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Developmental model for the pathogenesis of testicular carcinoma *in situ*: genetic and environmental aspects

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Carcinoma *in situ* testis (CIS), also known as intratubular germ cell neoplasia (ITGCN), is a pre-invasive precursor of testicular germ cell tumours, the commonest cancer type of male adolescents and young adults. In this review, evidence supporting the hypothesis of developmental origin of testicular germ cell cancer is summarized, and the current concepts regarding aetiology and pathogenesis of this disease are critically discussed. Comparative studies of cell surface proteins (e.g. PLAP and KIT), some of the germ cell-specific markers (e.g. MAGEA4, VASA, TSPY and NY-ESO-1), supported by studies of regulatory elements of the cell cycle (e.g. p53, CHK2 and p19-INK4d) demonstrated a close similarity of CIS to primordial germ cells and gonocytes, consistent with the pre-meiotic origin of CIS. Recent gene expression profiling studies showed that CIS cells closely resemble embryonic stem cells (ESCs). The abundance of factors associated with pluripotency (NANOG and OCT-3/4) and undifferentiated state (AP-2γ) may explain the remarkable pluripotency of germ cell neoplasms, which are capable of differentiating to various somatic tissue components of teratomas. Impaired gonadal development resulting in the arrest of gonocyte differentiation and retention of its embryonic features, associated with an increasing genomic instability, is the most probable model for the pathogenesis of CIS. Genomic amplification of certain chromosomal regions, e.g. 12p, may facilitate survival of CIS and further invasive progression. Genetic studies, have so far not identified gene polymorphisms predisposing to the most common non-familial testicular cancer, but this research has only recently begun. Association of CIS with other disorders, such as congenital genital malformations and some forms of impaired spermatogenesis, all rising in incidence in a synchronous manner, led to the hypothesis that CIS might be a manifestation of testicular dysgenesis syndrome (TDS). The aetiology of TDS including testicular cancer remains to be elucidated, but epidemiological trends suggest a primary role for environmental factors, probably combined with genetic susceptibility.

Key words: carcinoma *in situ*/germ cell differentiation/embryonic stem cells/testicular cancer/testicular dysgenesis syndrome

Introduction

Testicular cancer is in most cases considered a disease of adults. Seeing a young man presenting with a testicular tumour or with symptoms of disseminated cancer disease, few clinicians would think that their patient's disease had been initiated long time before, during fetal development. However, evidence gathered over the last three decades and the newest findings support this hypothesis, as will be critically discussed in this review.

The early origin is only one of the unique features of testicular germ cell cancer. This neoplasm is unlike any other solid tissue cancer for a number of reasons, including unusual epidemiological and biological features. Epidemiological hallmarks include the peak incidence in a very young adult age, a markedly increasing incidence worldwide but with striking geographic and ethnic differences, and association with other reproductive conditions. Among particular biological features are the unusual histology characterized by extreme heterogeneity with components mimicking any tissue type of the body, including caricatural reflection of early embryos in teratomas, and the extreme sensitivity to irradiation and cytotoxic treatment.

One of the possible explanations for the unique biology of testicular germ cell cancer is that it is derived from germ cells, which are different from any other cells in the body because of their special function of exchanging and transferring hereditary information as gametes. Germ cells are the only cells that use two different types of cell division (mitosis and meiosis), and for that they require different regulation of cell cycle and DNA repair. The regulation of gene expression appears to be different as well, including waves of epigenetic activation and silencing, and a final selective chromosomal condensation during the process of spermiogenesis. In contrast to other cell types, germ cells retain

embryonic stem cell (ESC)-like features and pluripotency for a long time during development. For reasons not yet fully understood, perhaps because of this special hereditary role, germ cells and the reproductive system serving them appear to be exquisitely sensitive to changes in micro- and macro-environment. Research on these aspects has been energized in recent years after adverse epidemiological trends in male reproduction were observed worldwide, with a rise in testicular cancer the first trend to be noted. As will be discussed in detail in this review, studies on the origin and biology of the early stage of this neoplasia played a key role for the understanding of the association between male reproductive disorders and their possible link to changing environment and lifestyle.

A bit of history: histopathology of germ cell neoplasia

Germ cell tumours have fascinated several generations of pathologists because of their histological heterogeneity and seemingly unlimited ability to differentiate into all somatic tissues (totipotency). Moreover, germ cell-like tumours were noticed in remote extragonadal locations, including intracranial sites, usually near the midline of the body. Histological complexity of germ cell tumours constituted a diagnostic conundrum and contributed to the chaos with numerous classifications and nomenclatures. Because classification is not the topic of this review, the readers are referred to specialist reviews and monographs (Grigor, 1993; Ulbright *et al*., 1999; Eble *et al*., 2004). An easy and logical division of testicular germ cell tumours follows three age groups: tumours of newborns and infants (teratomas and yolk sac tumours), tumours of adolescents and young adults (seminomas and non-seminomas, which may also occur simultaneously as combined tumours) and the spermatocytic seminoma of elderly men (Oosterhuis and Looijenga, 2005). In addition, individuals with intersexual phenotype and dysgenetic gonads can harbour gonadoblastoma, a clinically benign but potentially malignant lesion (Scully, 1970). The tumours of infants and elderly are very rare.

One of the most important advances in the understanding of the biology and natural history of germ cell neoplasms, which led to a substantial revision of previous classifications, was the first description of testicular carcinoma *in situ* (CIS) in patients who subsequently developed testicular cancer, by a paediatric endocrinologist with a keen interest in testicular development and function in various pathologies (Skakkebæk, 1972). The cells described by Skakkebæk as a precursor for overt germ cell tumours were seen previously, however, others did not recognize their biological significance and considered them as 'degenerate forms' secondary to a tumour or 'intratubular spread of tumour cells' (Azzopardi *et al*., 1961; Mark and Hedinger, 1965), even several years after the Skakkebæk's description of CIS (Teilum, 1976; Pugh and Parkinson, 1981). Skakkebæk himself acknowledged those earlier descriptions (Skakkebæk, 1981), but it required an intervention by Gondos (1990) and a recent gracious commentary by Parkinson and Harland (2002) to put the earlier history of the discovery of CIS in the correct context. After a few years of denials and discussions, CIS has been commonly accepted as a precursor for all germ cell tumours of the adolescents and young adults, both seminomas and non-seminomas (Ulbright *et al*., 1999). Other synonyms for CIS have been proposed: intratubular germ cell neoplasia (ITGCN), also called unclassified (ITGCNU) (Ulbright *et al*., 1999), testicular intraepithelial neoplasia (Loy and Dieckmann, 1990) and gonocytoma *in situ* (Grigor, 1993). As will be evident from the discussion below, the last term may be the most accurate from the biological point of view.

Already some of the early studies of Skakkebæk and his group provided evidence that CIS was the pre-invasive lesion for the tumours of the adolescents and young adults but not for the infantile tumours or spermatocytic seminoma (Müller *et al*., 1987; Skakkebæk *et al*., 1987; Jørgensen *et al*., 1995a). Biological differences in the pathogenesis of these rare tumours have been confirmed subsequently by studies of genomic aberrations and gene expression patterns (Hawkins *et al*., 1997; Kraggerud *et al*., 1999; Perlman *et al*., 2000; Schneider *et al*., 2001; Stoop *et al*., 2001; Rajpert-De Meyts *et al*., 2003b; Looijenga *et al*., 2006).

Phenotypic features of CIS in relation to germ cell differentiation

Morphological features of CIS cells (Figure 1) have been described in numerous previous articles and pathology textbooks (Skakkebæk, 1972; Ulbright *et al*., 1999; Rørth *et al*., 2000; Eble *et al*., 2004). Close morphological similarity between CIS cells and human fetal gonocytes (as well as seminoma cells and the neoplastic germ cells of gonadoblastoma) was noticed soon after the first description of this lesion and later confirmed by ultrastructural studies (Holstein and Körner, 1974; Nielsen *et al*., 1974; Gondos, 1993). Subsequent studies provided supporting evidence for these similarities based on the comparison of immunohistochemical markers (Hustin *et al*., 1987; Jørgensen *et al*., 1993, 1995b, 1997; Honecker *et al*., 2004). Over the years, more and more proteins/antigens were identified in CIS cells (a partial list is presented in Table I). The status of knowledge on the emerging phenotype of the CIS cell up to year 2002 was summarized in my previous review (Rajpert-De Meyts *et al*., 2003a). Here, only the most important earlier findings are briefly highlighted, whereas the most recent advances are described in greater detail.

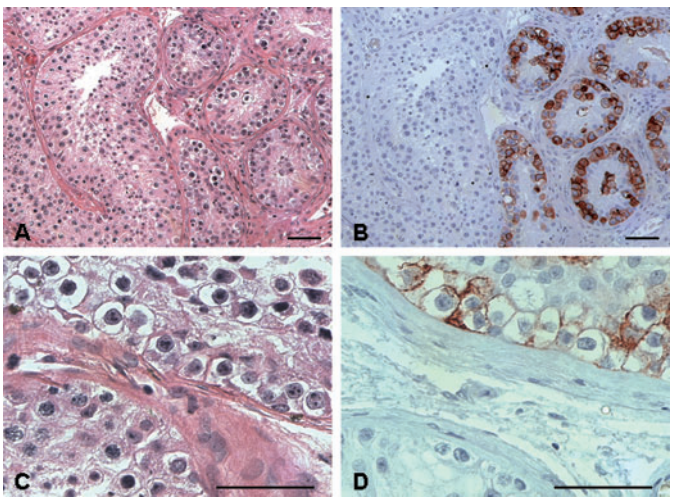

Figure 1. Histological appearance of human adult testis with carcinoma *in situ* (CIS), in cross-sections stained with haematoxylin–eosin (on the left). CIS cells are visualized by immunohistochemical staining for placental-like alkaline phosphatase (PLAP) in the same tissue samples (images on the right). (**A** and **B**) Low power images showing the different appearance of tubules with CIS in comparison with tubules with preserved spermatogenesis. (**C** and **D**) Higher magnification images showing details of CIS cells' morphology. Scale bar, 50 μm.

