 year	prepubertal	> 1 year
Position of OCT3/4 positive cells within the seminiferous tubule	luminally	luminally and on the basal lamina	on the basal lamina
Position of OCT3/4 positive cells throughout the gonad	widespread	confined to a specific region - rest of the gonad is free of positive cells or displays characteristics of maturation delay	confined to a specific region - rest of the gonad is free of positive cells or displays characteristics of maturation delay

ITGNU: intra tubular germ cell neoplasia unclassified

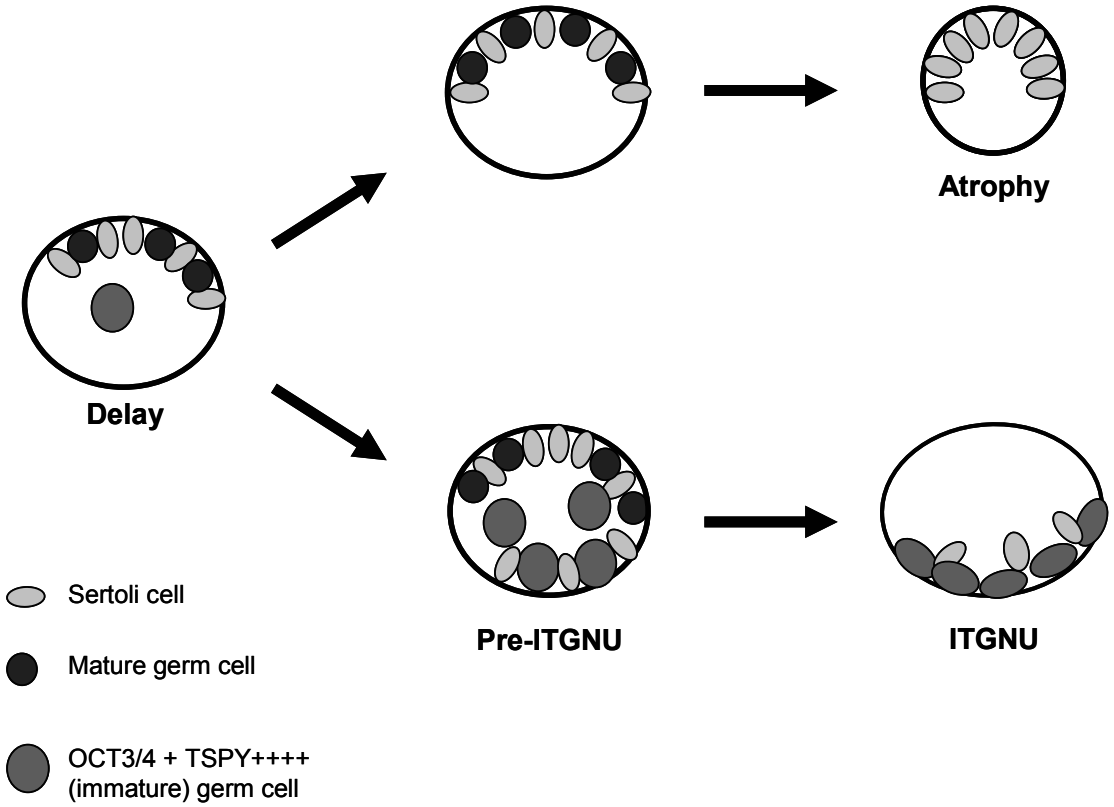

Figure 8-1: Development of ITGNU in the dysgenetic testis: Germ cells that are delayed in their maturation are characterized by prolonged OCT3/4 and increased TSPY expression. Due to the unfavourable environment, most of these germ cells will die, leading to tubular atrophy. However, surviving germ cells are prone to clonal expansion and the formation of an in situ neoplasia.

8.3.3 Progression towards malignancy

Our results suggest that maturation delay of germ cells may indeed result in a moderately increased risk for neoplastic transformation, due to the prolonged expression of OCT3/4 and increased TSPY expression, which provides the cell with an equipment to survive and proliferate. However, in most cases this is only a transient condition: in spite of their initial developmental delay, immature germ cells loose OCT3/4 expression and differentiate into a more mature cell type, or alternatively they die, due to the inappropriateness of their environment. However, in the testes of patients with undervirilization syndromes and GD, it was occasionally observed that OCT3/4 positive PGCs/gonocytes manage to make contact with the basal lamina and seem to definitively escape downregulation of OCT3/4 and further differentiation along the spermatogenic pathway. These OCT3/4 positive and TSPY positive germ cells in contact with the basal lamina can now undergo mitotic proliferation and are prone to clonal expansion. On immunohistochemical staining, this pre-neoplastic lesion is

characterized by the combined presence of OCT3/4 positive and OCT3/4 negative germ cells on the basal lamina of the seminiferous tubule. Luminally positioned OCT3/4 positive cells may also be present (Table 8-4 and Figure 8-1). This pattern was hitherto only seen in prepubertal patients (1, 26). Thus, histologically, a continuum can be identified, from maturation delay towards pre-neoplastic changes of the germ cells and final organisation into a typical ITGNU pattern.

Maturation arrest of tissue-determined stem cells has been proposed previously as a common underlying mechanism in all epithelial derived tumors as well as in tumors of hematopoietic origin (89). In this context, the recent observation that global loss of genomic imprinting in mouse embryonic stem cells predisposes these cells to malignancy by rendering them immortile is of interest (90). Indeed, type II GCTs of the testis have been found to consistently show biallelic expression of imprinted genes, i.e., they lack a somatic pattern of genomic imprinting (91), see Introduction). In spite of the fact that it possibly concerns an intrinsic characteristic of the cell of origin (a primordial germ cell/gonocyte), this loss of genomic imprinting might in fact represent a step in the pathogenetic process of malignant transformation. Extrapolation of these data to patients with DSD creates a model in which PGCs, insufficiently nourished by an inappropriate environment undergo a severe delay or arrest in their development, which is most pronounced in those tissues with the lowest degree of differentiation, thereby maintaining (or inducing) a status of erased genomic imprinting and prolonged OCT3/4 expression of the germ cells (immortalization). Increased TSPY expression may rapidly lead to expansive proliferation of this immortal cell type (Figure 8-2).

