

Chapter 2

Germ Cell Cancer, Testicular Dysgenesis Syndrome and Epigenetics

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Abstract Testicular dysgenesis syndrome (TDS) comprises a series of disorders, which are linked by an aberrant development of the testis, including abnormal differentiation of somatic cells and delayed maturation of germ cells. Environmental factors (including endocrine disruptors) are thought to impair development of the testis. It is likewise thought that epigenetic patterns can be regulated by environmental stimuli and we here review the current knowledge on epigenetic patterns in testicular dysgenesis, which to a large extent is limited to testicular cancer. Testicular Germ Cell Tumors (TGCTs) of young adults originate from a precursor cell, carcinoma in situ testis, which has features of arrested and transformed gonocytes, including high expression of embryonic pluripotency genes and low genome methylation. Overt TGCTs are capable of a marked re-programming with loss of germinal phenotype and increased somatic differentiation. These features suggest a role for epigenetic changes in the pathogenesis and progression of TGCT. In addition to chromatin modifications and gene imprinting, other regulatory mechanisms have been emerging, i.e., miRNAs, which are instrumental in the regulation of gene transcription. It is possible that epigenetic reprogramming during early development may also be a cause of genital malformations and reduced spermatogenesis, but more research is needed to identify the causative environmental factors, their precise targets and affected pathways.

Keywords Epigenetics · Testicular Cancer · Testicular Dysgenesis Syndrome

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2.1 Introduction

Germ cell cancer in human males occurs predominantly in the testis, although in very rare instances it may also occur in extragonadal positions. Testicular germ cell tumors (TGCTs) are quite unique, as they occur predominantly in young men, and their initiation begins most likely during early development of gonads. Another unique feature of these tumors is their ability to transform into virtually any type of somatic tissue, which is strongly linked to both genomic and epigenetic changes that affect gene expression.

Testicular tumors are epidemiologically and clinically linked to other disorders of male reproduction, with a common feature of developmental origins. These observations led to the proposed grouping of these disorders within the so-called Testicular Dysgenesis Syndrome (TDS). Growing evidence suggests that the majority of cases of TDS are caused predominantly by external factors, which may act directly on numerous pathways involved in testis development, and are also suspected of inflicting epigenetic changes that may be instrumental in reprogramming of germ cell differentiation. The factors remain to be identified and proven to be causal, but factors related to modern lifestyle, including endocrine disruptors have been under growing scrutiny as possible culprits. These aspects are reviewed in this chapter, with emphasis on germ cell tumors, where most information is available. The chapter focuses on human disorders, however, very little is known about the epigenetic reprogramming during human testis development and germ cell differentiation, therefore we discuss relevant findings gathered from studies in mice as well.

2.2 Epigenetics in Testicular Development: From Specification of Primordial Germ Cells to Formation of the Gonads

During embryonic development, embryonic ectoderm gives rise to somatic germ layers and primordial germ cells (PGCs) which are first observed at embryonic day E7.25 (mice) in the posterior end of the primitive streak. However already before specification of PGC's, pre-PGC's at E6.25 starts to express BLIMP1 (Ohinata et al. 2005), which is crucial for suppression of somatic programming of *Hox* genes (Ohinata et al. 2005). Around embryonic day E8 (mice), the PGCs start to migrate to the genital ridge and form a primitive gonad, tightly associated with the mesonephros, which later develops into kidney and epididymis. At the colonization-stage, the primitive gonad is still sexually bipotential and sex-determination first takes place between E10.5 and E11.5. While female germ cells subsequently enter meiosis, male germ cells, gonocytes, continue to proliferate until they differentiate to pre-spermatogonia that enter mitotic arrest. It is thought that sex-determination is determined by the expression of the male specific gene *Sry* in precursors of Sertoli cells. If *Sry* is expressed, this will trigger expression of *Sox9* and several

other masculinising genes, which are essential for Sertoli cells differentiation (recently reviewed by Koopman 2009). Sertoli cells then embed the PGC and form cords, which in mice are seen at E12.

Already during specification and migration, PGCs undergo epigenetic reprogramming, including genome-wide DNA de-methylation (starting at E8), progressive erasure of H3K9me2 (starting at E7.5) and subsequently establishment of H3K27me3 (starting at E8.25) in a progressive, cell-by-cell manner, presumably depending on their developmental maturation (Seki et al. 2005, 2007). These are all modifications that repress the DNA and the PGC is thus free of repressive chromatin between E7.5 and E8.25. As the loose chromatin structure thus would allow massive transcription, in this period PGCs are kept transcriptionally quiet by transient repression of RNA polymerase II dependent transcription (Seki et al. 2007). In addition, prior to H3K9 de-methylation PGCs repress the histone methyltransferase GLP, which mediates H3K9 methylation (Tachibana et al. 2008). In migrating PGCs, BLIMP1 (expressed from E6.25) is found in the nucleus is complexed with PRMT5, which mediates symmetrical methylation of histones H2A and H4 at arginine 3 (H2AR3me2, H4R3me2). When PGCs arrive in the genital ridge this complex translocates to the cytoplasm leading to reduced levels of H2AR3me2 and H4R3me2, resulting in widespread epigenetic modifications and transcriptional repression (Ancelin et al. 2006). Translocation of the BLIMP1/PRMT5 complex leads to down-regulation of *Blimp1*, which coincides with re-expression of pluripotency-associated genes like *Nanog* and *Sox2* (Ohinata et al. 2005; Yabuta et al. 2006).

When PGCs colonize the genital ridge (E11.5), extensive chromatin and epigenetic reprogramming occurs. This includes a second wave of DNA de-methylation, which includes erasure of parental imprints (Hajkova et al. 2002). Some parental imprints are already set again starting at E14.5, while others are first acquired at the newborn stage (Li et al. 2004). Linker histone H1, H3K9ac, H3K9me3, H3K27me2, DAPI chromocenters and, as mentioned above, H4/H2A R3me2, all disappear around gonadal colonization at E11.5 (Hajkova et al. 2008) as illustrated in Fig. 2.1. Using FACS sorting of dissected urogenital ridges in an *Oct4-GFP* transgenic mouse, Hajkova et al. (2008) were also able to show two *Oct4+* (GFP) populations of PGCs at E11.5: One population of E11.5 PGC was at a more advanced stage, with low levels of H2A.Z, linker H1, H3K9ac, H3K9me3 and H3K27me3 as well as H2A/H4R3me2, when compared to the other more primitive population, which showed higher levels of these marks. The primitive population of E11.5 PGCs showed variations in the levels of DNA methylation (5-methylcytosine levels), whereas the advanced population was practically devoid of DNA methylation (Hajkova et al. 2008). It is thus evident that extensive epigenetic modifications of PGC occur at the time of gonadal colonization.