Table I. A list of selected proteins/antigens, which are expressed in carcinoma *in situ* (CIS) cells, presented in relation to the expression pattern in normal human male germ cells during their differentiation and maturation and in overt testicular germ cell tumours

Protein/antigen (gene)	ESC	PGC	Gonocytes	Sp-gonia	Sp-cytes	Sp-tids	CIS	SEM	N-SEM		SpSEM
									EC	TER	
NANOG	+	+	+	–	–	–	+	+	+	–	–
OCT3/4 (*POU5F1*)	+	+	+	–	–	–	+	+	+	–	–
AP-2γ (*TFAP2C*)	+	+	+	–	–	–	+	+	+	–/+	–
TRA-1-60	+	+	+/–	–	–	–	+/–	+/–	+	–	–
PLAP *(ALPL)*	–	+	+	–	–	–	+	+	+/–	–	–
M2A *(PDPN)*	?	+	+	–	–	–	+	+	–	–	–
KIT	+	+	+/–	–/+	–	–	+	+	–	–	–
DAZL1	?	+	+	+/–	+	–	+	+/–	–	–	?
VASA	?	+/–	+/–	+	+	+	+	+/–	–	–	+
Hiwi	?	+	+	+	+	+/–	+	+/–	–	–	?
TSPY	?	?	+	+	–	–	+	+	–	–	–
Cyclin D2 (*CCND2*)	?	?	+	–	–	–	+	+/–	+/–	+/–	+?
MAGE-A4	?	–	+	+	+/–	–	+/–	+/–	–	–	+
NY-ESO-1	?	–	+	+	+	–	+/–	–	–	–	+/–

EC, embryonal carcinoma; ESCs, embryonic stem cells; N-SEM, non-seminoma; PGC, primordial germ cells; SEM, seminoma; Sp-cytes, spermatocytes; Sp-gonia, spermatogonia; SpSEM, spermatocytic seminoma; Sp-tids, spermatids; TER, teratoma.
A strong expression is marked by +, a heterogeneous expression by +/-. A minus sign means that a protein is not detectable by immunohistochemistry, but it may be present in a given cell type in extremely low quantities, and the gene may be highly expressed at the RNA level. A question mark means that there is no information concerning the protein presence. Modified and updated from Rajpert-De Meyts *et al*. (2003a).

CIS markers, including the KIT receptor, are also expressed in human gonocytes

Early studies focussed on finding clinically useful marker to facilitate the detection of CIS in testicular biopsies. A classic example is placental-like alkaline phosphatase (PLAP, Figure 1), the first identified marker of murine primordial germ cells (PGCs) with still unknown biological function, which remains to this day the most commonly used marker for CIS and seminoma in testicular biopsies and other pathological tissue samples (Jacobsen and Nørgaard-Pedersen, 1984; Hustin *et al*., 1987; Rajpert-De Meyts *et al*., 2003a; references therein).

Over the years, the list of markers for CIS steadily grew; the early markers were usually identified serendipitously, e.g. by testing of an antibody against a glycoprotein abundant in a tumour cell line. Two of these markers, TRA-1-60 (Giwercman *et al*., 1993; Badcock *et al*., 1999) and M2A (Giwercman *et al*., 1988; Marks *et al*., 1999), which are abundant in CIS but undetectable in the normal adult testis, were detected in normal fetal and infantile germ cells, thus giving the first evidence supporting the hypothesis of the prenatal origin of CIS (Jørgensen *et al*., 1993, 1995b).

Further evidence for our hypothesis was provided by investigations of the expression of *c-KIT* in germ cell neoplasms. This gene encodes a cell membrane tyrosine kinase receptor for stem cell factor, a signalling system essential for early germ cell survival, as was first observed in mutant mice with either *W* or *Sl* phenotype (Chabot *et al*., 1988; Huang *et al*., 1990; Yarden *et al*., 1987). Differential expression of *KIT* was first described in germ cell tumours by Strohmeyer *et al*. (1991a) and detected in CIS cells (Figure 2) by Rajpert-De Meyts and Skakkebæk (1994), followed by several other studies (Izquierdo *et al*., 1995; Strohmeyer *et al*., 1995; Bokemeyer *et al*., 1996). As expected, KIT was also strongly expressed in fetal and infantile gonocytes (Jørgensen *et al*., 1995b; Robinson *et al*., 2001; Gaskell *et al*., 2004; Honecker *et al*., 2004) but very low or undetectable in adult spermatogonia in the adult human testis, although this has been somewhat dependent on the specificity of the antibodies and tissue fixation used (Rajpert-De Meyts *et al*., 2003b). The ontogeny of expression of KIT in the human testis demonstrated that it is present at a very high level in the majority of gonocytes during the first trimester of gestation, thereafter the KIT expression was gradually down-regulated (Jørgensen *et al*., 1995b; Gaskell *et al*., 2004; Honecker *et al*., 2004). The retention of a very high expression of KIT beyond a normal window was noted in dysgenetic fetal gonads of some intersex cases (Rajpert-De Meyts *et al*., 1996a). As KIT is a potent pro-survival factor, its prolonged expression could give a growth advantage to the surviving undifferentiated cells. This observation, along with a known association of CIS with poor gonadal development (Table II), led to a new hypothesis that a delay in differentiation could be of one of the mechanisms of neoplastic transformation of germ cells (Rajpert-De Meyts *et al*., 1998a). This is in the line with reports on 'gain-of-function' mutations in the *c-KIT* gene in virtually all sporadic bilateral tumours, both seminomas and non-seminomas (Looijenga *et al*., 2003a), and in a subset of familial and sporadic unilateral testicular tumours but, interestingly, less frequently in non-seminomas (Tian *et al*., 1999; Madani *et al*., 2003; Kemmer *et al*., 2004; Rapley *et al*., 2004). The high frequency of mutations of KIT in bilateral tumours suggests that the mutations most probably had occurred in PGCs, before their migration to the gonadal regions has taken place (Looijenga *et al*., 2003a).

Stem cell-like features: is CIS a fossil from the embryonic past?

The high expression of KIT (the receptor for the stem cell factor), which is present in different types of tissue-specific stem cells, turned our attention into stem cell-like characteristics of CIS

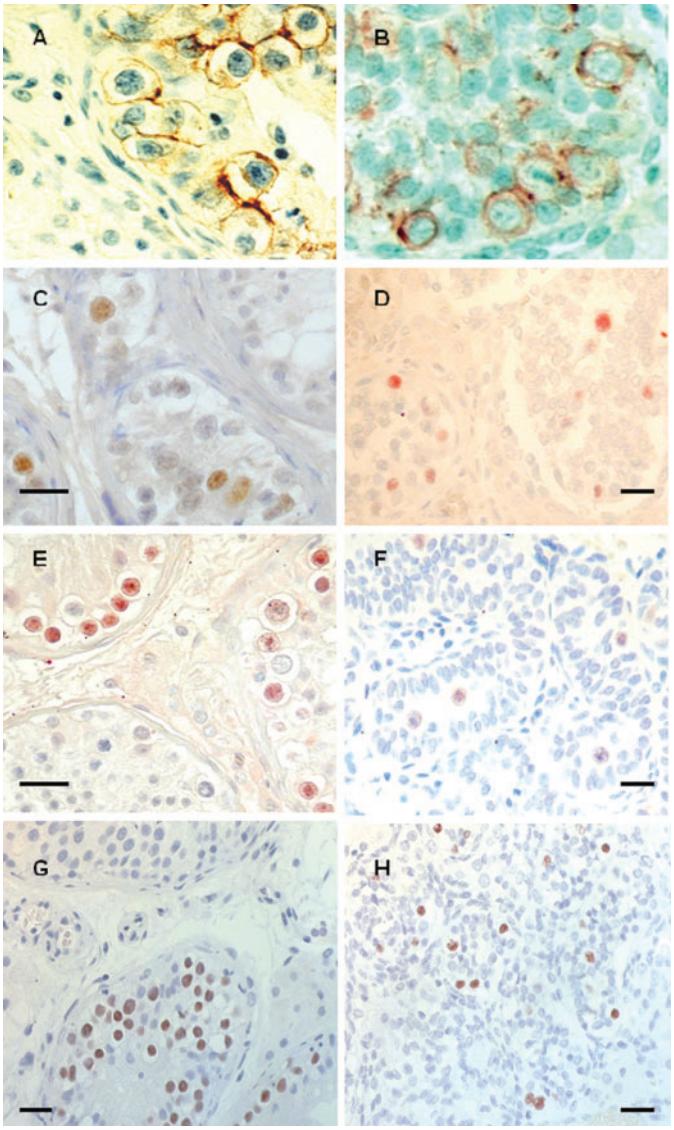

Figure 2. Examples of immunohistochemical staining for proteins highly expressed in carcinoma *in situ* (CIS) cells in adult testicular specimens (left) and in fetal gonocytes in normal fetal testes (right). Scale bar, 20 μm. (**A** and **B**) KIT, (**C** and **D**) p53, (**E** and **F**) OCT-4 and (**G** and **H**), AP-2γ.

cells. Previous studies of embryonal carcinoma-derived cell lines have demonstrated that they closely resemble human ESCs, including such hallmark features, as pluripotency and ability to differentiate when stimulated with retinoic acid (Andrews, 1984, 1998). Among the above-mentioned early markers for CIS cells was TRA-1-60, one of the best known markers for embryonal carcinoma and human ESC (Andrews *et al*., 1984; Giwercman *et al*., 1993; Badcock *et al*., 1999; Henderson *et al*., 2002; Park *et al*., 2004). More recently, OCT-4 (or OCT-3/4) encoded by *POU5F1*, the first transcription factor associated with pluripotency and specific for ESC (Schöler *et al*., 1989) was detected in CIS cells, gonadoblastoma and overt germ cell tumours, with the exception of differentiated teratomas (Palumbo *et al*., 2002; Gidekel *et al*., 2003; Looijenga *et al*., 2003b; Jones *et al*., 2004; Rajpert-De Meyts *et al*., 2004). Interestingly, OCT-4 was highly expressed by virtually all CIS cells in all these studies, whereas other markers, TRA-1-60 and to lesser extent KIT, were present in a subset of CIS cells only, preferentially in those in the vicinity of non-seminomas or seminomas, respectively, thus demonstrating a remarkable heterogeneity of CIS cells (Rajpert-De Meyts *et al*., 1996b). Heterogeneity of the expression of certain embryonic and germ cell-specific markers in CIS cells indicates plasticity of the phenotype of CIS cells, which may begin invasive transformation while still *in situ*.