8.4 ITGNU or gonadoblastoma?

Why do some patients with DSD develop an ITGNU and others a GB as an *in situ* neoplastic lesion? From Table 8-2 becomes apparent that ITGNU is the almost exclusive precursor lesion in undervirilized patients, while GB is predominant in patients with GD. Undervirilization syndromes are characterized by the presence of bilateral, well-differentiated testes. Thus, in the various undervirilization syndromes, as well as in the (more frequently occurring) ITGNU lesion of the normal adult male, it is clear that the gonadal tissue in which

these lesions originate always consists of well differentiated testicular tissue (see also Figure 8-2).

Our data suggest that GB result from surviving PGCs residing in UGT, structures that are only encountered in the context of GD and that remained unrecognized so far, see chapter 5 (26).

A model for the development of GB and ITGNU in patients with GD is proposed in Chapter 5 and schematically represented in Chapter 5, Figure 5-5.

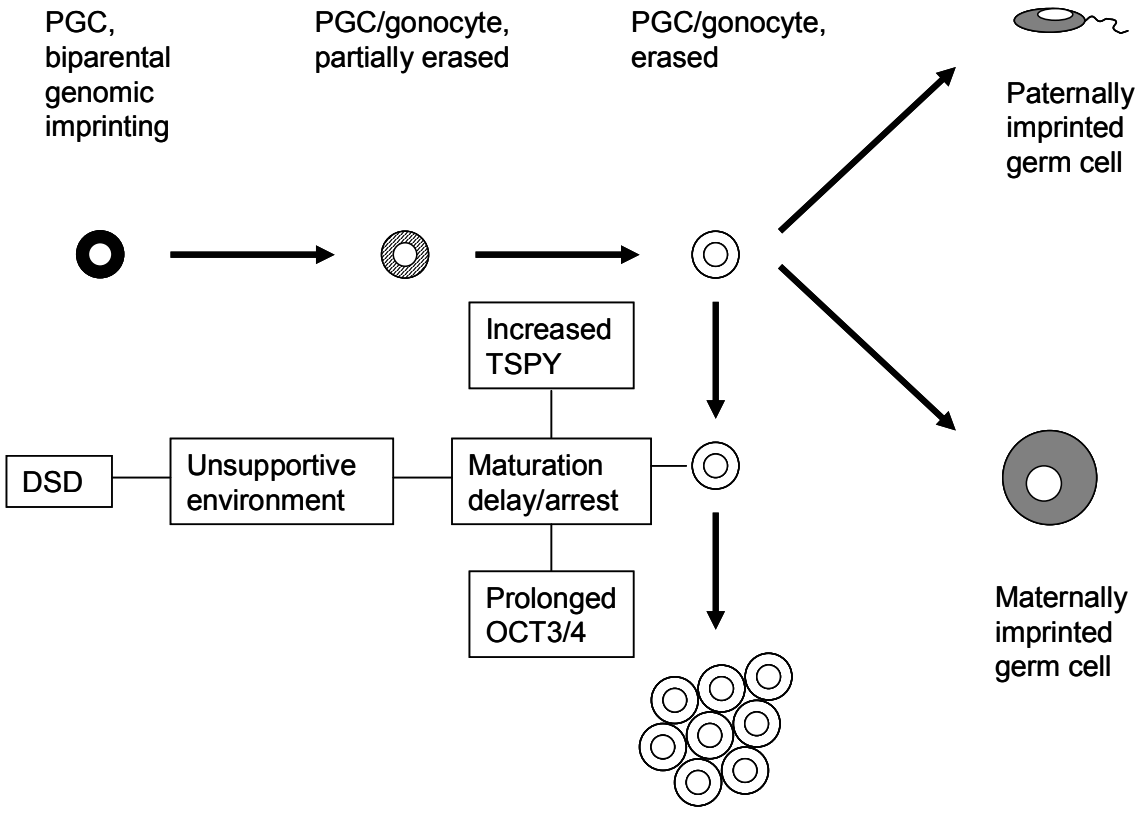

Figure 8-2: Overview of the pathogenetic mechanisms leading to clonal expansion of germ cells in patients with DSD. The original bipaternal pattern of genomic imprinting present in the zygote has to be erased during PGC development to install a uniparental pattern of maternal genomic imprinting in the oocyte and paternal genomic imprinting in the spermatozoon. Loss of genomic imprinting (or bialellic expression of imprinted genes, as is seen in the PGS/gonocyte) may lead to immortalization. In DSD, due to the unsupportive environment, the normal maturation of germ cells is interrupted. Maturation delay/arrest of the germ cells, characterized by prolonged OCT3/4 expression (increased survival of the PGC) and a maintained or induced status of erased genomic imprinting may lead to immortalization of the cell. Increased TSPY expression may cause rapid proliferation of this immortalized cell type. (PGC: primordial germ cell; DSD: disorders of sex development)

8.5 Proposal for a new classification of patients with disorders of sex development according to their risk for the occurrence of germ cell tumors and future perspectives

In recent years, patient advocacy groups and medical staff emphasize on the need for a postponed decision taking regarding sex of rearing and genital surgery and a more

conservative approach towards gonadectomy (92-97). However, before safe guidelines for including patients in watchful-waiting protocols can be established, a good insight in the individual risk for tumor development is required. A review of the literature, as outlined in the first part of this chapter, reveals that actually, this goal cannot be achieved for the reasons stated above. Therefore, we propose an adaptation to the recently proposed classification system for patients with DSD (98), taking into account the affected level of sex development which is important from a comprehensive point of view, and the differentiation type of the gonad, which is directly related to the risk for tumor development (Figure 8-3 and Table 8-5).

A major disadvantage of this classification system is that obtaining representative gonadal tissue - at least a bilateral gonadal biopsy is needed - becomes essential for classifying the patient, which makes a surgically restrictive patient care impossible at once. Therefore, we would like to consider it as a temporary tool to re-classify detailed patient series from the literature and additionally, newly diagnosed patients, in whom a bilateral gonadectomy is as a rule performed at present.