Around E13, the proliferative PGCs have reached sufficient numbers, and female germ cells enter meiotic prophase while male germ cells (gonocytes) arrest in the G0/G1-phase of the cell cycle until after birth. After birth, gonocytes give rise to primitive spermatogonia (spermatogonial stem cells) that start to proliferate and develop into spermatocytes at days 10–14 post natal (Fig. 2.1).

By E12.5 most DNA methylation is lost (Reik and Walter 2001) and de novo methylation is initiated in males at E14.5 and thereafter in females such that the mature gametes of both sexes will eventually become highly methylated. However, an additional de-methylation wave takes place in pubertal and adult male germ cells after meiosis during spermatogenesis (Bernardino et al. 2000; del Mazo et al. 1994; Norris et al. 1994), where also histones are exchanged with transition proteins and finally protamines (Fig. 2.1). In addition, remethylation and further chromatin modifications may possibly occur as the spermatozoa mature in the epididymis (Xie et al. 2002).

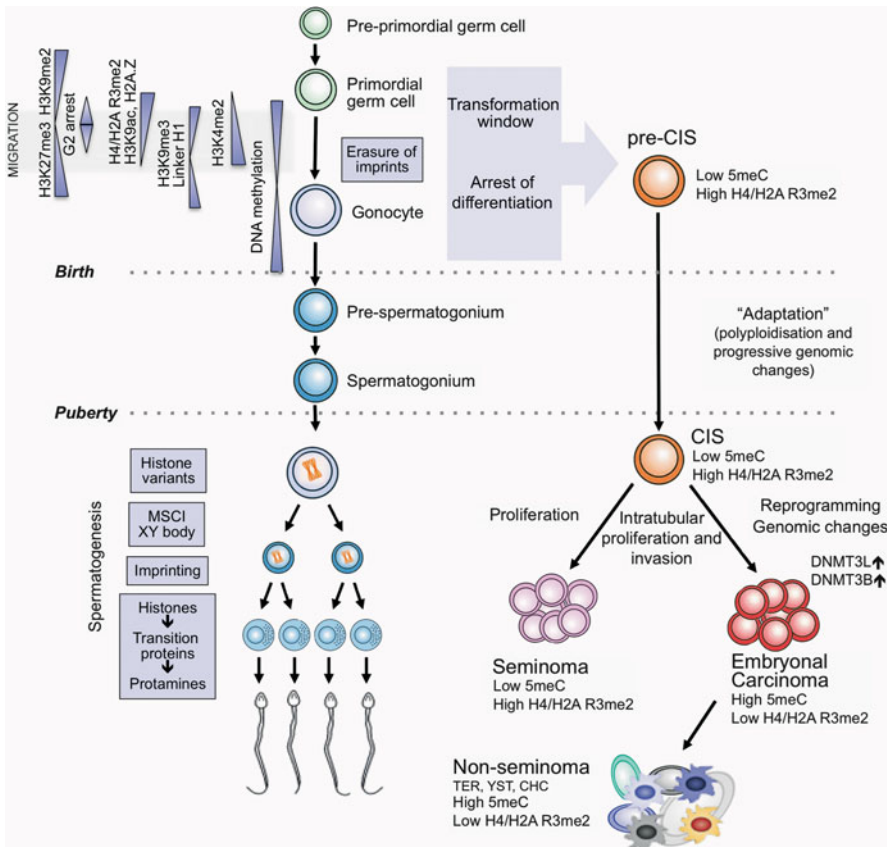


Fig. 2.1 Schematic drawing of major epigenetic events during male gonadal development and development of testicular germ cell tumors derived from CIS cells (modified from Rajpert-De Meyts 2006). DNA methylation patterns are greatly simplified as a number of different categories of sequence specific methylation (CpG, non-CpG, repeat elements, X chromosome, imprinted genes etc.) are independently regulated. Likewise, represented regulations are not exact but are intended as an overview. Data on H3K27me3 (Hajkova et al. 2008; Seki et al. 2007) is somewhat divergent and the depicted pattern is adapted from Seki et al. *MSCI* Meiotic sex chromosome inactivation, *TER* teratoma, *YST* Yolk sac tumor, *CHC* choriocarcinoma

2.3 TDS: A Collection of Reproductive Disorders with a Possible Common Pathogenesis

TDS was proposed by Skakkebaek and co-workers (2001) as a set of developmental disorders that affect male reproduction. The concept was mainly based on testicular germ cell cancer but studies of this cancer suggested that several other disorders may have a similar aetiology and pathogenesis. These disorders (very briefly summarized below) include some forms of undescended testis, hypospadias and reduced spermatogenesis, all linked together by common risk factors that operate in utero or early infancy. The TDS concept is outlined in Fig. 2.2.