Recent development of high throughput methods sped up markedly the characterization of gene expression in germ cell tumours and CIS at the RNA level. Most of the published studies analysed gene expression profiles in overt tumours or tumour-derived cell lines, focusing first on genes on certain chromosomal regions, e.g. 17q and 12p (Skotheim *et al*., 2002; Rodriguez *et al*., 2003), and later on a genome-wide analysis (Okada *et al*., 2003; Sperger *et al*., 2003; Skotheim *et al*., 2005). The Norwegian group investigated also gene expression at the protein level in a large array of tissues, including CIS, and confirmed the expression of *JUP* (plakoglobulin) in all CIS samples studied (Skotheim *et al*., 2003).

The first study that focussed on the expression profile of CIS (Hoei-Hansen *et al*., 2004a) used differential display and identified several genes that function in fetal life and thus supported the hypothesis of fetal origin of CIS. A substantial advance was the study by Almstrup *et al*. (2004), which using a genome-wide cDNA microarray, identified a large number of genes not previously reported in CIS. Importantly, the gene expression profile of CIS revealed a remarkable similarity to ESC (Almstrup *et al*., 2004). Among the genes over-expressed in CIS were *NANOG*, *POU5F1* (OCT-3/4), *KIT*, *SFRP1*, *TFAP2C* and several members of the *DPPA* family, which all have been identified in human ESC (Sato *et al*., 2003; Sperger *et al*., 2003; Clark *et al*., 2004), and more recently, also in embryonal carcinoma (Skotheim *et al*., 2005). A more detailed analysis of NANOG in CIS and germ cell tumours demonstrated a pattern of expression essentially identical to that of OCT-3/4 (Hart *et al*., 2005; Hoei-Hansen *et al*., 2005b). A common feature of these genes is their link to pluripotency; they prevent further differentiation of the cell and ensure a 'stock' of undifferentiated cells to renew the tissue. Outside the early embryonic development, NANOG and OCT-3/4 are only found in immature germ cells. A high expression of these genes is a probable explanation of the ability of CIS cells to undergo reprogramming to pluripotent embryonal carcinoma and further differentiation to teratomas, which may contain all types of somatic tissues.

Some of the genes associated with 'stemness' are present not only in ESC but also in various tissue-specific stem cells, e.g. *KIT* and *TFAP2C*. *TFAP2C* (mapped to chromosome 20q13.2), which encodes the transcription factor activator protein-2 (AP-2γ), was previously known as a possible oncogenic factor in other neoplasms, e.g. breast cancer (Turner *et al*., 1998) but never detected in testis. We established AP-2γ as a novel marker for fetal gonocytes and neoplastic germ cells, including testicular CIS (Figure 2), with a role in pathways regulating cell differentiation and a possible involvement in testicular oncogenesis (Hoei-Hansen *et al*., 2004b). This was confirmed by another study (Pauls *et al*., 2005). Thanks to its abundance in nuclei of CIS cells; AP-2γ is currently under investigation as a possible tool for the identification of CIS cells in semen samples in a clinical setting (Hoei-Hansen *et al*., 2005a).

Studies of the pattern of expression during development (Figure 3) demonstrated that OCT-4, AP-2γ, NANOG, as well as KIT, and

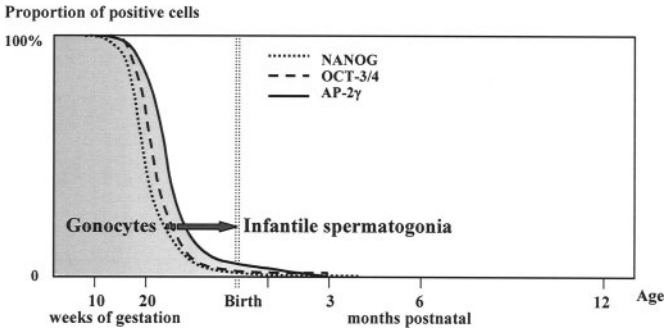

Figure 3. Developmental pattern of the expression of three markers of carcinoma *in situ* (CIS): NANOG, OCT-3/4 (POU5F1) and AP-2γ. The image shows approximately smoothed curves based on the combined results of several studies (Gaskell *et al*., 2004; Hoei-Hansen *et al*., 2004b, 2005b; Honecker *et al*., 2004; Rajpert-De Meyts *et al*., 2004).

probably a number of other CIS markers are abundant in early fetal gonocytes and the expression gradually decreases while gonocytes differentiate to infantile spermatogonia (Jørgensen *et al*., 1995b; Gaskell *et al*., 2004; Hoei-Hansen *et al*., 2004b, 2005b; Honecker *et al*., 2004; Rajpert-De Meyts *et al*., 2004). During human fetal testicular development, a rapid transition from PGCs (which in the testis are germ cells not yet enclosed in seminiferous cords) to gonocytes first takes place, later followed by much slower differentiation of gonocytes into pre-spermatogonia (also called infantile spermatogonia). At that time, germ cells gradually loose their embryonic characteristics while acquiring features of germ cells manifested by the expression of male-specific genes. It is important to underline here the continuum of the expression profile of germ cells, which are the only cell type in the body that retains for such a long time the high expression of genes necessary to maintain ESC-like pluripotency.

Germ cell-specific genes

In addition to ESCs and early fetal germ cells, CIS cells have also a lot in common with normal germ cells of the adult testis. Numerous of proteins/antigens present in normal spermatogonia were also found in CIS cells. The list of such proteins is growing practically by the day. Among the first published were globotriazol ceramide, Gb3 (Kang *et al*., 1995), and neuron-specific enolase, NSE (Kang *et al*., 1996), followed by many others, including some found also in spermatocytes and even in haploid spermatids, as listed in Table I (and reviewed in Rajpert-De Meyts *et al*., 2003a). One recent example is *VASA*, a gene-encoding DEAD-box RNA helicase, which is present in human germ cells throughout their development and maturation (Castrillon *et al*., 2000; Honecker *et al*., 2004) and is also expressed in CIS and overt tumours that retain germ cell-like morphology, such as testicular seminomas and ovarian dysgerminomas (Zeeman *et al*., 2002).

Recent advances in studies on germ cells uncovered a large number of genes that are germ cell-specific, but their biological function has not yet been elucidated, except that many of these genes appear to be involved in RNA processing and regulation, which is essential for spermatogenesis. As expected, quite a few of male germ cell-specific genes are located on the Y chromosome (Lahn and Page, 1997). Very little is known about the expression and function of these genes during early development of germ cells and even less about possible changes in testicular dysgenesis. An early study reported the expression of *RBMY* gene family both in the fetal and in the adult testis (Elliot *et al*., 1997), however, in more recent studies, RBMY was not detected by immunohistochemistry neither in CIS cells nor in overt tumours (Lifschitz-Mercer *et al*., 2000; Schreiber *et al*., 2003). Whether or not down-regulation of this gene family has something to do with neoplastic transformation of early germ cells into CIS remains to be elucidated. Another germ cell-specific gene family includes *DAZ* (on the Yq, usually consist of four copies) and closely related autosomal genes *DAZL* and *BOULE*. DAZ and DAZL have been described in mitotic germ cells, including PGCs and gonocytes (Reijo *et al*., 2000; Xu *et al*., 2001). Consequently, DAZL protein was detected in CIS, in seminomas but not in non-seminomas, consistent with its germ cell-specific function (Lifschitz-Mercer *et al*., 2002). Another multicopy gene, *TSPY*, was suggested as a candidate gene for gonadoblastoma (Salo *et al*., 1995; Tsuchiya *et al*., 1995). *TSPY* in the adult testis is expressed in spermatogonia, and its protein product was also described in immature germ cells in undifferentiated tubules of dysgenetic testes, CIS, seminoma (Schnieders *et al*., 1996) and gonadoblastoma (Lau *et al*., 2000; Kersemaekers *et al*., 2005). The function and biological role of TSPY remains to be elucidated. Likewise, it remains to be proven that *TSPY* is the only gene responsible for gonadoblastoma, as this tumour is frequently seen in mixed gonadal dysgenesis where there is a mosaic aneuploidy of sex chromosomes (46,XY/45,X). The presence of gonadoblastoma is thus most probably a result of male germ cells developing in an insufficiently masculinized gonad because of the lack of function of the Y-chromosome genes in somatic cells in the vicinity. As it will be discussed further, a similar pathogenesis is most probably responsible for CIS, except that CIS occurs in testes with development impaired to much lesser degree than is the case in mixed gonadal dysgenesis.