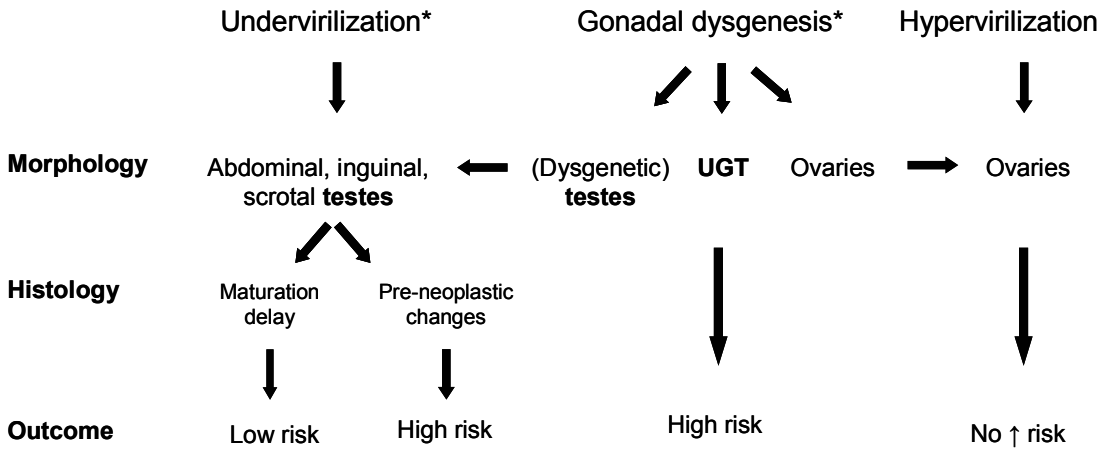

Figure 8-3: Classification of patients with disorders of sex development (UGT: undifferentiated gonadal tissue, PBL: peripheral blood)

Table 8-5: New classification system for patients with disorders of sex development: The combined presence of testicular tissue or undifferentiated gonadal tissue/sex cords and the TSPY gene contains an increased risk for the development of germ cell tumors. (next page)

Affected level	Relation to karyotype	Underlying defect	Gonadal differentiation pattern	Testis	UGT	Ovary	Streak	Presence of Y/TSPY
Gonadal sex: Chromosomal sex (Sex chromosome DSD)	45,X (Turner syndrome)	Sex chromosome: numerical	Complete GD[1] (or ovaries)	-	-	+/-	+/-	-
	47,XXY (Klinefelter syndrome)	Sex chromosome: numerical	Testes	+	-	-	-	+
	45,X/46,XY and variants	Sex chromosome: numerical	Complete GD	-	-	-	+	+/-
			Testicular dysgenesis[2]	+	-	-	+/-	+/-
			Ovotesticular dysgenesis[3]	+	-	+	-	+/-
			Undifferentiated GD[4]	+/-	+	+/-	+/-	+/-
	46,XX/46,XY and variants	Sex chromosome: numerical	Complete GD	-	-	-	+	+/-
			Testicular dysgenesis	+	-	-	+/-	+/-
			Ovotesticular dysgenesis	+	-	+	-	+/-
			Undifferentiated GD	+/-	+	+/-	+/-	+/-
Gonadal sex: Mutation in sex determining gene	46,XY GD	Mutation in sex determining gene	Complete GD	-	-	-	+	+
			Testicular dysgenesis	+	-	-	+/-	+
			Ovotesticular dysgenesis	+	-	+	-	+
			Undifferentiated GD	+/-	+	+/-	+/-	+
			Gonadal regression	-	-	-	-	+
	46,XX GD	Mutation in sex determining gene	Complete GD	-	-	-	+	+/-
			Testicular dysgenesis	+	-	-	+/-	+/-
			Ovotesticular dysgenesis	+	-	+	-	+/-
			Undifferentiated GD	+/-	+	+/-	+/-	+/-
Phenotypic sex	46,XY (Undervirilization)	Disorders in T and DHT biosynthesis*	Testes	+	-	-	-	+
		End organ resistance for T action**	Testes	+	-	-	-	+
		Testicular unresponsiveness to LH and hCG	Testes	+	-	-	-	+
		Disorders of AMH and AMH receptor	Testes	+	-	-	-	+
	46,XX (Hypervirilization)	Fetal: Disorders in adrenal steroid biosynthesis	Ovaries	-	-	+	-	-
		Fetoplacental: aromatase deficiency	Ovaries	-	-	+	-	-
		Maternal androgens	Ovaries	-	-	+	-	-
		Mutations in genes related to ovarian differentiation	Complete GD or ovaries	-	-	+/-	+/-	-
Other	46,XY	Severe hypospadias, cloacal extrophy,…	Testes	+	-	-	-	+
	46,XX	Cloacal extrophy, vaginal atresia,…	Ovaries	-	-	+	-	-

* Patients with 5α-reductase II deficiency should be considered separately within this group since this defect, in contrast to the other enzyme defects, only affects the development of the male external genitalia.
** A strict subclassification of patients in groups CAIS *versus* severe AIS and PAIS, based on the combination of clinical data, histology of Wolffian duct derivatives, sequencing of the androgen receptor and androgen receptor binding studies, is essential to demonstrate the expected major difference in tumor risk between these groups.
[1] Normal female phenotype in the presence of bilateral streak gonads (devoid of germ cells) in a 46,XX or 46,XY individual
[2] (Mostly ambiguous) phenotype resulting from the presence of one (dysgenetic) testis on one side and a streak on the other side, or of two dysgenetic testes
[3] (Mostly ambiguous) phenotype resulting from the presence of both testicular tissue consisting of seminiferous tubules, and ovarian tissue, containing germ cells that are all enclosed in primordial and eventually growing follicles in the same individual, either in a single gonad or in opposite gonads
[4] (Mostly ambiguous) phenotype resulting from the presence of undifferentiated gonadal tissue or sex cords, whether or not in combination with testicular or ovarian tissue or a streak.
DSD: disorder of sex development; UGT: undifferentiated gonadal tissue; TSPY: testis specific protein-Y encoded; GD: gonadal dysgenesis; LH: Luteinizing hormone; hCG: human chorionic gonadotrophin; DHT: dihydrotestosterone

Important questions and remarks are:

- To what degree is a gonadal biopsy representative for the whole gonad in patients with undervirilization syndromes and GD? How many biopsies (at the same time and/or with a given time interval) are necessary to generalize data on the presence or absence of germ cells or their malignant transformation in a biopsy specimen? How to exclude the (eventually microscopical) presence of testicular or undifferentiated gonadal tissue in patients with GD and predominant ovarian differentiation based on limited biopsy material?
- Are patients with CAIS – provided that the diagnosis is made on very strict criteria – at risk for germ cell tumor development at all? If the answer is negative, it is preferable to leave their gonads in place, which allows these patients to profit from their endogenous hormone production. In this context, the suggestion of some authors (20, 21) to re-classify AIS patients into PAIS, severe AIS and CAIS would be very relevant, the true CAIS patients – without pubic hair growth at puberty and without Wolffian duct derivatives – being the only category in which such a policy would be defendable at present.
- Very limited or no data at all exist at present regarding the incidence and age of occurrence of germ cell tumors in patients with PAIS, 17β-HSD deficiency, Leydig cell hypoplasia and *WT-1* and other gene mutations. However, answering this question is of major importance since these patients more and more will be reared as males, preferably with their gonads preserved and fixed in the scrotum. The establishment of safe follow-up protocols for this patient population by non-invasive techniques (e.g. ultrasound) is mandatory. In adult males diagnosed with ITGNU, it was shown that local low-dose irradiation prevents progression towards invasiveness and allows the preservation of hormonal function in most patients (99). To what extent these data also apply to ITGNU lesions in the testes of young patients with DSD remains to be examined but is of high relevance since it possibly offers to them an alternative to gonadectomy.
- Is the incidence of germ cell tumors in patients with ovotesticular dysgenesis as low as it appears to be? A low incidence would indeed be expected, since their gonads consist of well-differentiated ovarian tissue, with no risk for germ cell neoplasia, and of well-

differentiated (cryptorchid) testicular tissue, eventually displaying maturation delay. Moreover, 60% of ovotesticular dysgenesis patients have a 46,XX karyotype (100) and theoretically these patients have no increased risk. However, to what extend the PBL karyotype in these patients corresponds to their gonadal karyotype is actually unknown.

- In the light of its suspected role in the development of germ cell tumors, the gonadal presence of the TSPY gene and/or protein should always be ruled out in dysgenetic testes, UGT or in ovotesticular dysgenesis, even if the PBL karyotype does not reveal a Y bearing cell line.
- The diagnosis of complete GD should only be made on very strict criteria, since the total absence of germ cells rules out the possibility of germ cell tumors. The presence of rare isolated germ cells in otherwise ovarian-type fibrous stroma should always be designated as UGT and contains a high risk for GB formation.

Table 8-6: Summary of the risk of germ cell malignancy in the various forms of DSD, subdivided into high, intermediate, low and possibly no risk. Recommended actions are indicated, as well as the number of studies and patients included in the survey. In case of PAIS, 17β-HSD and ovotestis, the decision regarding gonadetomy is largely determined by sex of rearing.

Risk group	Disorder	Risk (%)	Action needed	# Studies	# Patients
High	GD[a] (+Y)[b] intra-abd	15-35	gonadectomy[c]	12	>350
	PAIS non-scrotal	15	gonadectomy[c]	3	80
	Frasier	60	gonadectomy[c]	1	15
	Denys-Drash (+Y)	40	gonadectomy[c]	1	5
Intermediate	Turner (+Y)	12	gonadectomy[c]	11	43
	17β-HSD	28	watchful waiting and possible biopsy	2	7
Low	CAIS	0.8	biopsy[d] and possible irrad/gonadectomy	3	120
	ovotest. DSD	3	testicular tissue removal in case of ♀ rearing?	3	426
	Turner (–Y[e])	1	None	11	557
Unknown[f]	5α-reductase	0	unresolved	1	3
	Leydig cell hypopl	0	unresolved	1	2
	GD (+Y)[b] scrotal	unknown	biopsy[d] and irrad?	0	0
	PAIS scrotal gonad	unknown	biopsy[d] and irrad?	0	0

Relevant data from the recently published study by Hannema and co-workers (88) are not included in this table since it is at present unclear to us to what extent patient series from this study show overlap with patient series from a previously published study by the same group (16).
[a]Gonadal Dysgenesis (including not further specified, 46XY, 46X/46XY, mixed, partial, complete)
[b]GBY region positive, including the TSPY gene
[c]at time of diagnosis
[d]at puberty, allowing investigation of at least 30 seminiferous tubules, preferentially diagnosis based on OCT3/4 immunohistochemistry
[e]PCR detection of Y-chromosomal sequences (in particular the GBY region) is implicated if a marker is identified by karyotyping
[f]based on current knowledge (single study including limited number of patients, or no studies reported at all)
Intra-abd: intra-abdominal located gonad; non-scrotal: non-scrotally located gonad; scrotal: scrotally located gonad; hypopl: hypoplasia; irrad: local irradiation with 18 Gy; ovotest. DSD: formally ovotestis (true hermaphrodite)

In view of the limited patient numbers, an intense and multicenter collaboration is needed to answer these questions in a restricted time period. A first step to achieve this goal was recently made during an international consensus meeting (Chicago, USA, October 2005), where the implementation of the data presented in this thesis led to the proposal of new recommendations for the management of patients with DSD (Table 8-6) (98). In the future, the conclusions drawn from these guidelines may serve as a jumping-off board towards the establishment of safe, evidence-based protocols for a definitively revised management of patients with DSD, in accordance with the possibilities offered by modern diagnostic and surgical techniques and with the aspirations from patient advocacy groups all over the world.

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9

Summary/Samenvatting

Summary

This thesis describes the origin, identification and correct diagnosis of the earliest stages of malignant germ cell tumors, i.e. intra tubular germ cell neoplasia unclassified (ITGNU) and gonadoblastoma, in patients with disorders of sex development. Special attention is given to the possible pathogenetic mechanisms underlying the development of these lesions, in order to identify high-risk patients. Ultimately, this study aims to contribute to the actual process of re-thinking the clinical management of patients with disorders of sex development, in accordance with the intense aspirations of these patients to evolve towards a safe, evidence-based, but more conservative approach regarding gonadectomy and other surgical procedures in the future wherever possible.