2.3.1 Testicular Cancer

The vast majority of human testicular tumors are derived from germ cells, and most of these tumors occur in young men (between 17 and 40 years of age). Germ cell tumors can also occur in infants (yolk sac tumor or teratoma) and older men (spermatocytic seminoma), but these are very rare; moreover they are thought to

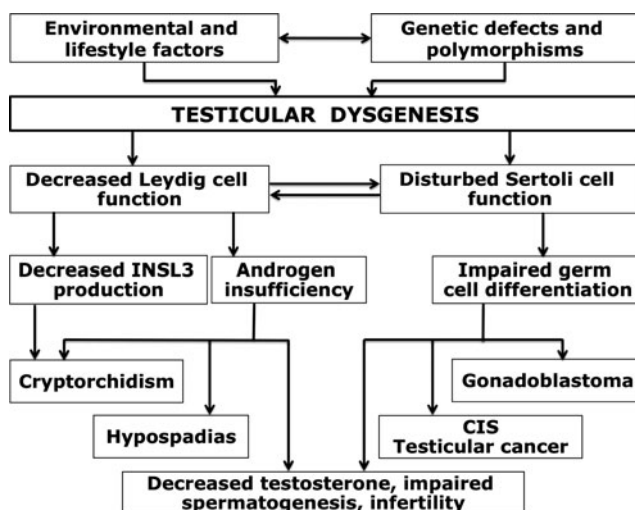


Fig. 2.2 Schematic depiction of the TDS concept. Environmental factors together with genetic aberrations or polymorphisms that increase sensitivity to those factors cause testicular dysgenesis, which results in an impaired differentiation and function of somatic cells that in turn leads to a delayed germ cell maturation and decreased secretion of reproductive hormones. This cascade of events is manifested by several clinical outcomes, including impairment of testicular descent, hypospadias, neoplastic transformation of germ cells (CIS in testes or gonadoblastoma in intersex gonads) or some forms of impaired testicular function and spermatogenesis later in life. Adapted from Skakkebaek et al. (2007)

be of purely genetic origin and not part of TDS (Oosterhuis and Looijenga 2005). Somatic cell tumors of the testis (sex cord–stromal neoplasms and Leydig cell tumors) are much less frequent, have also predominantly a genetic background, and are not considered as part of TDS. Thus in this chapter we shall focus on TGCTs of young adults, which can be divided into two main types: seminoma (also called classical seminoma to distinguish it from spermatocytic seminoma) and non-seminoma (Fig. 2.1). Seminoma is a homogeneous tumor composed of mitotically dividing germ cells, whereas non-seminomas are heterogeneous tumors that may contain varying proportions of undifferentiated embryonal carcinoma (EC), partially differentiated somatic tissues (teratoma), and extra-embryonic elements such as choriocarcinoma and yolk sac tumor (Ulbright et al. 1999).

A characteristic feature of TGCTs of young adults is their origin from a pre-malignant lesion called carcinoma in situ (CIS) testis (Skakkebaek et al. 1987), which is also known as intratubular germ cell neoplasia (ITGCN) or transepithelial intratubular neoplasia (TIN). A similar lesion, called gonadoblastoma, which consists of germ cells virtually identical to CIS cells, but surrounded by undifferentiated somatic cells in structures that do not resemble tubules, occurs in gonads of patients with more severe disorders of sexual differentiation, who most often are phenotypic female (Cools et al. 2006; Jorgensen et al. 1997).

Comparative studies of protein markers (reviewed in Rajpert-De Meyts et al. 2003) and the global gene expression profile of CIS cells (Almstrup et al. 2004, 2007; Sonne et al. 2009) provided evidence that CIS cells are transformed gonocytes that failed to mature during development (Rajpert-De Meyts 2006). The transformation of delayed gonocytes to CIS cells most likely occurs during adaptation to the changed endocrine environment of the peri- and post-pubertal testis, when the cells undergo polyploidisation (Oosterhuis et al. 1989) and acquire a characteristic pattern of genomic aberrations, including gain of chromosome 12p (Ottesen et al. 2003; Rosenberg et al. 2000), often manifested as the isochromosome 12p (Atkin and Baker 1982). In analogy to PGCs and gonocytes, CIS cells highly express transcription factors associated with embryonic stem cell pluripotency and somatic tissue-specific stem cell lineages, including e.g., *POU5F1*/*OCT-3/4*, *NANOG*, *GDF3*, *KIT* and *AP-2 γ* (Almstrup et al. 2004; Biermann et al. 2007; Hoei-Hansen et al. 2004, 2005; Looijenga et al. 2003; Skotheim et al. 2005; Sperger et al. 2003). Seminoma is very similar to CIS and shows expression of virtually the same genes (Almstrup et al. 2005; Gashaw et al. 2007; Hoei-Hansen 2008). By contrast, non-seminomas display a very different profile, which is characterized by the lack of expression at the protein level of the commonly used CIS/seminoma markers, e.g., PLAP, KIT, PDPN as well as germ cell-specific genes, e.g., VASA or DAZL1 (Korkola et al. 2005, 2006). This is consistent with the reprogramming of nonseminomas into a more embryonic cell type (Oosterhuis and Looijenga 2005; Rajpert-De Meyts 2006). The embryonic pluripotency genes are highly active in EC but down-regulated in differentiated components of teratomas, in line with the latter recapitulating the embryonic development (Korkola et al. 2006; Skotheim et al. 2005).

2.3.2 Undescended Testis and Hypospadias

Undescended testis (cryptorchidism) and hypospadias are common urogenital abnormalities, usually diagnosed in newborn or infantile boys. These abnormalities have a broad spectrum of severity (especially hypospadias) and the most severe manifestations can also occur in combination with other malformations. Importantly, cryptorchidism is the most significant risk factor for testicular cancer (Dieckmann and Pichlmeier 2004).

Testicular descent in humans first occurs trans-abdominally by enlargement of the gubernaculum and then as the inguino-scrotal phase when the testis gradually glides to its final position (Hutson and Hasthorpe 2005). In rodents, and most likely also in humans, the trans-abdominal phase is dependent on INSL3, a peptide hormone acting via its receptor, RXFP2, previously known as LGR8 (Nef and Parada 1999; Zimmermann et al. 1999). The second phase is mainly dependent on androgens; thus, different forms of androgen insufficiency (e.g., androgen receptor mutations) are often associated with undescended testes. Undoubtedly, other factors are also involved in both phases of descent, but the pathways involved are yet to be elucidated. The aetiology of hypospadias is likewise multi-factorial and not well understood, but, in the milder forms, androgen insufficiency is one of the known factors (Ferraz-de-Souza and Achermann 2007).