According to traditional knowledge, genes on the Y chromosome were considered to play the principal role in male reproduction, whereas the X chromosome was more linked to the female fertility. Female ovarian failure is frequently caused by the monosomy (Turner syndrome) or deletions of the X chromosome (reviewed in Zinn and Ross, 2001; Laml *et al*., 2002; Schlessinger *et al*., 2002). Recent years provided new evidence that the X chromosome contains a large number of genes expressed in male germ cells and is apparently essential not only for the female but also for the male germ cell function (Wang *et al*., 2001; Wang, 2004). Only a few of these genes have been studied so far in germ cell neoplasms. Of particular interest is large family of the so-called ‘cancer/testis’ genes, most of them mapped to the X chromosome, which were given this name because—apart from germ cells—they were only detected in various somatic cancers, e.g. melanoma and breast cancer (reviewed in Scanlan *et al*., 2002). Two members of this family, MAGE-A4 and NY-ESO-1, are highly expressed at the protein level in normal fetal gonocytes at the transition period to infantile pre-spermatogonia, in adult spermatogonia as well as in a subset of CIS cells and germ cell tumours, including in spermatocytic seminoma but not in non-seminomas (Jungbluth *et al*., 2000; Aubry *et al*., 2001; Yuasa *et al*., 2001; Satie *et al*., 2002; Rajpert-De Meyts *et al*., 2003b). Such a pattern of expression is consistent with a physiological

function of these genes in germ cells, in analogy to the above-mentioned germ cell-specific genes of the Y chromosome. The lack of expression of MAGE-A4 and NY-ESO-1 in non-seminomatous tumours is poorly understood but may be explained by differences in the genome methylation, which is much more pronounced in non-seminomas (Koul *et al*., 2002; Smith-Sorensen *et al*., 2002; Smiraglia *et al*., 2002; Honorio *et al*., 2003). The re-expression of cancer/testis genes in somatic tumours is probably also linked to changes in DNA methylation of promoter regions (Maio *et al*., 2003) but may be a result of other regulatory mechanisms. The X chromosome is the most tightly controlled in this aspect because of the need to compensate for the double dosage effect in females. The process is controlled by the X-inactivation centre, which produces the *XIST* transcript, which in turn triggers chromatin changes by Polycomb group proteins and DNA methylation (Csankovszki *et al*., 2001; Heard, 2004). In male germ cells, *XIST* is transcribed, but the X chromosome remains largely active. Interestingly, the *XIST* transcript is also over-expressed in testicular germ cell tumours and in CIS cells, perhaps partly because of a frequent increase in the copy number of X chromosomes in aneuploid neoplastic germ cells (Looijenga *et al*., 1997; Kawakami *et al*., 2003; Hoei-Hansen *et al*., 2004a).

Studies of the cell cycle and DNA repair are consistent with the pre-meiotic origin of CIS

Profound differences in the biology of germ cell neoplasms in comparison with the somatic tumours are undoubtedly related to a very special feature of germ cells—their ability to switch from mitotic cell division to the meiotic division, which is required for gamete formation. Regulatory mechanisms involved in the two types of cell division differ, and a number of studies provided evidence supporting the pre-meiotic origin of germ cell tumours, including CIS. Cell division is a final step in the cell cycle, which has to be exquisitely regulated to maintain the balance between proliferation and differentiation, a disturbance of this balance may lead to cancer or cell death. Closely related to the cell cycle regulation are the mechanisms of DNA repair, which are essential to prevent cell death or neoplastic transformation, especially in cells subjected to adverse environmental effects. Germ cells appear to have inherently high sensitivity to cytotoxic drugs and irradiation. This feature is further magnified in germ cell-derived tumours (reviewed in Masters and Koberle, 2003; Spierings *et al*., 2003). This is, of course, with great benefit for the patients with germ cell neoplasms, who can be efficiently treated by cisplatin-based regimens (Einhorn, 1997) or, in certain cases of isolated CIS, even by irradiation alone (Von der Maase *et al*., 1986). The processes of DNA repair are regulated differently in mitotically dividing immature germ cells during testicular development, and different mechanisms are specifically triggered when the meiotic division starts at puberty, because the meiotic crossover requires double-strand DNA breaks. As far as CIS is concerned, the evidence accumulated so far unequivocally demonstrates that a high expression of the key tumour suppressors involved in the DNA repair, such as p53 (Bartkova *et al*., 1991) and CHK2 (Bartkova *et al*., 2001), is a persistent developmental feature. Both proteins are abundant in normal fetal gonocytes (see p53 in Figure 2); p53 is then down-regulated in spermatogonia, whereas CHK2 remains highly expressed in spermatogonia but disappears at the onset of meiosis (Quenby *et al*., 1999; Bartkova *et al*., 2001; Rajpert-De Meyts *et al*., 2003b). A recent study demonstrated that after the onset of meiosis, a rapid activation of the ATM kinase takes place in spermatocytes to process multiple DNA double-strand breaks (Bartkova *et al*., 2005).

A wealth of evidence indicates that the G1/S-phase transition of the cell cycle is primarily controlled by the retinoblastoma protein (pRB) pathway, which is commonly involved in the pathogenesis of various malignancies (Mihara *et al*., 1989; Bartek and Lukas, 2001; Sherr, 2004; references therein). The pRB pathway regulation appears to be different in germ cells and deregulated in germ cell tumours but without structural aberrations (mutations) typical for somatic cancers (reviewed in Bartkova *et al*., 2003b). The observed changes are most likely due to a direct transcriptional regulation, an increased promoter methylation, or a more recently discovered regulatory mechanism by micro-RNAs (reviewed in Ambros, 2001; Zamore and Haley, 2005). As far as the CIS cells are concerned, the first interesting observation was the lack of pRB in CIS, seminoma and embryonal carcinoma, with a normal expression in teratomas (Strohmeyer *et al*., 1991b). This surprising finding is consistent with developmental regulation of pRB, which is apparently physiologically down-regulated in fetal gonocytes but active in mature spermatogonia (Bartkova *et al*., 2003a). As pRB is a tumour suppressor, the lack of pRB in fetal germ cells and CIS may render these cells more vulnerable to oncogenic stimuli but simultaneously also more prone to apoptosis (Bartkova *et al*., 2003b).

The second interesting feature of CIS and overt germ cell tumours is the over-expression of a protooncogenic cyclin D2 (encoded by *CCND2* mapped to chromosome 12p), significance of which will be discussed below (Sicinski *et al*., 1996; Houldsworth *et al*., 1997; Bartkova *et al*., 1999; Schmidt *et al*., 2001). The third feature, important for our discussion on the origin of germ cell neoplasms in relation to the meiotic switch, is the lack of the cyclin-dependent kinase (CDK) inhibitor p19-INK4d in CIS and overt germ cell tumours. P19-INK4d is abundant in normal spermatocytes and detectable in spermatids but completely absent from fetal gonocytes (Bartkova *et al*., 2000). Similarly, cyclin A1—which was described in spermatocytes—has not been detected in CIS or seminomas (Liao *et al*., 2004). Taken together, the studies of the regulatory machinery of the cell cycle strongly support the origin of CIS from early fetal and pre-meiotic germ cells.

Genomic aberrations in CIS: 12p or not 12p?

The question addressed soon after the discovery of a remarkable resemblance of CIS cells and fetal germ cells was whether CIS cell is a truly neoplastic cell or simply an immature gonocyte persisting in an adult testis. While substantial knowledge concerning genomic aberrations of the overt germ cell tumours was accumulated, the studies of CIS lagged behind, mainly because of technical difficulties due to a low number of CIS cells, their relatively low rate of proliferation (Höfken and Lauke, 1996) and poor growth in culture (Rajpert-De Meyts *et al*., 1998b). Only after the advent of a new technology of the comparative genomic hybridization (Kallioniemi *et al*., 1992), the genome of CIS cells has been better characterized. A detailed overview of genomic aberrations in the germ cell neoplasms, including CIS, is beyond the scope of this article, therefore, the reader is referred to recent excellent review articles on this topic (Skotheim and Lothe, 2003; von Eyben, 2004). I shall discuss here only the aberrations that are

probably the most informative with regard to the possible mechanism of neoplastic transformation, namely polyploidization and regional amplification of chromosome 12p.

Like nearly all neoplasms, CIS cells found in the adults are aneuploid with a mean DNA content in the hyper-triploid to hypo-tetraploid range (Skakkebæk, 1972; Müller and Skakkebæk, 1981; de Graaff *et al*., 1992). The longest lasting controversy concerned the presence in CIS of an isochromosome of the short arm of chromosome 12, i(12p), an aberration first described by Atkin and Baker (1982) and considered a hallmark of overt germ cell tumours (Castedo *et al*., 1988; Rodriguez *et al*., 1992; Van Echten *et al*., 1995a). Even in germ cell tumours without apparent presence of i(12)p, some amplification of the 12p material have been reported (Castedo *et al*., 1988; Rodriguez *et al*., 1993; Suijkerbuijk *et al*., 1993). The i(12)p has usually identical arms and is probably caused by an erroneous centromeric division during mitotic anaphase (Sinke *et al*., 1993). However, some loci on 12q in i(12)p-positive tumours retain heterozygosity, and thus polyploidization has to precede the formation of i(12p) (Geurts van Kessel *et al*., 1989).

The i(12p) in CIS was sporadically demonstrated by karyotyping (Vos *et al*., 1990; Van Echten *et al*., 1995b), but this has been disputed as the majority of the subsequent molecular studies did not detect genomic amplification of that region in CIS (Rosenberg *et al*., 2000; Summersgill *et al*., 2001). It was, therefore, proposed that the formation of i(12p) was not involved in the early pathogenetic process, but the relative gain of 12p sequences was associated with survival of CIS independently of Sertoli cells leading to their transformation to invasive tumours (Looijenga *et al*., 2003c). Our own study performed on the microdissected CIS cells by the comparative genomic hybridization added a missing link in this puzzle: we demonstrated that there indeed was no gain of 12p in two cases of CIS found as an isolated pre-invasive lesion, however, a clear genomic amplification in this region was detected in nearly all cases of CIS present in the vicinity of invasive tumours (Figure 4), suggesting clonal heterogeneity and possibly genomic instability of CIS cells (Ottesen *et al*., 2003). A subsequent analysis performed on CIS cells flow-sorted according to the DNA ploidy (Ottesen *et al*., 2004a) supported a hypothesis first suggested by Oosterhuis *et al*. (1989, 1990) that the polyploidization (tetraploidization) probably precedes the gain of 12p and other chromosomal aberrations. Some allelic losses detected in CIS resemble quite closely those in seminoma and, to a lesser extent, those in non-seminomas (Faulkner *et al*., 2000). However, the pattern of chromosomal aberrations/imbalances in overt germ cell tumours reported in numerous studies is quite similar despite morphological differences among germ cell tumour types (reviewed in Van Echten *et al*., 1995a; Skotheim and Lothe, 2003; von Eyben, 2004). A recent analysis of a large number of germ cell tumour karyotypes proposed that a multipolar cell division with non-disjunction of a tetraploid precursor cell, combined with some secondary imbalances/structural changes, is the most likely model of the karyotypic evolution of germ cell tumours (Frigyesi *et al*., 2004). Overall, genetic evidence gathered so far supports the progression of these tumours from a polyploid precursor cell, such as CIS (Oosterhuis *et al*., 1989, 1990), but the mechanisms of polyploidization remain to be elucidated.