Chapter 1 situates the ITGNU and gonadoblastoma lesions within the group of malignant germ cell tumors. It offers an overview of our actual knowledge on normal gonadal and sexual development and describes the recent hypotheses on how this process can be disturbed by environmental influences. The different phenotypes in the various disorders of sex development are described from a developmental point of view, with emphasis on the biology and functioning of the malformed gonad.

In **chapter 2**, the normal gonadal development is studied in female embryos: the sequential expression of protein markers for germ cells, detectable by immunohistochemisty, in relation to the ultrastructure and cellular organization within the developing ovary is described. Together with a similar study on male gonadal development (Honecker et al, J Pathol 2004, 203 (3): 849-57), this study forms the basis for further research on the process of maturation delay of germ cells, as described in the following chapters.

Chapter 3 examines if and to what extent maturation delay of germ cells is present in patients with trisomy 21, a condition unrelated to disorders of sex development but also characterized by an increased risk for germ cell tumors in males. Accordingly, a delay in germ cell maturation was mainly found in the male trisomy 21 population. This adds evidence to the "testicular dysgenesis syndrome" hypothesis, suggesting that a disturbed gonadal development is a common underlying mechanism in various, apparently unrelated conditions that are characterized by male subfertility and increased risk for germ cell cancer.

The origin and progression of ITGNU lesions in patients with undervirilization syndromes are studied in **chapter 4**. Aberrant expression of immunohistochemical germ cell markers, a typical finding in young patients with undervirilization has for a long time been interpreted as "a pre-pubertal carcinoma *in situ* pattern" (i.e. ITGNU), but is now placed in the context of maturation delay of germ cells. This study presents diagnostic tools to discriminate between the benign condition of developmental delay of germ cells and the malignant ITGNU and offers a model for the stepwise progression from the first condition into the second.

Chapter 5 focuses on the origin of gonadoblastoma lesions in patients with gonadal dysgenesis. Within these gonads, gonadal differentiation patterns and germ cells which are most prone to malignant transformation are sought, with the aim of identifying high-risk patients. This approach led to the identification of the hitherto undescribed "undifferentiated gonadal tissue" (UGT), a pattern only found in dysgenetic gonads. In this chapter it is hypothesized that gonadoblastomas originate from surviving germ cells residing in UGT. A model for the differential development of the two types of *in situ* malignant lesions (ITGNU and gonadoblastoma) in patients with disorders of sex development is presented.

Chapter 6 describes the progression from the *in situ* malignant gonadoblastoma cells towards the invasive dysgerminoma. It underscores the heterogeneity of the germ cells regarding their maturation status within a gonadoblastoma lesion, and identifies those germ cells that will escape proliferation-regulating mechanisms and will become invasive. The value and possible pathogenetic relevance of the diagnostic markers OCT3/4 and TSPY in this context becomes apparent from this study.

In **chapter 7** it is examined to what extent gonadal development and germ cell tumor formation are influenced by the presence of the Y chromosome in patients with gonadal dysgenesis. The results indicate that the peripheral blood and gonadal karyotypes are not a suitable indicator to predict the differentiation patterns present in the dysgenetic gonad, nor do they identify high-risk patients.

Chapter 8 offers an extensive and updated review of the literature in order to gain insight in the risk for germ cell cancer in the various types of disorders of sex deelopment. This goal cannot be achieved at present and specific problems in this context are discussed. The results from the studies in the present thesis are interpreted in the light of overcoming these

problems in the future. This thesis concludes with the presentation of a new classification system for patients with disorders of sex development, opening perspectives for a better estimation of the risk for germ cell tumor development in the individual patient and thus providing a basis for optimised medical care in accordance with aspirations from patient advocacy groups in the future.

Samenvatting

Dit proefschrift beschrijft het ontstaan, het herkennen en het correct diagnosticeren van de vroegste stadia van kwaadaardige kiemceltumoren, met name "intra tubular germ cell neoplasia unclassified" (ITGNU) en gonadoblastoma, bij patiënten met stoornissen in de geslachtsontwikkeling. Bijzondere aandacht gaat uit naar de onderliggende mechanismen voor de ontwikkeling van deze tumoren, om zo patiënten met een sterk verhoogd risico te kunnen identificeren. Uiteindelijk heeft deze studie tot doel een bijdrage te leveren aan de actuele pogingen om het medische beleid bij patiënten met stoornissen in de geslachtsontwikkeling te herzien en in overeenstemming te brengen met de intense wil van patiënten om geleidelijk te evolueren naar een meer terughoudend beleid met betrekking tot het verwijderen van de gonaden en andere chirurgische interventies waar mogelijk.

Hoofdstuk 1 plaatst de ITGNU en gonadoblastoma afwijkingen binnen de groep van kwaadaardige kiemceltumoren. Het biedt een overzicht van onze huidige kennis van de normale ontwikkeling van de geslachtsorganen en beschrijft recente hypothesen over hoe dit proces verstoord kan worden door omgevingsfactoren. Voorts wordt aangetoond hoe de verstoorde ontwikkeling en functie van de gonade (geslachtsklier) resulteert in een afwijkend fenotype bij patiënten met stoornissen in de geslachtsontwikkeling.

In **hoofdstuk 2** wordt de normale ontwikkeling van de gonade bestudeerd in vrouwelijke embryo's: Er wordt gekeken naar de opeenvolgende expressie van eiwit markers voor kiemcellen met behulp van immuunhistochemie, in relatie tot de microscopische structuur en de organisatie van de verschillende celtypes binnen de zich ontwikkelende eierstokken. Deze studie vormt, samen met een gelijkaardige studie betreffende de ontwikkeling van de mannelijke gonade (Honecker et al, J Pathol 2004, 203 (3): 849-57) de basis voor het bestuderen van de vertraagde rijping van kiemcellen zoals wordt beschreven in de volgende hoofdstukken.

Hoofdstuk 3 onderzoekt of en in welke mate een vertraagde rijping van kiemcellen aanwezig is in trisomie 21 patiënten, een aandoening die niet gerelateerd is aan stoornissen in de geslachtsontwikkeling, maar die ook gekenmerkt wordt door een verhoogd risico voor de ontwikkeling van kiemceltumoren bij de mannelijke patiëntenpopulatie. Een vertraging in het rijpingsproces van de kiemcellen werd dan ook voornamelijk gevonden in de mannelijke trisomie 21 groep. De resultaten van deze studie zijn in overeenstemming met de "testiculaire dysgenesie syndroom" hypothese, waarin verondersteld wordt dat een verstoorde ontwikkeling van de geslachtsklier een gemeenschappelijk onderliggend mechanisme is in verschillende ogenschijnlijk niet aan elkaar gerelateerde aandoeningen, allen gekenmerkt door mannelijke subfertiliteit en een verhoogd risico op kiemceltumoren.