2.3.3 Impaired Spermatogenesis with Developmental Aetiology

Quantitative or qualitative reduction of spermatogenesis can be caused by a very large number of factors, making this disorder one of the most heterogeneous diseases, and also one of the least recognized as it is only diagnosed when it begins to affect a man's fertility. We would like to stress that only the forms of spermatogenesis impairment that are caused by aberrations in testis development are considered part of TDS. The proportion of men that are affected is not known, because the only currently existing evidence for developmental pathogenesis is the presence of histological changes, such as distorted tubules, undifferentiated Sertoli cells, persisting gonocytes (CIS cells), patchy distribution of Sertoli-cell-only tubules or microlithiasis (Jørgensen et al. 2010). Another important point to make is that semen quality is not only defined by sperm density and total sperm count, but these variables are the easiest to establish and to correlate with clinical outcomes, so most of the existing evidence is based on the sperm production. According to several studies, male fecundity begins to be affected (usually documented as increasing waiting time to pregnancy) when sperm concentration falls down to around 40 mill/ml (Bonde et al. 1998; Guzick et al. 2001; Slama et al. 2002). However, signs of impaired development can be seen in the testis producing sperm even within the normal range.

2.4 Endocrine Disrupters and Their Possible Influence on Epigenetic Patterns

Endocrine disrupters are chemicals that interfere with the endocrine balance in higher mammals and were at first thought to be chemicals that mimic intrinsic hormones i.e., synthetic estrogens (Sharpe and Skakkebaek 1993; Toppari et al. 1996). Later it has become clear that chemicals that interfere with all aspects of steroid metabolism i.e., aromatase inhibitors, androgen receptor blockers, can disrupt the endocrine function (Sharpe and Skakkebaek 2008). Even more importantly, the ubiquitous presence of numerous chemicals present in the environment (food, home, workplace) can lead to synergistic effects of compounds acting in a similar fashion or affecting different targets in co-operating pathways (Kortenkamp 2008). The male reproductive system appears to be especially vulnerable to endocrine disrupters because most of the known compounds in this category are known to target key pathways in testis development, i.e., anti-androgens, such as DDE, vinclozolin (Gray et al. 1994; Wilson et al. 2008) or phthalates (Mylchreest et al. 1999).

2.4.1 Evidence from Human Studies

There is very little direct evidence from human studies for a role of endocrine disrupters in testicular cancer, and only a very limited recent evidence for a role in cryptorchidism. Most of the data come from indirect epidemiological studies that are consistent with a possibility of a disruption of the early testis development by xenobiotic chemicals.

The first indication of a predominant role of external factors in the aetiology of testicular cancer was the dramatic increase observed in the incidence of this cancer in the second half of the twentieth century in many countries around the world, but with marked geographic or ethnic differences, suggestive of the greatest risk among white men of Scando-Germanic origin in well developed countries (Huyghe et al. 2003; McGlynn et al. 2005; Parkin et al. 1997; Richiardi et al. 2004; Shah et al. 2007).

Similar trends in other manifestations of TDS were noted first in Denmark and then in other countries (Aschim et al. 2004; Boisen et al. 2004; Carlsen et al. 1992; Hotelling and Walsh 2009; Moller et al. 1996; Paulozzi 1999), although some studies questioned the existence of such trends (Akre and Richiardi 2009).

These epidemiological observations point to the primary importance in the pathogenesis of factors acting during the intrauterine or early postnatal developmental window (Rajpert-De Meyts and Skakkebaek 1993; Skakkebaek et al. 2001; Weir et al. 2000). Initially, the excess of estrogens was suspected (Henderson et al. 1979; Moss et al. 1986; Sharpe and Skakkebaek 1993; Toppari et al. 1996), later anti-androgenic effects were indicated as of primary importance (Wilson et al. 2008),

which is in line with an increased risk of germ cell neoplasia in individuals with the androgen insensitivity syndrome. Few studies so far investigated the exposure of patients with TGCT/TDS to xenobiotics. Beside the long time interval from the fetal exposure to the disease manifestation, a big problem is the possible chemical interactions of mixtures, which are very difficult to identify in the human setting (Kortenkamp 2008). Studies of exposure to DES and other estrogens, although consistent with an increased risk of TGCT and poor semen quality in males, were not conclusive (Dieckmann and Pichlmeier 2004; Storgaard et al. 2006; Strohsnitter et al. 2001; Swan 2000). A few studies investigated exposure to other persistent endocrine disruptors, among them Hardell et al. (2003) found higher serum levels of polychlorinated biphenyls, DDE and several other persistent organic pollutants in mothers of men with testicular cancer, but no significant changes in the serum levels were found in men themselves (Hardell et al. 2003, 2006). Another study from the US demonstrated a greater burden of DDE and some chlordane-related compounds in the TGCT cases (McGlynn et al. 2008). More recently, a comparative study of these chemicals in milk samples of Danish and Finish mothers demonstrated a very different profile in the two countries, consistent with a higher risk of TDS in Denmark as compared to Finland (Krysiak-Baltyn et al. 2009).

Another group of ubiquitous pollutants, which in laboratory animals cause a TDS-like syndrome are phthalates (Hutchison et al. 2008). Some studies linked phthalate exposure to under-masculinization of boys, including changes in the androgenic behavioral programming (Matsumoto et al. 2008; Swan et al. 2005, 2009). However, there is no evidence as yet from the human studies if any of the above mentioned endocrine disruptors cause epigenetic changes. Some evidence begins to emerge from animal studies, which are briefly summarized below.

2.4.2 Evidence from Studies in Animals

There are numerous toxicological studies of endocrine disrupters in animals, but few explored possible epigenetic effects. In mice exposed to DES, lesions of the rete testis and some genitourinary tumors (but not TGCTs) were observed and these effects were reported to be transmitted transgenerationally, suggesting an epigenetic mechanism (Newbold et al. 2000).

Exposing rats in utero to phthalates caused a TDS-like phenotype including cryptorchidism, hypospadias, and a delay of gonocyte maturation (reviewed in Foster et al. 2001; Gray et al. 2000; Hutchison et al. 2008; Mylchreest et al. 1999), but epigenetic changes were not investigated in those studies.