Why the gain of 12p is so interesting? A look at the list of genes located there explains that. A number of genes associated with pluripotency of ESC and human teratocarcinoma cell lines, e.g.. *NANOG*, *STELLAR*, *DPPA-5* and *GDF3* (Caricasole *et al*., 1998; Sato *et al*., 2003; Sperger *et al*., 2003; Clark *et al*., 2004; Skotheim *et al*., 2005), and with germ cell proliferation or increased survival, e.g. *CCND2* and *K-RAS* (Sicinski *et al*., 1996; Houldsworth *et al*., 1997; Roelofs *et al*., 2000), are localized to the 12p region. This region constitutes also one of the hot spots of highly expressed genes in the profiling study of CIS (Almstrup *et al*., 2004). Interestingly, non-random gains of chromosomal material in the same region have been reported in human ESC maintained for a prolonged period in culture (Draper *et al*., 2004). That study, and a more recent investigation by Maitra *et al*. (2005), reported also non-random aberrations in cultured ESC in 17q, a region frequently rearranged in germ cell tumours (Kraggerud *et al*., 2002; Skotheim *et al*., 2002) where a cluster of genes highly expressed in CIS was detected as well (Almstrup *et al*., 2004). The observation of chromosomal aberrations in cultured ESC indicates that the microenvironment of growing ESC may be important for genomic stability. The molecular mechanisms are though poorly understood, and it is not known whether 12p and 17q are especially sensitive to chromosomal rearrangements. An alternative hypothesis is that the genome of CIS cells undergoes many random aberrations, and only the aberrations that render the cells better adapted to a changed microenvironment survive. This hypothesis postulates that the regions 12p, 17q and probably parts of X harbour genes with oncogenic potential, perhaps particularly oncogenic for germ cells. Some of the genes in these regions are indeed highly expressed in CIS cells, and we listed these candidate genes in a recent review article (Almstrup *et al*., 2005). I speculate that a similarity between ESC and CIS could indicate that CIS cells perhaps may originate from PGCs or gonocytes through a similar mechanism of 'natural selection' of cells that adapted themselves to their disturbed microenvironment in the developing gonad. How the development of the early gonad may be disturbed is the matter discussed in the remaining part of this review.

Who is at risk for germ cell cancer? The importance of prenatal events and the concept of testicular dysgenesis syndrome

Conditions associated with germ cell cancer and factors which increase the risk of this cancer are numerous and surprisingly variable.

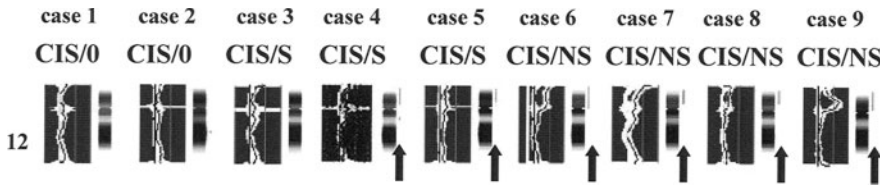

Figure 4. The mean ratio profiles of chromosome 12 analysed by comparative genomic hybridization in carcinoma *in situ* (CIS) cells microdissected from testes with CIS alone (CIS/0) or CIS adjacent to overt seminomas (CIS/S) or non-seminomas (CIS/NS). The relative gains in 12p regions are shown as light-grey vertical bars, marked with arrows on the right side of the ideograms of chromosomes (Ottesen et al., 2003; reprinted with permission from Wiley & Sons).

A systematic and critical analysis of clinical epidemiology of testicular cancer was recently published by Dieckmann and Pichlmeier (2004). Here, only a partial list of the best documented risk factors is listed in Table II, and a short summary of this topic is presented, mainly to illustrate the concept of the testicular dysgenesis syndrome (TDS).

Severe but relatively rare genetic abnormalities which cause testicular dysgenesis and the intersex syndrome (e.g. 45X/46XY and androgen insensitivity) are associated with a high risk of testicular cancer, often in combination with undescended testis and hypospadias (Aarskog, 1970; Scully, 1981; Savage and Lowe, 1990). Skakkebæk was the first to notice CIS in the dysgenetic testes of children with the intersex syndrome (Skakkebæk, 1979; Müller and Skakkebæk, 1984; Müller *et al*., 1985). Subsequently, several reports described the presence of CIS or gonadoblastoma in dysgenetic gonads of subjects with various forms of the intersex syndrome with or without structural aberrations of chromosomes (Cassio *et al*., 1990; MacMahon and Cussen, 1991; Rutgers and Scully, 1991; Jacobsen and Henriques, 1992; Ramani *et al*., 1993; Slowikowska-Hilczer *et al*., 2001; Slowikowska-Hilczer *et al*., 2003). In addition to linking gonadal dysgenesis with germ cell neoplasia, these observations support the notion that CIS and CIS-derived germ cell tumours may occur in the pre-pubertal testes and speak against an alternative hypothesis that the post-pubertal zygotene–pachytene spermatocyte is the cell of origin for CIS (Chaganti and Houldsworth, 2000).

Among more common urogenital abnormalities, cryptorchidism (undescended testis) is the best documented risk factor for testicular neoplasia, including CIS (Campbell, 1942; Morrison, 1976; Krabbe *et al*., 1979; Batata *et al*., 1982; Giwercman *et al*., 1989; Prener *et al*., 1996; Coupland *et al*., 1999; Weir *et al*., 2000). A recent meta-analysis evaluated the relative risk (RR) of testicular cancer in subjects with a history of cryptorchidism as 4.8 (95% CI = 4.0–5.7) (Dieckmann and Pichlmeier, 2004). There is also evidence for an association between testicular cancer and inguinal hernia or hypospadias (Morrison, 1976; Klein *et al*., 1996; Prener *et al*., 1996). Testes in cases with congenital urogenital malformations often are associated with some degree of maldevelopment, including clusters of poorly differentiated Sertoli-cell-only tubules and hyaline bodies (Sohval, 1954; Huff *et al*., 1993). More conspicuous but surprisingly common are histological signs of poor testicular development and function in adult patients with sporadic testicular tumours (Sohval, 1956), even in the seemingly 'normal' contralateral testes in patients with unilateral testicular cancer (Berthelsen and Skakkebæk, 1983; Hoei-Hansen *et al*., 2003). The degree of differentiation of Sertoli cells in adults with testicular cancer is variable depending on the grade of dysgenesis, but even morphologically immature Sertoli cells in most cases with complete spermatogenesis present elsewhere in the testis do not retain expression of the anti-Müllerian hormone, which is highly expressed before puberty (Rey *et al*., 1996; Rajpert-De Meyts *et al*., 1999). Hyaline bodies are frequently (but not always) seen on the ultrasound as testicular microlithiasis (reviewed in Holm *et al*., 2001). An association of microlithiasis with CIS and even testicular masses in the contralateral testis is so common that this ultrasonic abnormality should alert the attending physician about a possibility of testicular neoplasia, especially in patients with atrophic testes (Bach *et al*., 2003; Holm *et al*., 2003; de Gouveia Brazao *et al*., 2004).

Several studies documented that men with testis cancer had significantly reduced fertility before the development of their tumour, with a lower proportion of male children (decreased offspring sex ratio), and abnormal semen characteristics (Berthelsen and Skakkebæk, 1983; Møller and Skakkebæk, 1999; Jacobsen *et al*., 2000a,b; Richiardi *et al*., 2004c). On the contrary, men with subfertility have often a history of genital malformations and may harbour histological signs of testicular maldevelopment, including CIS, thus confirming an association between these conditions (Skakkebæk *et al*., 2003). Furthermore, an analysis of risk factors, such as low birthweight or intrauterine growth retardation (Depue *et al*., 1986; Morley and Lucas, 1987; Francois *et al*., 1997; Cicognani *et al*., 2002; English *et al*., 2003), suggested that the pathogenesis might be, at least partially, shared by germ cell tumours, cryptorchidism and male subfertility. Recently, a Norwegian study of risk factors for hypospadias found also, among others, a low birthweight and inguinal hernia (Aschim *et al*., 2004a). The epidemiological associations outlined above constituted the basis for a hypothesis of an aetiological link between the male reproductive disorders that are associated with impaired testicular development, within the so–called TDS presented schematically in Figure 5 (Skakkebæk *et al*., 2001; Asklund *et al*., 2004). The assumption that prenatal or perinatal factors are responsible for growing incidence of germ cell cancer and TDS is additionally corroborated by the birth cohort effects, meaning that the epidemiological trends are associated with the year of birth, and each subsequent cohort is more affected that the previous one. A birth cohort effect was, e.g., demonstrated for a decline in sperm concentrations of Scottish men (Irvine *et al*., 1996), one of the studies that followed the report on the possible decline of semen quality worldwide (Carlsen *et al*., 1992). One exception to the rule of the consecutive decline, which at the same time is a striking example of a birth cohort effect, was an unexplained decrease of the prevalence of testicular cancer among Scandinavian men born during wartime (Møller, 1993; Bergström *et al*., 1996).