De oorsprong en de progressie van ITGNU letsels in patiënten met ondervirilizatie worden bestudeerd in **hoofdstuk 4**. De afwijkende expressie van immuunhistochemische markers voor kiemcellen, een typische bevinding bij jonge patiënten met ondervirilizatie, werd vroeger geïnterpreteerd als een "prepubertair carcinoma *in situ* letsel" (i.e. ITGNU), maar wordt nu in de context van vertraagde rijping van kiemcellen geplaatst. De studie reikt dan ook diagnostische hulpmiddelen aan om deze goedaardige aandoening te onderscheiden van het kwaadaardige ITGNU letsel, en stelt een model voor dat de stapsgewijze evolutie van het eerste naar het tweede letsel beschrijft.

Hoofdstuk 5 spitst zich toe op het ontstaan van gonadoblastoma letsels in patiënten met abnormaal aangelegde gonaden. In de gonaden van deze patiënten wordt gezocht naar het type differentiatie patroon en de kiemcellen die de grootste kans hebben om kwaadaardig te worden, met de bedoeling patiënten met een hoog risico te identificeren. Deze benaderingswijze heeft geleid tot de beschrijving van het zogenaamde "ongedifferentieerde gonadale weefsel", een differentiatie patroon van de geslachtsklier dat totnogtoe niet was beschreven en dat uitsluitend gevonden wordt in abnormaal aangelegde gonaden. In dit hoofdstuk wordt de hypothese gepresenteerd die veronderstelt dat gonadoblastomas ontstaan vanuit kiemcellen die erin slagen in dit "ongedifferentieerde gonadale weefsel" te overleven. Ten slotte wordt een model gepresenteerd dat de verschillende ontwikkeling verklaart van de 2 types *in situ* kwaadaardige letsels die worden gevonden bij patiënten met geslachtsdifferentiatie stoornissen, namelijk ITGNU en gonadoblastoma.

Hoofdstuk 6 beschrijft de evolutie van de niet invasieve gonadoblastoma cellen tot het resulterende invasieve dysgerminoom. Het heterogene karakter van de kiemcellen binnen een gonadoblastoma letsel wordt benadrukt. Deze studie identificeert de kiemcellen die uiteindelijk zullen ontsnappen aan groeiregulerende mechanismen en een invasief groeipatroon zullen vertonen en toont de waarde en mogelijk oorzakelijke rol aan van de diagnostische merkers OCT3/4 en TSPY.

In **hoofdstuk 7** wordt onderzocht in welke mate de ontwikkeling van de geslachtsklier en het ontstaan van kiemceltumoren wordt beïnvloed door de aanwezigheid van het Y chromosoom bij patiënten met abnormaal aangelegde geslachtsklieren. De resultaten van deze studie wijzen erop dat de verschillende gonadale differentiatie patronen niet kunnen worden afgeleid uit het karyotype dat gevonden wordt, noch in perifeer bloed, noch in de geslachtsklier zelf. Bovendien laat de bepaling van het karyotype niet toe patiënten met een sterk verhoogd risico te herkennen.

Hoofdstuk 8 ten slotte biedt een uitgebreid en recent overzicht van de literatuur, om zo een beter inzicht te verschaffen in het risico op kiemceltumoren voor de verschillende categorieën van patiënten met stoornissen in de geslachtsontwikkeling. Dit doel kan momenteel echter niet bereikt worden en de problemen die in dit verband opduiken worden aangehaald. De resultaten die voortkwamen uit de verschillende studies van dit proefschrift worden besproken en kunnen aangewend worden om deze problemen op te lossen in de toekomst. Dit proefschrift besluit met de voorstelling van een nieuw classificatie systeem voor patiënten met stoornissen in de geslachtsontwikkeliing, wat perspectieven opent voor een betere inschatting van het individuele risico op de ontwikkeling van kiemceltumoren. Op deze manier legt het onderzoek gepresenteerd in dit proefschrift de fundamenten voor een geoptimaliseerde medische behandeling van patiënten met stoornissen in de geslachtsontwikkeling in de toekomst, rekening houdend met de opmerkingen en verzuchtingen van verschillende patiënten verenigingen.

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Martine, 21.12.2005.

Curriculum Vitae

Martine Cools was born in 1971 in Antwerp, Belgium. For two years she studied Roman Philology at the University of Antwerp and graduated *cum laude* in 1991 (Bachelor). Afterwards, she initiated her medical studies at the University of Leuven and continued subsequently at the University of Antwerp. She graduated *cum laude* in 1998. She was a fellow in Pediatrics from 1998 to 2003 in the Queen Paola Children's Hospital and the University Hospital of Antwerp, Belgium, and was registered as a pediatrician in 2003. As a laureate of the ESPE (European Society for Pediatric Endocrinology) Research Fellowship, she started to work in the Laboratory for Experimental Patho-Oncology of Prof Leendert Looijenga in January 2004. Her research focused on the earliest steps in the malignant transformation of germ cells in patients with disorders of sex development, with the aim of identifying high risk patients. She started a clinical fellowship in Pediatric Endocrinology at the Sophia Children's Hospital Rotterdam under the supervision of Prof Sten Drop in January 2006.

Martine Cools lives together with Björn Debaillie, with whom she travelled for several months through Asia before starting the research described in this thesis. Together they have a daughter: Lies, born in 2004.