The most striking evidence for the involvement of epigenetic regulation in effects of some endocrine disrupters came from studies of animals exposed in utero to the anti-androgenic fungicide vinclozolin, which caused defects of spermatogenesis that persisted in a large proportion of the progeny over four generations (Anway et al. 2005). In animals that were allowed to age, numerous other disorders appeared, including kidney and prostate disease, immune abnormalities as well as an increased

frequency of tumors, but testicular malignancies were not observed (Anway et al. 2006). Interestingly, all abnormalities were transmitted via male germ line, suggesting that in contrast to oocytes, in male germ cells exposed during embryonic sex determination some epigenetic regulation (i.e., DNA methylation) appeared to be permanently reprogrammed (Skinner 2007).

2.5 Epigenetics of Germ Cell Tumors and Testicular Dysgenesis

Normal germ cells undergo extensive chromatin changes during the process of maturation and gametogenesis. In particular the process of homologous recombination during the meiotic division, where double-strand DNA breaks are induced, requires the activation of specialized DNA repair machinery (Longhese et al. 2009). Constitutive activation of the DNA damage response process, which may be brought upon by various defects of this network, e.g., p53 mutations, is commonly found in numerous types of somatic solid tumors (Bartkova et al. 2006). By contrast, these pathways are largely silenced in TGCTs, including the pre-invasive CIS, as demonstrated by the lack of activation of the ATM kinase and phosphorylation of the downstream target histone γ H2AX (Bartkova et al. 2005), or downstream effectors MDC1 and 53BP1 (Bartkova et al. 2007a) in CIS cells. This feature of TGCTs most likely reflects the physiological silencing of DNA repair pathways in foetal germ cells and may – at least to some extent – explain the exquisite sensitivity of TGCT (and normal germ cells) to irradiation and chemotherapy, which inflict DNA damage (Bartkova et al. 2007b).

Much of the current knowledge about epigenetic changes in testicular cancer has been gained from studies on primary tissue, but to date no line representing the CIS cell has been identified. Techniques such as restriction landmark genomic scanning (RLGS) that require isolation of bulk DNA have, however, been used to study epigenetic changes in the overt tumor types derived from CIS, seminoma, and non-seminomas.

2.5.1 *Chromatin Changes*

Several studies have shown striking differences between seminomas and non-seminomas: there is little or no methylation in seminomas, whereas hypermethylation of specific gene promoters was observed in non-seminomas, increasing together with higher differentiation of the tumor. This was first shown by Peltomäki et al. by HpaII/MspI analysis (Peltomäki 1991). Smiraglia et al. later confirmed this pattern by the use of RLGS and found a similar pattern, with seminomas greatly de-methylated, while the level of DNA methylation in non-seminomas was relatively high and comparable to that of solid tumors derived from somatic cells (Smiraglia et al. 2002). More recently, a matching pattern of global DNA

methylation (low in seminoma and high in non-seminomas) was confirmed by immunohistochemistry and extended to CIS cells, which also showed global DNA de-methylation (Netto et al. 2008). The Low CpG methylation level in CIS was indeed confirmed in our laboratory as shown in Fig. 2.3, where immunohistochemical staining of CIS cells for 5-methylcytosine is shown. In line with these data, a comparative gene expression profiling of CIS, seminomas and non-seminomas revealed that the de novo DNA methyltransferase DNMT3B and the related protein DNMT3L were upregulated in non-seminomas (Almstrup et al. 2005). Interestingly, DNMT3L is known to interact directly with histone H3 when unmethylated at K4 (Ooi et al. 2007). De novo methylation consequently may be prevented at these CpG islands due to methylated H3K4 and is consistent with a strong negative correlation between DNA methylation and the presence of H3K4 methylation in several cell types (Weber et al. 2007).

Typical conditions for altered epigenetic control in cancer are reduced DNA methylation of most of the genome, which may lead to genomic instability, and hypermethylation of CpG islands located in the promoter regions of tumor suppressors and tumor-related genes (Esteller 2002). CIS and TGCTs, however, represent an exception probably due to their origin from foetal germ cells, most likely from PGCs or gonocytes, the cell types in which genomes are physiologically under-methylated (Trasler 1998). This again points to the foetal origin of CIS as probably a key event in the pathogenesis of TGCT and indicates that the loss of epigenetic control may be implicated in the pathogenesis of TDS-related disorders. Poor sperm have been reported to show altered epigenetic profiles compared to normal sperm (Hammoud et al. 2009; Houshdaran et al. 2007; Poplinski et al. 2009).

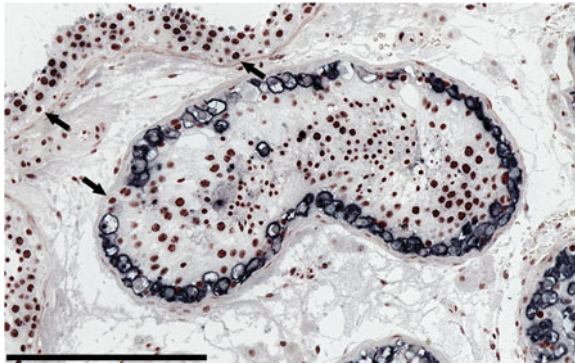


Fig. 2.3 Immunohistochemical double staining for 5-methyl-cytosine (5 mC, *reddish brown*) and placental-like alkaline phosphatase (PLAP, *dark blue*) in carcinoma in situ (CIS) testis. PLAP is a classical cytoplasmic marker for CIS cells, which show very low 5 mC staining in the nucleus indicating hypo-methylation of the genome. This is in line with a previous report showing low levels of 5 mC in CIS, varying amounts in seminoma and high levels in non-seminomas (Netto et al. 2008). Note that all other normal germ cells, including spermatogonia (marked with *arrows*) as well as Sertoli and Leydig cells show high 5 mC levels and no PLAP signal. *Bar* represents 200 μ m

However, it remains to be established whether these changes observed in adult men are the result of events occurring during development, and thus can be classified as TDS. Based on the findings of transgenerational effects and late-onset diseases transmitted by sperm of animals who were exposed in utero (Anway et al. 2005), it is likely that events occurring during early differentiation of germ cells may affect the quality of sperm later in life. We are, however, in need of developing molecular markers that would corroborate this hypothesis.