A strong corroborating evidence for the TDS concept—which simultaneously incriminates environmental factors—is the geographical association between various components of TDS. A very illustrative example is given by the comparison of the rates in Denmark and in Finland, and another nearby located Nordic country. The incidence of testicular cancer, which is high in Denmark, is markedly lower in Finland (Adami *et al*., 1994; Richiardi *et al*., 2004a). Studies of the incidence rates of testicular cancer in populations migrating from these two countries to Sweden, which is

Table II. Risk factors for carcinoma *in situ* (CIS)/testicular cancer

Contralateral testis tumour
Cryptorchidism
Other genital malformations (inguinal hernia and hypospadias)
Intersex, including the androgen insensitivity syndrome
Gonadal dysgenesis
Familial testicular cancer
Testicular atrophy
Subfertility/infertility
Low birthweight
Down syndrome
Birth order (first pregnancy)
Early puberty
Estrogen excess during gestation

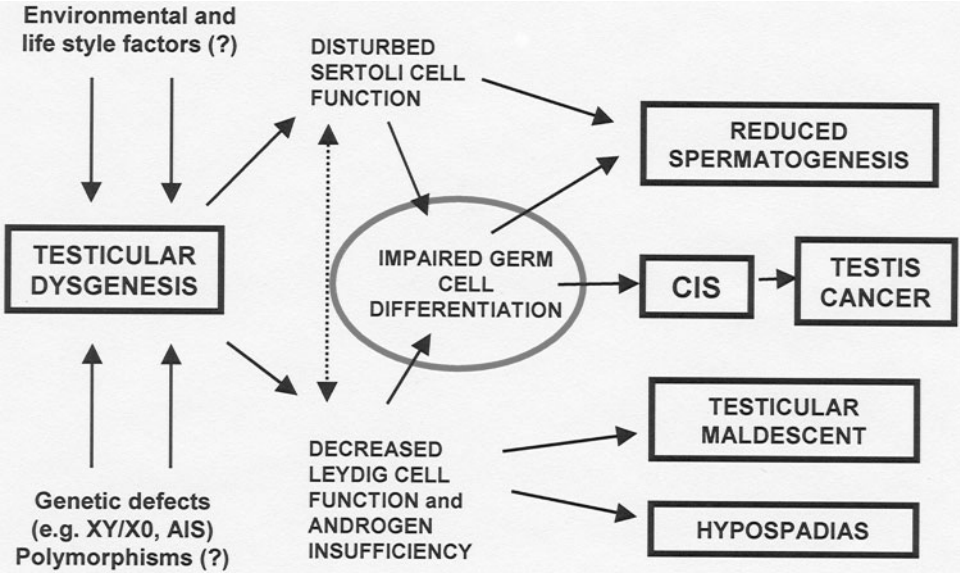

Figure 5. Schematic representation of the possible aetiology, pathogenesis and clinical manifestations of testicular dysgenesis syndrome emphasizing the key role of disturbed germ cell differentiation in the pathogenesis of testicular carcinoma *in situ* (CIS) (modified from Skakkebæk *et al*., 2001).

located in between, clearly demonstrated that the first generation immigrants retained the incidence as in their country of origin, whereas the second generation (born in Sweden) had the risk of testicular cancer similar to native Swedes (Hemminki and Li, 2002). Studies of semen quality found also all parameters better in Finland than in Denmark (Jensen *et al*., 2000; Jørgensen *et al*., 2001, 2002). The differences in rates of congenital genital malformations seemed also to be different, but less certain because of problems with definition and registry data (reviewed in Toppari *et al*., 2001). Data from other countries were confusing with some reporting an increase while other argued for a possible decline in cryptorchidism rates (Chilvers *et al*., 1984; Paulozzi, 1999; Toledano *et al*., 2003). Therefore, coordinated prospective studies of genital malformations have been launched in cohorts of infants, providing most telling evidence for the difference in the rates of cryptorchidism and hypospadias at birth in Denmark versus Finland (Boisen *et al*., 2004, 2005). At the same time, the Boisen *et al*. (2004) study demonstrated an increase of the incidence of cryptorchidism in Denmark over time (Buemann *et al*., 1961).

Geographical and ethnic differences have been noted much earlier for testicular cancer in other countries of the world, with unexplained high prevalence among Caucasians living in well-developed countries and notably lower prevalence among men of African descent and Asians, even inhabiting the same countries (English *et al*., 2003; Huyghe *et al*., 2003). The obvious question that arises is whether the reasons for the geographic and temporal differences in the prevalence of TDS are because of environmental differences or genetic variation/predisposition?

Genetic aspects of testicular cancer and TDS: can genetic polymorphisms explain geographic differences in the incidences?

Familial testicular cancer

Although familial testicular cancer is rare, this cancer has quite strong hereditary component. Sons and brothers of men with testicular cancer carry a four- and eight-fold increased risk of developing tumours, respectively (Lutke Holzik *et al*., 2004; references therein). However, a large proportion of familial cases, especially among brothers, may be explained, at least in part, by shared environment during early development (Hemminki and Li, 2004; Ottesen *et al*., 2004b). A few gene mutations have been reported in tumour tissues, but most of them have been linked to just one or two cases, with a notable exception of the activating mutation in the *KIT* gene, which has been detected in a subset of sporadic and familial tumours (Tian *et al*., 1999; Rapley *et al*., 2004) but which is present in virtually all bilateral testicular tumours (Looijenga *et al*., 2003a). So far, only one locus suspected for a germ cell cancer susceptibility gene has been reported at Xq27, but its importance is weakened by a simultaneous association with testicular maldescent (Rapley *et al*., 2000). Epidemiological observations suggest that most probably the majority of cases of testicular cancer are not because of a genetic mutation. Simple genetic polymorphisms, which are at the core of phenotypic diversity of human populations, may also be responsible for ethnic differences in the prevalence of human reproductive disorders. It is plausible that, e.g., genes that are involved in hormonal regulation of testicular development may contain polymorphic sequences that would slightly alter sensitivity to hormones, natural or synthetic. A similar phenomenon was observed in mice, where steroid hormones had vastly different effects in various mouse strains (Spearow *et al*., 1999). The human genotype is, of course, much more complicated that of an inbred laboratory mouse. Very few candidate genes relevant to humans have been studied so far, and the evidence is briefly reviewed here.

The androgen receptor

The androgen receptor gene is the most obvious candidate for a possible association of a polymorphism with disorders of male reproduction, in particular testicular cancer. Although the androgen function in the early fetal development has not been elucidated yet, the lack of function may impair genital development and increases

the risk of germ cell neoplasia considerably (Manuel *et al*., 1976; Quigley *et al*., 1995; Sultan *et al*., 2001). The opposite situation—an increased androgen signalling during development—may decrease the risk of testicular cancer (Rajpert-De Meyts and Skakkebæk, 1993). As mentioned above, the incidence of testicular cancer among Africans is very low, and by contrast they have a very high risk of prostate cancer, suggesting a possible role of higher testosterone levels *in utero* or other genetic predisposition (Henderson *et al*., 1988; Ross *et al*., 1998). One possible explanation could be a difference in the length of the polymorphic polyglutamine stretch in the androgen receptor, which is on average shorter among Africans and thus may be slightly more efficient in activating transcription (Sartor *et al*., 1999; Irvine *et al*., 2000).

The possible role of the two polymorphic trinucleotide (CAG and GGN) sequences, encoding polyglutamine and polyglycine stretches, has been extensively studied in all components of TDS. Expansion of the CAG repeat above 40 (normal range 8–37) causes spinal bulbar muscular atrophy, also known as the Kennedy syndrome, a serious neurodegenerative disease with progressive testicular atrophy and hypogonadism (La Spada *et al*., 1991). A series of studies, started by Tut *et al*. (1997) and Dowsing *et al*. (1999), reported a relation between the length of repeats and male infertility/subfertility while a similar number of studies failed to find such an association. It is impossible to cite in this review all studies investigating this problem in several centres around the world, but references can be found in meta-analyses and recent review articles (Rajpert-De Meyts *et al*., 2002a; Asatiani *et al*., 2003; Erasmuson *et al*., 2003; Ochsenkühn and de Kretser, 2003; Yong *et al*., 2003; Gottlieb *et al*., 2005). An association of the AR polymorphisms with testicular function (sperm production, sperm morphology and reproductive hormone profile)—both in infertile and fertile men—was also addressed by several studies (Mifsud *et al*., 2001; von Eckardstein *et al*., 2001; Härkönen *et al*., 2003; Milatiner *et al*., 2004). Most of these studies observed an inverse association between the number of CAG repeats and sperm production or quality. In our own study, a weak trend (not statistically significant) for a decrease in sperm concentrations with increasing $(CAG)_n$ was observed among fertile controls, but this trend disappeared after a greater number of subjects have been studied (Rajpert-De Meyts *et al*., 2002a). The whole issue remains open and debated; the reasons for the controversy include pathogenetic heterogeneity of clinical infertility, ethnical differences, poor characterization of control subjects in some studies and possible influences of confounding environmental factors. Possible differences in the mechanism of action of androgens within the testis in comparison with other parts of the male reproductive system should also be considered (Ochsenkühn and de Kretser, 2003).