List of publications

- **M Cools, SLS Drop, KP Wolffenbuttel, JW Oosterhuis, LHJ Looijenga**. Germ cell tumors in the intersex gonad: old paths, new directions, moving frontiers. Endocrine Reviews, In press.
- **M Cools, R van Gurp, M Boter, H Stoop, P Poddighe, YFC Lau, SLS Drop, KP Wolffenbuttel, LHJ Looijenga.** Analysis of Y containing cell lines in the gonads of patients with gonadal dysgenesis: impact on gonadal differentiation patterns and risk for gonadoblastoma formation. Submitted.
- **M Cools, H Stoop, AMF Kersemaekers, SLS Drop, KP Wolffenbuttel, JP Bourguignon, J Slowikowska-Hilzcer, K Kula, S Faratz, JW Oosterhuis, LHJ Looijenga.** Gonadoblastoma arising in undifferentiated gonadal tissue within dysgenetic gonads. J Clin Endocrinol Metab, In press.
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- **M Cools, E Duval, A Jespers.** Adverse neonatal outcome after maternal biliopancreatic diversion operation: report of 9 cases. Eur J Pediatr 2006; 165(3): 199-202.
- **M Cools, HP Van Bever, JJ Weyler, WJ Stevens.** Long-term effects of specific immunotherapy, administered during childhood, in asthmatic patients allergic to either house-dust mite or to both house-dust mite and grass pollen. Allergy 2000; 55: 69-73.

Legends for color illustrations

Chapter 2

Figure 2-2: Results of immunohistochemical analysis of different markers in fetal germ cells. The cortex (C) and medulla (M) are indicated. A) Foetal ovary (15.5 weeks of development) (magnification 200x); 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) Foetal ovary (same case) (magnification 200x); placental/germ cell alkaline phosphatase (PLAP; red cytoplasmic signal). Note high number of positive cells (oogonia) at a cortical localization. C) Foetal ovary (22 weeks of development) (magnification 400x); OCT3/4 (brown nuclear signal). Oogonia and immature oocytes, mainly cortically localized, are positive, whereas primordial follicles (indicated by an arrow) are negative. D) Foetal ovary (same case) (magnification 400x); 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 PLAP (indicated by an arrowhead). E): Foetal ovary (same case) (magnification 400x); 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 at is positive for both markers. F) Foetal ovary (same case) (magnification 400x); 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) Foetal ovary (same case) (magnification 400x); 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) Foetal ovary (15.5 weeks of development) (magnification 200x); 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 2-3: Results of immunohistochemical analysis of different markers in fetal germ cells. The cortex (C) and medulla (M) are indicated. A) Foetal ovary (28 weeks of development); c-KIT (red cytoplasmic and membranous signal), positive in both immature oocytes and oocytes involved in folliculogenesis. B) Foetal ovary (same case); c-KIT. Note staining of both immature oocytes (arrow) and primordial follicles (arrowhead). C) Foetal ovary (same case); E-cadherin (brown-reddish membranous signal). D) Foetal ovary (same case); E-cadherin. E) Foetal ovary (same case); ß-catenin (brown-reddish signal) is seen in oocytes at different stages of folliculogenesis. F) Foetal ovary (same case); ß-catenin. Note strong submembranous signal intensity in the primordial follicles (arrow), suggesting formation of an E-cadherin-catenin complex. G) Foetal ovary (22 weeks of development); VASA (red cytoplasmic signal), seen both in germ cells of all stages of development. H) Foetal ovary (same case); VASA. Left panels: magnification 200 x right panels: magnification 400x.

Chapter 3

Figure 3-2: A: OCT 3/4 expression in a male individual with trisomy 21, 18w GA (x 200) B: OCT 3/4 expression in a male control, 18w GA (x 200). The number of OCT3/4 positive cells per tubule cross-section is higher in the trisomy 21 fetus as compared to the age-matched control.

Figure 3-4: A: OCT 3/4 expression in the ovarian cortex of a trisomy 21 female, 20w GA (x200) B: OCT 3/4 expression in the ovarian cortex of a control, 20w GA (x200). OCT3/4 expression in the cortical germ cells of the trisomy 21 fetus is markedly higher as compared to the age-matched control. OCT3/4 positive cells are only found in the immature oogonia, mainly residing in the cortical region of the ovary (in trisomy 21 and control fetal gonads).

Figure 3-6: A: TSPY expression in a trisomy 21 male, 19w GA (x400). TSPY expression is frequently seen both in prespermatogonia on the basal membrane (arrows) and in the centrally located gonocytes (arrowheads). The intensity of the TSPY staining is very strong as compared to controls. B: TSPY expression in a male control, 18w GA (magnification X400). TSPY is mainly seen in prespermatogonia on the basal membrane (arrows), gonocytes are often found to be TSPY negative (arrowheads).

Chapter 4

Figure 4-2: A: OCT3/4 positive tubules scattered throughout the gonad in a 6 months old patient with 17 β-HSD deficiency. Note the central position of the OCT3/4 positive cells within the tubules (OCT3/4 staining, X 100). B: OCT3/4 positive tubules in a 13 years old PAIS patient. The OCT3/4 positive tubules are confined to one limited area of the gonad, separated from the OCT3/4 negative areas by fibrous septa (OCT3/4 staining, X 100).

Figure 4-5: Abundant TSPY expression in a 10 months old CAIS patient (A) as compared to a control, 18 months of age (B). TSPY staining, X400

Figure 4-6: A: OCT3/4 (blue) - VASA (red) double staining (X 200) in a 1 month old CAIS patient: OCT3/4 positive cells are separated from the basal membrane by at least one layer of Sertoli cells, VASA positive cells are mainly found on the basal lamina. B: OCT3/4 (blue) - VASA (red) double staining (X 200) in a 13 year old PAIS patient: OCT3/4 positive cells are found almost exclusively along the basal lamina. Tubules expressing OCT3/4 have almost completely lost VASA expression. C: OCT3/4 (blue) - VASA (red) double staining (X 200) in a 4 year old 17 β-HSD deficiency patient: OCT3/4 positive cells are found centrally in the tubule and along the basal lamina. VASA expression is maintained in OCT3/4 positive tubules. Some germ cells co-express OCT3/4 and VASA.