2.5.2 Methylation Profiles of Specific Genes Important for Pluripotency and Tumorigenesis

A hallmark of CIS cells is expression of a range of genes known to be responsible for stemness and pluripotency (Almstrup et al. 2004; Rajpert-De Meyts et al. 2003). One of the best-known pluripotency markers is POU5F1, better known as OCT3/4. OCT3/4 is a key transcriptional factor expressed in pluripotent stem and germ cells as well as in CIS, seminomas and EC, the undifferentiated stem cell for non-seminomas. However, if EC differentiates into yolk sac tumor, choriocarcinoma or teratoma, expression of OCT3/4 becomes repressed (Looijenga et al. 2003). De Jong et al. compared the methylation pattern of the upstream region of the OCT3/4 gene in different types of TGCTs and in normal tissue. Seminoma and EC showed a hypomethylated upstream region whereas differentiated non-seminomas, teratoma and yolk sac tumor, which lack OCT3/4 expression, were found to be hypermethylated in the upstream region (De Jong et al. 2007).

Methylation profiles of tumor-related genes in TGCTs differ from those in neoplasms of somatic cell origin, in particular testicular malignant lymphomas (Kawakami et al. 2003a). Methylation of E-cadherin gene (*CDH1*), *CDKN2B*, *CDKN2A*, *BRCA1*, *RBI*, *VHL*, *RASSF1A*, *RARB*, and *GSTP1* was not detected in any of the investigated TGCT tissue samples, whereas tissue samples of testicular lymphoma showed hypermethylation of *CDH1*, *RASSF1A*, and *RARB* (Kawakami et al. 2003a). A study performed by Honorio et al. (2003) agrees with these findings, showing the absence of, or infrequent, promoter methylation at *CDKN2A*, *GSTP1*, *RARB*, *DAPK* and *CDH1*, in both seminomas and non-seminomas. However, in the same study, some non-seminomatous TGCTs displayed frequent methylation of *RASSF1A*. This disagreement may be caused by the different CpG sites of *RASSF1A* analysed in the two studies.

In addition, studies of methylation status of the O6-Methylguanine-DNA Methyltransferase (MGMT), showed methylation in some of the non-seminomas but not in seminomas (Smith-Sørensen et al. 2002). Likewise, Zhang et al. (2005) found that the 5' ends of MAGE-A1 and MAGE-A3 were unmethylated in seminomatous tumors regardless of their expression, whereas methylation of these loci in the non-seminomas apparently prevented their expression. *SYCP1* on the other hand remained unmethylated, regardless of expression in both tumor types.

This implies that 5' upstream methylation patterns do not always dictate whether the downstream gene is expressed, which is in line with recent reports of the different usage of DNA methylation in CpG-rich and poor promoters (Weber et al. 2007).

Expression of the BLIMP1/PRMT5- complex is responsible for suppression of the somatic differentiation program via Hox genes and leads to dimethylation of arginine 3 on histone H2A and H4 (H2AR3me2, H4R3me2). The BLIMP1/PRMT5 complex, as well as H4/H2A R3me2, was detected in CIS and most seminomas and, at the same time, down regulated in EC and other non-seminomas. These results led to the suggestion that H4/H2A R3me2 is a mechanism by which CIS and seminomas maintain their undifferentiated form, while loss of H4/H2A R3me2 causes differentiation of non-seminomatous tumor cells into various somatic tissue components (Eckert et al. 2008).

The emerging picture of DNA methylation in TGCTs is thus an overall under-methylated CIS genome, which is retained in seminomas but becomes highly methylated in non-seminomas.

2.5.3 *X-Linked Genes and XIST Expression*

The possible involvement of the X chromosomes in the pathogenesis of TGCT has been implicated by several studies (Hasle et al. 1995; Lykkesfeldt et al. 1983; Rapley et al. 2000; van Echten et al. 1995). The number of X chromosomes is often increased in TGCTs (Rosenberg et al. 1999; Summersgill et al. 2001; van Echten et al. 1995), although this is most likely a reflection of their polyploidy. Moreover patients with Klinefelter's syndrome (47 XXY) more often develop extragonadal germ cell tumors (Hasle et al. 1995). A TGCT susceptibility gene (TGCT1), also associated with cryptorchidism, was identified on Xq27 by a genome-wide linkage study, with only fragile X mental retardation 1 (*FMR1*) gene characterized in this region so far (Rapley et al. 2000) but this association has not been confirmed in subsequent studies (Rapley 2007).

In mammalian cells with more than one X chromosome, nature has evolved a system for dosage-compensation of specific alleles by transcriptional inactivation. The X-inactive specific transcript, *Xist* is transcribed from inactive X chromosome (s) (Brown et al. 1991), and functions as a structural RNA covering the inactive X in a cis-like manner inactivating its "own" chromosome (Penny et al. 1996). After *Xist*-mediated inactivation, *Xist* is no longer needed to maintain the inactive state (Brown and Willard 1994), but instead additional factors, including variant histones and hypo-acetylation of histones H2A, H3 and H4 maintains X-inactivation. (Belyaev et al. 1996; Costanzi and Pehrson 1998; Hoyer-Fender et al. 2000; Jones et al. 1998; Mermoud et al. 1999; van der Vlag and Otte 1999; Wang et al. 2001). The silent non-expressed *Xist* allele on the active X-chromosome is methylated, while the expressed allele on the inactive X is unmethylated (Bernardino et al. 2000; Norris et al. 1994), and *Xist*-expression seems dependent on the methylation

status of the X-chromosomes. However, expression of *Xist* is also regulated by another noncoding transcript, *Tsix*, that is transcribed antisense to *Xist* (Lee et al. 1999). *Tsix* has a dual function as both a repressor of the steady-state level of *Xist* transcription and as a regulator of the distribution of *Xist* transcripts within the nucleus (Lee et al. 1999; Morey et al. 2001; Stavropoulos et al. 2001).

Xist is essentially undetectable in somatic male XY-cells but it is expressed in spermatocytes in the late stages of first meiotic prophase in the testis (De La Fuente et al. 1999; Farazmand et al. 2001; McCarrey and Dilworth 1992; Richler et al. 1992; Salido et al. 1992). Even though sex chromosomes are inactivated at the same stage of spermatogenesis by formation of the meiotic sex chromosome inactivation (MSCI) complex, *Xist* is not essential for proper MSCI (McCarrey et al. 2002; Turner et al. 2002). As reviewed by Turner (2007) MSCI is regulated by coating the large unpaired meiotic X-Y bivalent with BRCA-1 which attracts the ATR kinase to phosphorylate H2AX at serine-139 (γ H2AX) leading to condensation of chromatin on the X and Y chromosomes. MSCI is further substantiated by additional chromatin modifications such as H2A ubiquitylation, deacetylation of H3 and H4, sumoylation and H3K9me2.