Testicular cancer was investigated for the androgen receptor polymorphism in three studies only (Rajpert-De Meyts *et al*., 2002b; Giwercman *et al*., 2004; Garolla *et al*., 2005). Neither found an association of the cancer risk with the length of the CAG repeat alone, however, Giwercman *et al*. (2004) reported a possible link between the longest CAG repeats and the tumour progression to non-seminomas as well as clinically more aggressive disease, whereas Garolla *et al*. (2005) found that the combination CAG=20/GGC=17 was significantly more frequent in patients with testicular cancer than in controls. As far as genital malformations and undermasculinization are concerned, there is a better but not perfect consensus among the few published studies. Most of the European studies reported an association of these phenotypes with longer CAG (Lim *et al*., 2000, 2001) or GGN stretches or with certain combinations of CAG/GGN (Aschim *et al*., 2004b; Ferlin *et al*., 2005). By contrast, reports from Japan did not find any association, however, all these studies were performed by the same centre (Sasagawa *et al*., 2000; Ishii *et al*., 2001; Muroya *et al*., 2001), thus it would be beneficial for the final conclusion to have some confirmation from other Asian research groups.

Despite the controversy, a consensus slowly emerges that the AR-$(CAG)_n$ may play a role in the function of androgen-related pathways and their pathologies, especially outside the testis. However, this polymorphism should not be investigated in isolation, but a number of contributing factors (e.g. other diseases, lifestyle or environmental influence) should be considered (Hughes *et al*., 2001).

Possible role of deletions and polymorphisms of the Y chromosome

Individuals with the intersex syndrome and a relative reduction of the Y chromosome genetic material carry a high risk of germ cell neoplasia (Scully, 1981; Savage and Lowe, 1990; Peltomäki *et al*., 1991). The genes on the human Y chromosome that are most likely to be involved in germ cell differentiation and spermatogenesis are clustered in the so-called azoospermia factor (AZF) region of Yq11 (Tiepolo and Zuffardi, 1976; Vogt *et al*., 1992; Vogt, 1996), which is a part of the recently proposed male-specific Y-chromosome region (MSY) (Kuroda-Kawaguchi *et al*., 2001). This region is especially prone to interstitial deletions, which are associated with variable grade of testicular failure and impaired spermatogenesis and were first identified in infertile men (Tiepolo and Zuffardi, 1976; Vogt *et al*., 1992; Reijo *et al*., 1995; Vogt, 1996; Lahn and Page, 1997; Krausz *et al*., 2000; Kuroda-Kawaguchi *et al*., 2001; Frydelund-Larsen *et al*., 2002; Luetjens *et al*., 2002; Repping *et al*., 2002). It was long suspected that the propensity of the Yq region to those microdeletions may be caused by intrachromosomal recombination due to a presence of repetitive sequences, including those of ancient retroviruses, e.g. HERV (Kamp *et al*., 2000; Sun *et al*., 2000), high expression of which was reported in germ cell tumours (Herbst *et al*., 1996; Roelofs *et al*., 1998). Indeed, the sequencing of the entire Y chromosome and subsequent studies demonstrated that much of the sequence in MSY region consists of long palindromic repeats called amplicons, though most of them are not associated with retrotransposons (Kuroda-Kawaguchi *et al*., 2001; Tilford *et al*., 2001; Skaletsky *et al*., 2003). A deletion of a large amplicon usually removes a huge amount of DNA and is associated with very severe spermatogenic failure, with an exception of rare cases of subfertile men with large AZFc deletions who can occasionally produce sperm but still demonstrate testicular failure, manifested both by histological abnormalities and changes in the reproductive hormone profiles (Krausz *et al*., 2001a; Frydelund-Larsen *et al*., 2002).

More recently, smaller palindromes were discovered within the large amplicons in AZFc region. Deletions of these sequences remove several copies of multicopy gene families and are associated with a variable clinical and histological phenotype, although there is growing evidence that, e.g., gr/gr deletion may be a significant risk factor for decreased spermatogenesis, whereas b2/b3 is

probably neutral for testicular function (Fernandes *et al*., 2002; Repping *et al*., 2003, 2004; de Llanos *et al*., 2005; Giachini *et al*., 2005; Lynch *et al*., 2005). Some of these partial AZFc deletions, including gr/gr, can also be found in fertile men with normal spermatogenesis (Hucklenbroich *et al*., 2005), so it remains to be resolved whether these aberrations may play a role alone or only as a confounding factor predisposing to subfertility in the presence of other deleterious factors.

It is important to keep in mind that lack of recombination of the substantial part of the Y chromosome led to the formation of haplogroups which differ among populations, and these can contain single-nucleotide polymorphisms defining haplotypes (McElreavey and Quintana-Murci, 2003). A correlation between some of the Y-chromosome haplogroups and reduced sperm concentrations was found in Japan (Kuroki *et al*., 1999) and in Denmark (Krausz *et al*., 2001b). What is the mechanism leading to impaired spermatogenesis is not fully understood yet, but some haplotypes may segregate with rearrangements/inversions which may generate different types of the aforementioned partial AZFc deletions (Krausz *et al*., 2004; Machev *et al*., 2004). In some populations, e.g. Japan or Finland, certain Y chromosomes with partial AZFc deletions may have acquired compensatory mutations which would change the phenotype (Krausz *et al*., 2004; Vogt, 2005). This hypothesis is supported by observations from Finland, where there is no evidence of problems with spermatogenesis at the population level (Vierula *et al*., 1996; Jørgensen *et al*., 2002) despite a high prevalence of haplogroup N which is strongly associated with g1/g3 deletion (Krausz *et al*., 2004; Vogt, 2005).

The frequency of AZF deletions in the Danish population appears to be similar to that in other European countries and is not increased in patients with testicular cancer, thus the high prevalence of TDS in Denmark cannot be explained by a high incidence of such deletions (Krausz *et al*., 2001a; Frydelund-Larsen *et al*., 2003). A similar study was performed in Dutch patients and confirmed our observation of the absence of constitutional large AZF deletions in patients with testicular cancer (Lutke Holzik *et al*., 2005). This is also supported by the lack of association between Y lineages or haplotypes and testicular germ cell cancer (Quintana-Murci *et al*., 2003; Richard *et al*., 2004). Evidence gathered so far suggests that the molecular aetiology of TDS and sporadic testicular germ cell cancer most likely does not involve the same pathways as male infertility caused by deletions of genes located in the AZF region. A very recent study reported though that gr/gr deletions might confer susceptibility to the familial testicular germ cell cancer (Nathanson *et al*., 2005). However, the final conclusion awaits more detailed structural analysis of the Y chromosome in larger numbers of patients and controls.

Genes regulating testicular descent

As mentioned above in this review, failure of testicular descent (cryptorchidism) is a risk factor for testicular cancer, and the two disorders are probably closest associated with each other within the TDS. Aetiology of the majority of cases of cryptorchidism is unknown, indicating the involvement of a large number of factors in the pathogenesis of this complex disorder (Hutson *et al*., 1997). Among others, a lack of proper function of the androgen signalling pathways and anti-Müllerian hormone have been long known as capable of disturbing testicular descent, but more recently additional pathways have been unravelled. Detailed discussion on the genetic background of cryptorchidism is beyond the scope of this article, and the reader is referred instead to recent excellent reviews (Ivell and Hartung, 2003; Klonisch *et al*., 2004; Kolon *et al*., 2004). Here, only one pathway is briefly mentioned, that of insulin-like factor 3 (INSL3), as an example of a possible involvement of a genetic polymorphisms in the pathogenesis of some forms of TDS. INSL3, a testicular hormone (also known as relaxin-like factor, RLF), acts through a receptor named (G-protein-coupled receptor) LGR8/GREAT (Kumagai *et al*., 2002), and this system was first linked to testicular descent after targeted gene disruption in mice (Nef and Parada, 1999; Zimmerman *et al*., 1999; reviewed in Ivell and Bathgate, 2002). A large number of studies of the INSL3/LGR8 system in human subjects with cryptorchidism followed, some authors finding mutations, others failing to do so, but identifying several gene polymorphisms (Ferlin *et al*., 2003; references therein). Most of the identified mutations/polymorphisms were heterozygous, moreover in some cases the same genotype was linked to variable phenotypes, suggesting the involvement of multiple other factors, probably also environmental. Unravelling of the complex interplay between the structural changes of the INSL-3/LGR8 system and environmental impact on the regulatory pathways will require further studies, and this is only an example of one pathway. There are, undoubtedly, many more to investigate.

Are environmental or lifestyle factors responsible for increasing problems in male reproductive system including testicular cancer?

The rapid increase in the incidences of male reproductive problems indicates that environmental or lifestyle factors may play the primary role. A large number of epidemiological studies support this hypothesis. Probably the best documented and most illustrative are studies from Scandinavian countries, where excellent registries exist and where the reproductive problems were first noted. Among them, the finding of an association between a decreased incidence of testicular cancer and the year of birth, especially at wartime, clearly indicated the importance of external factors acting prenatally or perinatally (Møller, 1993; Bergström *et al*., 1996). This was supported by the aforementioned studies examining incidences of testicular cancer among migrating Scandinavian populations. (Hemminki and Li, 2002; Ekbom *et al*., 2003).

Which environmental or lifestyle factors can impair development of the reproductive system? Recent years provided growing evidence that the number and variability of contributing factors may be much greater than what we thought when the rise in testicular cancer was noted a few decades ago. The first hypothesis came from Henderson, and his group suggested a possible link between excessive exposure to bioavailable estrogens *in utero* (associated, e.g., with first pregnancy or maternal obesity) and reproductive abnormalities in men, in particular germ cell cancer (Henderson *et al*., 1979; Depue *et al*., 1983). The influence of parity was later confirmed by others (English *et al*., 2003; Richiardi *et al*., 2004b). Estrogens play an important role in spermatogenesis (Kula, 1988; Couse *et al*., 2001; Carreau *et al*., 2003), and a variant estrogen receptor β is present in fetal gonocytes (Gaskell *et al*., 2003), but the function of estrogens during early development of the testis is poorly understood. An ability to mimic estrogens and disturb hormonal pathways *in vitro* and *in vivo* in experimental animals

was discovered for a number of environmental chemicals, and endocrine hormone disrupters were suggested as possible inducers of reproductive problems in men (Sharpe and Skakkebæk, 1993; Toppari *et al*., 1996). However, the issue of endocrine disrupters appears to be very complex when viewed from the perspective of more recent evidence.