Chapter 5

Figure 5-1: Differentiation patterns encountered in dysgenetic gonads without neoplastic transformation: A: Patient 30r. Dysgenetic testicular tissue, containing germ cells (arrowheads). HE staining, 400X. B: Patient 18r. Testicular tissue. OCT3/4 positive cells are located in the center of the tubule but are also found on the basal lamina. OCT3/4 staining, 200X. C: Same patient. A subpopulation of germ cells (recognized by the VASA staining) expresses OCT3/4. OCT3/4 positive cells are located in the center of the tubule (arrows) but are also found on the basal lamina (arrowheads). OCT3/4 (blue)-VASA (red) double staining, 400X. D: Patient 19r. Testicular (left part) and ovarian (right part) differentiation, the testicular tissue is devoid of germ cells; ova are enclosed in primordial and primary follicles. HE staining, 200X. E: Patient 14l. Undifferentiated gonadal tissue. Germ cells align in clusters together with Sertoli/granulosa cells or lay isolated in fibrous stroma. HE staining, 200X. F: Same patient. A subpopulation of germ cells within the UGT area express OCT3/4. OCT3/4 (blue)-VASA (red) double staining, 200X. G: Patient 13l. Streak. Remnants of cords that have lost their germ cells and have undergone a fibromatous reaction are recognizable. HE staining, 200X.

Figure 5-2: A: Patient 3l. Ova in a primordial follicle (arrows) are enclosed in seminiferous tubules containing spermatogonia (arrowhead). HE staining, 200X. B: Same patient. TSPY negative ova (arrow) next to TSPY positive spermatogonia (arrowhead), TSPY staining, 400X. C: Same patient. Ova (arrow) and spermatogonia (arrowhead) both express VASA. VASA staining, 200X.

Figure 5-3: A: Patient 37r. UGT with isolated germ cells (arrows) adjacent to gonadoblastoma lesions (left). HE staining, 200X. B: Patient 32r. Rectangle: Isolated germ cells in the tissue adjacent to gonadoblastoma. HE staining, 100X. C: Same patient, enlargement of Figure 3B. HE staining, 400X. D: Same patient. Isolated OCT3/4 positive germ cells in UGT adjacent to gonadoblastoma. OCT3/4 staining, 400X. E: Patient 39l. Clonal expansion of OCT3/4 positive cells within UGT in the close proximity of a gonadoblastoma lesion. OCT3/4 staining, 50X. F: Same patient. A subpopulation of germ cells within UGT adjacent to gonadoblastoma expresses OCT3/4 (red) and TSPY (blue). OCT3/4-TSPY double staining, 200X. G: Same patient. Most germ cells within a gonadoblastoma lesion express OCT3/4 (red) and TSPY (blue). OCT3/4 - TSPY double staining, 200X.

Figure 5-4: A: Patient 5I, biopsy performed at 4 months. Germ cells are clearly recognizable in the biopsy sample of the left gonad (arrows). HE staining, 400X. B: Same patient. Germ cells enclosed in cords are encountered in a PLAP staining of the biopsy sample. PLAP staining, 200X. C: Same patient, now 14 years old. Gonadoblastoma of the left gonad (lower part of the image), sporadic germ cells are still present in the UGT adjacent to the gonadoblastoma lesion (arrow). HE staining, 200X.

Chapter 6

Figure 6-1: Representative examples of histology and immunohistochemistry of a gonadoblastoma included in this study (A-D case 4, Table6-1). Parallel sections were used, allowing direct comparison of the different staining results. A: H&E staining. Note the presence of supportive cells Sertoli/granulosa cells (big 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 small black arrow), whereas mature germ cells show the opposite characteristics (indicated throughout this Figure with an arrowhead)). 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 show 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 x 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 embryonal carcinoma. Note that the CIS cells are specifically stained, and 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 loss of TSPY, but only of OCT3/4 in the invasive cells, i.e., TSPY is lost upon invasive growth. Co-expression 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). CIS cells stain stronger than spermatogonia. (E-H: magnifcation factor 200 x)

Figure 6-2: 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 dysgerminoma (case 3, Table). Similar to seminoma, the vast majority of dysgerminoma cells show expression of both markers. Magnification factor x 400; B: gonadoblastoma (case 1, Table). 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 small black arrow, and the germ cells only positive for TSPY by an arrowhead. The double positive cells are indicated by a big black arrow. Magnification factor x 400. C: normal fetal germ cells, gestational age 21 weeks. Note that whereas TSPY is predominantly expressed in prespermatogonia (located on the basal membrane, arrowhead), OCT3/4 is seen more frequently in gonocytes in a more central localization of the tubule (small black arrow). Only a minority of germ cells is positive for both factors (big black arrow). Magnification factor x 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. Magnification factor x 200; E: CIS adjacent to an invasive tumor. Note the presence of CIS cells with both markers (big black arrow) as well as CIS cells without TSPY, but staining for OCT3/4 (small black arrow). Magnification factor x 400; F: CIS before invasiveness. All cells show co-expression of both factors. Magnification factor x 200; G: Double-staining for TSPY (blue cytoplasmic and nuclear signal) and Ki-67 (red nuclear signal) in gonadoblastoma (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. Magnification factor x 400; H: normal fetal germ cells (same case as shown in 2C). Only a minority of germ cells (around 30%) is positive for both factors (indicated by an arrow). Magnification factor x 400.

Chapter 7

Figure 7-2: Representative results for morphological examination and TSPY stainingA: Patient 3, left gonad: UGT displaying many germ cells (arrows) organized in cord-like structures. HE staining, 200X. B: Patient 8, left gonad: Most germ cells in the UGT of this patient are TSPY positive, however, a subpopulation of germ cells does not reveal TSPY expression (arrows). TSPY staining, 200X. C: Patient 13, right gonad: Most germ cells in the testis of this patient are negative for TSPY (arrows). TSPY staining, 200X.

Figure 7-3: Representative results for FISH analysis. A: Patient 18, PBL karyotype 45,X/46,XX/46,XY: The left gonad contains well-differentiated ovarian and testicular portions. HE staining, 200X. B: FISH analysis of the same gonad: green signal: centromere X, red signal: centromere Y. 250X. C: Same gonad: In spite of the virtual absence of Y containing cells, the testis is nicely differentiated but contains no germ cells. FISH: green signal: centromere X, red signal: centromere Y. 250X.

Figure 7-4: Representative results for non fluorescent in situ hybridization. Patient 8. Non-FISH analysis of the UGT portion of the left gonad: black signal: centromere X, red signal: centromere Y. Germ cells are indicated with arrows, Sertoli/granulosa cells and interstitial cells can not be distinguished from each other within UGT. 1000X.

Personal notes