XIST has also been found expressed in practically all TGCT subtypes including CIS and spermatocytic seminoma (Kawakami et al. 2003b; Looijenga et al. 1997). In fact, detection of the unmethylated 5'-end of *XIST* in plasma has been suggested as a possible diagnostic tool for the identification of germ cell tumors (Kawakami et al. 2004). The 5'-end of *Xist* in XY males is methylated while it is found unmethylated in TGCTs (following *Xist* expression) and methylation-specific PCR of this fragment on plasma samples thus represents a possible diagnostic tool. The downside is however that this is only possible for disseminated tumors where already well-established and easy measurable plasma markers as HCG, AFP and LDH exist.

Kawakami et al. (2003b) showed by bisulphite genomic sequencing, that the methylation status of three X-linked genes (AR, FMR1 and GPC3), which normally do not escape X-inactivation (Allen et al. 1992; Huber et al. 1999; Kirchgeßner et al. 1995), was unmethylated- and presumably active- on super-numerical X-chromosomes in both seminomas and non-seminomas, which was not influenced by *XIST* expression (Kawakami et al. 2003b). The role of *XIST* expression in TGCTs is thus probably different from its X-inactivation function observed in XX females and whether it actually has a function in chromosome inactivation in TGCTs is at present doubtful.

2.5.4 Gene Imprinting

Two genes often investigated to elucidate imprinting patterns are Insulin-like Growth Factor 2 (*IGF2*) and *H19* both found at the 11p15 locus. While *IGF2* is expressed from the paternal allele, *H19* is expressed from the maternal allele (Rainier et al. 1993). The upstream region of *H19* gene in human consists of

seven CTCF (CCCTC binding factor)-binding sites (Bell and Felsenfeld 2000; Hark et al. 2000) with the sixth binding site demonstrated to be the key regulatory factor for *IGF/H19* genes (Takai et al. 2001). CTCF is able to bind only unmethylated alleles. The unmethylated maternal allele is thus occupied with CTCF, which in turn blocks the *IGF2* promoter and, consequently, prevents *IGF2* expression. On the contrary, CTCF binding is prevented at the methylated paternal allele, which makes the enhancer region available, and *IGF2* expression is stimulated (Bell and Felsenfeld 2000; Hark et al. 2000).

Expression of *H19* and the other imprinted genes becomes biallelic at E11.5 in developing germ cells in mice (Szabó and Mann 1995; Villar et al. 1995), which indicates that imprinting patterns are erased at this time of development.

Biallelic expression of *H19* has also been demonstrated in TGCTs (van Gurp et al. 1994). Kawakami et al. (2006) investigated methylation status of the promoter and CTCF-binding site upstream from *H19* gene in TGCTs and testicular lymphomas. Both seminomatous and non-seminomatous TGCTs showed a biallelic unmethylated promoter and CTCF-binding site upstream of *H19*. Peripheral blood lymphocytes and testicular lymphoma on the other hand showed differential or monoallelic methylation. Similar results were reported by Sievers et al. (2005) by methylation-sensitive single nucleotide primer extension (MS-SNuPE).

In other studies, hypomethylation of the sixth CTCF-binding site was detected in somatic tissue-derived cancers, such as osteosarcoma or bladder cancer (Takai et al. 2001; Ulaner et al. 2003), whereas the promoter region of *H19* in osteosarcomas was hemi-methylated (Ulaner et al. 2003) and CpG regions surrounding the CTCF-binding sites in bladder cancer were significantly methylated (Takai et al. 2001).

It has become clear that the methylation patterns of the promoter region and CTCF-binding site upstream of *H19* are different in TGCTs than in somatic-tissue derived cancers. TGCTs exhibit thorough unmethylation of both promoter and CTCF-binding site regions, while levels of methylation in somatic neoplasms differ in those regions. Kawakami et al. (2006) suggested that the methylation status of *H19* in TGCTs derives from “erasure of methylation”, whereas in somatic cancers, it is from “gain of hypomethylation”. Likewise, human foetal germ cells have a predominantly unmethylated region of *H19*, but in mature germ cells in adults, this region becomes significantly methylated (Kerjean et al. 2000). Taken together, these data suggested that the erasure of methylation at the promoter region and CTCF-binding site upstream of *H19* in TGCTs recapitulates that of foetal germ cells or PGCs, in line with a presumably foetal origin of these tumors.

Very recently, poor-quality sperm was also shown, by pyrosequencing, to have significantly different methylation profiles at the sixth CTCF binding site (Boissonnas et al. 2010). Similar altered epigenetic patterns are thus found both in poor semen and TGCTs emphasizing the suggested possible common aetiology of testicular dysgenesis (TDS). However, the methylation profile in sperm may be a reflection of de-regulation that occurred around puberty, in addition to that persisting from the foetal life. As to the aetiology, very little is known, but recently, oestrogen responsive elements have been found in the *IGF2/H19* locus (Pathak et al. 2010)

leaving a possible connection to endocrine disruption and altered methylation pattern at the *IGF2/H19* locus.