European epidemiological trends pointed at factors acting predominantly in highly developed countries, including those with an intensive agricultural industry, such as Denmark and Switzerland. Pollution of ground waters and food with potent synthetic hormones, e.g. estrogens, gestagens and androgenic anabolics used for meat production, has been suspected (Daxenberger *et al*., 2001). In addition, a large number of chemicals are components of pesticides, herbicides and food additives. For example, polychlorinated biphenyls, hexachlorobenzene and chlordanes elevated levels of which have been detected in blood from the mothers of men with testis cancer (Hardell *et al*., 2003). Studies of the mechanism of action of these compounds broadened the definition of endocrine disrupters to include chemicals interfering with various hormone pathways, most notably with androgen signalling and production (Gray *et al*., 2001; Williams *et al*., 2001). Recently, the attention of researchers has been focussed on phthalates, which are produced and utilized as plasticizers and softeners around the world in enormous quantities. Some phthalates, if administered *in utero*, can induce testicular dysgenesis and a TDS-like phenotypes in rats and rabbits (Foster *et al*., 2001; Fisher *et al*., 2003; Higuchi *et al*., 2003). The existing mechanistic evidence suggests that phthalates exert antiandrogenic effects (Fisher, 2004).

Apart from environmental chemicals, a host of lifestyle factors have also been indicated. Many of them are related to maternal habits, which may adversely influence the developing fetus, such as smoking (Jensen *et al*., 2004; Pettersson *et al*., 2004), maternal obesity and delayed childbearing (reviewed in Sharpe and Franks, 2002). The aetiology of testicular cancer and TDS is most likely multifactorial, and a relative importance of maternal factors and external exposures may be difficult to pinpoint.

Developmental model for the pathogenesis of CIS

Our current model of the pathogenesis of early stages of germ cell neoplasia is depicted schematically in Figure 6. Developmental arrest of germ cell differentiation is the core pathogenetic event leading to the origin of CIS. Most of this review discussed evidence indicating that CIS cells may be considered as transformed gonocytes. The initiation of the malignant transformation is most probably caused by the disturbance in the microenvironment of the differentiating fetal germ cells. Gonadal microenvironment during early development is very tightly regulated and exquisitely sensitive to hormones and paracrine factors. If this regulation is disturbed, a gonad may develop as a testis or as an ovary or something in between. Histological changes associated with CIS and evidence from animal models clearly indicate that somatic cells in the fetal testis, Sertoli and Leydig cells, or perhaps their precursors, are the mediators of hormonal and paracrine factors and are largely responsible for the differentiation of germ cells. However, direct influence on germ cells has also to be considered.

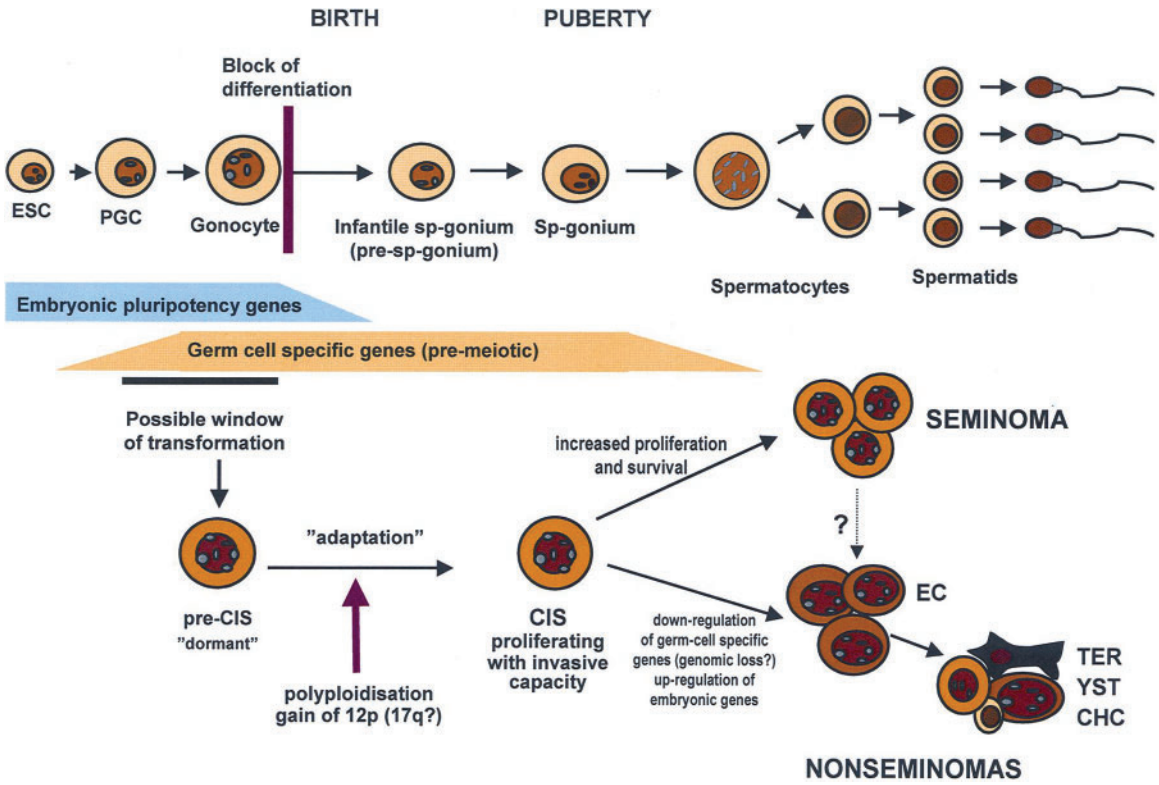

Figure 6. A scheme illustrating current understanding of the pathogenesis of testicular carcinoma *in situ* (CIS) in relation to germ cell differentiation. EC, embryonal carcinoma; TER, teratoma; YST, yolk sac tumour; CHC, choriocarcinoma.

In contrast to rodents, in the human fetus, differentiation of gonocytes into infantile spermatogonia is a relatively long and slow process. During this transition, embryonic traits disappear while germ cell-specific genes with a role in spermatogenesis are switched on (Figure 6). If something goes wrong and testicular differentiation is impaired, due to either an inherent genetic defect (e.g. in the androgen insensitivity syndrome) or an exposure to one or more environmental chemicals, this programme may be delayed or arrested, leading to the retention of embryonic features in germ cells outside the normal window of expression. Hormonal imbalance in the cellular microenvironment may lead to errors in mitosis–meiosis switch in 'sexually confused' germ cells and result in polyploidization. The mechanisms remain unknown, but we know that the processing of replicated sister chromatids and histone modification differ between mitosis and meiosis. One can speculate that a premature activation of some of the meiosis-specific mechanisms would somehow impair division of the replicated genome and cause polyploidization. Subsequently, other errors in cell division and progressive genomic aberrations would lead to further genomic instability and formation of transformed 'pre-CIS cells'. Most of these abnormal cells are probably eliminated, but some genomic changes may lead to the amplification of oncogenic pathways, which in combination with a high expression of anti-apoptotic pathways (e.g. KIT signalling) may favour survival of a subset of these cells. Recent observations in human ESCs undergoing chromosomal aberrations during long culture *in vitro* suggest that certain chromosomal gains (e.g. 12p, 17q or X) may be favourable for their adaptation and survival. A similar mechanism may be operating in transformed gonocytes *in vivo*; as mentioned earlier in this review, the pattern of chromosomal aberrations in CIS cells adjacent to overt tumours includes gains in the same chromosomal regions and may be responsible for their invasive ability. These adaptive changes and invasive progression are most probably triggered by the drastic change of testicular hormone production associated with puberty, since after puberty an explosive rise in the age-specific incidence of germ cell tumours is observed. Indeed, testicular germ cell tumours seem to be very rare in patients with severe hypogonadotrophic hypogonadism. Likewise, in patients with complete androgen insensitivity, undifferentiated gonocytes resembling CIS may persist for years without progressing to overt tumours (Manuel *et al*., 1976; Rutgers and Scully, 1991; Cools *et al*., 2005; Hannema *et al*., 2006).

Conclusions and future perspectives

The evidence summarized in this review demonstrates that testicular germ cell cancer is a developmental disease. Despite being manifested in young adults, this cancer is a result of disturbed gonadal development and germ cell differentiation. Our studies indicate that CIS cells are transformed gonocytes, which failed to differentiate and subsequently underwent non-random genomic aberrations facilitating their survival and further invasive progression, while retaining a high expression of embryonic genes linked to self-renewal and pluripotency. The ESC-like phenotype may explain the remarkable ability of CIS-derived tumours to differentiate to a variety of teratomatous somatic tissues. Further studies of the embryonic features of CIS cells and mechanisms of tumour progression may have broader implications for better understanding of basic pathways of PGC differentiation.

As shown in the Scandinavian countries, the prevalence of testicular cancer appears to be a good biomarker of testicular dysgenesis and may be used as a proxy to estimate the prevalence of other components of TDS in any given population. Differences between populations and between individuals within a given population may reflect differences in environment or lifestyle but may also be a result of genetic predisposition to reproductive problems. We have only begun to identify genes involved in the regulation of human gonadal development, and very few genes have so far been studied for possible polymorphisms. Environmental aetiological factors have not yet been elucidated, but testicular cancer and TDS are undoubtedly complex multifactorial diseases. To untangle this knot of possible factors will require further studies both at the level of populations and at the level of cells and their molecular pathways. Epidemiological trends in testicular cancer and TDS suggest that these disorders may be added to the list of the so-called 'civilization diseases', which includes diabetes, obesity and a number of cancers. These diseases increased around the world while the populations changed markedly their lifestyle and environment. However, the attention given to these diseases by researchers will hopefully elucidate their aetiology and provides tools for very early detection of negative trends and help to implement preventive measures.

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