2.5.5 Possible Involvement of miRNAs in TGCTs and Neoplastic Transformation

A range of studies has identified miRNAs to be abnormally expressed in TGCTs, indicating a possible mechanism in the pathogenesis of these tumors. One of the first indications of miRNAs crucial role for proper testicular development came from a targeted inactivation of protein Dicer1, which is responsible for digestion of double stranded pre-miRNAs to yield single stranded mature miRNAs. Dicer1 knockout mice showed among other things infertility due to lack of PGCs (Bernstein et al. 2003). More specific clues came later from a functional genetic screen, which identified miR-372 and miR-373 as miRNAs implicated in TGCT oncogenesis (Voorhoeve et al. 2006). In response to mitogenic signals from oncogenes, cells undergo a growth arrest. However, cells that lack functional p53 efficiently overcome this arrest, which is a prerequisite for full transformation into tumor cells. It was shown that expression miR-372 & 3 gave human primary cells the ability to circumvent the need to mutate p53 in order to drive oncogenic cell divisions. In addition, miR-372 & 3 were found highly expressed in TGCTs and in TGCT cell lines together with a normal non-mutated p53 (Voorhoeve et al. 2006) suggesting that these miRNAs might be more instrumental in the pathogenesis of TGCTs than the p53. P53 was initially suspected to play a role because of its high accumulation in a subset of CIS cells and overt tumors (Bartkova et al. 1991; Lutzker and Levine 1996), but mutations have never been convincingly demonstrated. Expression of the miR-371-373 cluster was later confirmed by expression profiling of 156 miRNAs in 69 TGCT samples (Gillis et al. 2007). This study showed, moreover, that samples clustered together depending on their maturation status, indicating involvement of altered miRNA expression in tumorigenesis (Gillis et al. 2007). One miRNA identified by Gillis et al. to be highly expressed in seminomas and EC was miR-302. Interestingly, miR-302 was recently identified as a potent suppressor of the tumor suppressor homologue p63 (Scheel et al. 2009).

So far the only results concerning miRNA expression in CIS cells come from a study by Novotny et al. (2007). It was shown that expression of the transcription factor E2F1 depends on the expression levels of pri-miR-17-5p in germ cells. A similar regulation was observed in CIS cells, which express the *E2F1* mRNA, but not E2F1 protein, concomitant with strong expression of the pri-miR-17-5p transcript (Fig. 2.4). As E2F1 is a key regulator of apoptosis and cell cycle progression, expression of pri-miR-17-5p could thus reduce apoptosis of CIS cells leading to sustained proliferation and ultimately neoplastic transformation. However, miR-17-5p might also be involved directly in the pathogenesis of testicular cancer as the miR-17 family recently was shown to be implicated in

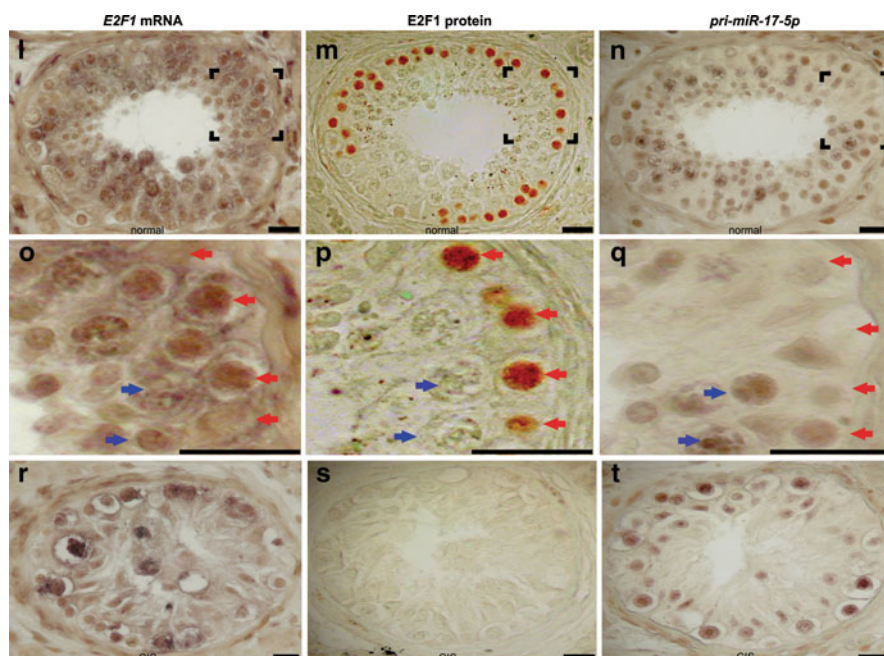


Fig. 2.4 Expression of E2F1 mRNA and protein in germ cells and CIS together with expression of the pri-miR-17-5p. Adapted from Novotny et al. (2007). Serial sections of normal human testis with in situ hybridization of *E2F1* mRNA (l), immunohistochemical detection of E2F1 protein (m) and in situ detection of *pri-miR-17-5p* (n). Corner marks indicate areas magnified underneath (o, p and q). Red arrows indicate cells with expression of E2F1 protein, whereas the same cells have no or low expression of *pri-miR-17-5p*. Blue arrows indicate examples of cells with no expression of E2F1 protein and high expression of miR-17-5p. (r, s and t) show serial sections of a tubule containing CIS cells. Bars correspond to 20 μ m

regulating stem cell differentiation in mice, probably by regulating STAT3 expression (Foshay and Gallicano 2009).

Still, very little is known about the complete regulatory network of miRNAs in testicular cancer and identification of many more miRNAs implicated in testicular cancer will probably be revealed in the near future. Yet questions of whether developmental mis-regulation of these miRNAs exist will also need to be elucidated. Especially remnants of embryonic miRNAs in CIS cells could be implicated in creating the neoplastic phenotype of CIS cells.

2.6 Open Questions and Perspectives

Based on the existing evidence – although it is so far limited – it seems that epigenetic changes are abundant in TGCTs and are likely to be involved in the pathogenesis of other symptoms of TDS. Much remains, however, unknown about

the epigenetic status of CIS, TGCTs and TDS, if we consider the huge number of possible epigenetic modifications. In addition, the consequences of changes of epigenetic status remain to be elucidated and compared to other features of TGCTs, i.e., genomic aberrations, and phenotypic features, such as pluripotency and invasiveness. The phenotypic features are of importance for possible clinical applications of this knowledge: can the tumor type, ability to metastasise, and the response to treatment be predicted from the epigenetic profile of the precursor CIS cell? A key question concerning the aetiology of TDS and TGCT is whether endocrine disruptors indeed are responsible for aberrant epigenetic patterns, as they seem to be in some experimental animals. Here a very important avenue of research is to identify the molecular targets for each chemical, both acting alone and in mixture, and to establish the most vulnerable cell types. This research may in the future lead to the more precise preventive measures.

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