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Epigenetic Modifications in Testicular Germ Cell Tumors

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1. Introduction

Over the past decade, accumulating evidence has implicated the phenomenon of epigenetic dysregulation in a variety of cancers (Biermann and Steger, 2007). Defined as the heritable changes in gene function that are not attributable to changes in DNA sequence, epigenetics involve biochemical modifications to DNA regulatory elements as well as to the tail regions of histone proteins (Maekawa and Watanabe, 2007). Lysine and arginine residues may be acetylated and methylated, with their effect being to activate or repress gene expression in the vicinity of that specific histone modification. Promoter regions of DNA are modified through the methylation of cytosine in CpG dinucleotides, resulting in the downregulation of those genes. Recent data suggest that these epigenetic modifications play a role in the development and progression of testicular germ cell tumors (TGCTs). This chapter will focus predominantly on adult TGCTs, along with their precursor *carcinoma in situ* (CIS), and will largely exclude the distinctive pediatric/prepubertal TGCTs. Many excellent reviews describing the classification of adult TGCTs exist (see Bahrami et al., 2007), and their content will not be duplicated here. Suffice it to say that the two main categories of TGCTs will be addressed in this chapter: the relatively undifferentiated seminomas, and the more differentiated non-seminomas (ranging from the less differentiated embryonal carcinomas to the highly differentiated yolk sac tumors, teratomas, and choriocarcinomas). Examination of histone modifications, histone modifying enzymes, genes exhibiting promoter DNA hypermethylation, and DNA methyltransferases in the context of CIS, seminomas, and non-seminomas should increase our understanding of TGCT biology and potentially generate new clinical applications.

2. Histone modifications and modifying enzymes

Histone modifications greatly influence the transcription status of nearby genes in a highly specific manner. Lysine and arginine residues on the tails of histones H3 and H4 may be methylated to generate signatures that associate with active chromatin, such as the trimethylation of H3 lysine 4 (H3K4me3), or with repressed chromatin, such as the

dimethylation and trimethylation of histone H3 lysine 9 (H3K9me2 and H3K9me3). Histones may also be acetylated and deacetylated through histone acetyltransferases and histone deacetylases (HDACs), which correspond to active and repressed chromatin, respectively. Histone modifying enzymes that transfer methyl groups to target residues include EZH2, SUV39H1/2, and G9a, while enzymes that remove methyl groups include JMJD3 and UTX.

2.1 H3K9 methylation

Dimethylation and trimethylation of histone H3 lysine 9 (H3K9me2 and H3K9me3) are associated with transcriptional repression. These marks are established by the histone methyltransferases SUV39H1/2 and G9a, which are critical to establish and maintain regions of heterochromatin (Peters et al., 2001; Tachibana et al., 2007). When global H3K9 methyl marks were examined in human CIS, Almstrup and colleagues found their presence to be very weak, revealing patterns similar to those in 20-40 week old normal fetal gonocytes (Almstrup et al., 2010). Interestingly, these marks were absent from the undifferentiated components of adult non-seminomatous embryonal carcinomas, but strongly detected in the undifferentiated seminomas (Almstrup et al., 2010). Godmann et al. reported the abundance of H3K9 methyl marks in both non-seminomas and seminomas, though they did not specify which nonseminomatous TGCTs they examined, nor their extent of differentiation (Godmann et al., 2009). In normal juvenile and adult mouse spermatogonia, the H3K9me2 mark is notably absent from the undifferentiated cells that exhibit stem cell activity; its presence coincides with the lineage commitment to differentiation (Payne and Braun, 2006). H3K9me3, meanwhile, exhibits a distinct perinuclear distribution in the undifferentiated germ cells that shifts and broadens to abundant punctate foci in the differentiating Type A spermatogonia, localizing to heterochromatin-rich regions (Payne and Braun, 2006). While these patterns have not been extensively evaluated in human seminiferous tubules, it is tempting to speculate that the transformation from normal primordial germ cells/gonocytes to CIS, and then subsequently to seminomas, involves the progressive upregulation of histone methyltransferase activity that results in more abundant H3K9me2 and H3K9me3 marks.

2.2 H3K27 methylation

Trimethylation of histone H3 lysine 27 (H3K27me3) is associated with transcriptional repression; this mark is established by the histone methyltransferase EZH2, a member of the Polycomb group protein family (Cao and Zhang, 2004). Similar to H3K9 methyl marks, H3K27me3 distribution is weak in CIS and abundant in seminomas (Almstrup et al., 2010). Surprisingly, EZH2 is strongly detected in the cytoplasm of CIS. Given that this enzyme normally functions in the nucleus to methylate histones, its cytoplasmic accumulation likely reflects misregulation in CIS. EZH2 is absent from embryonal carcinomas, as is H3K27me3 (Almstrup et al., 2010).

2.3 H3K4 methylation

Methylation of histone H3 lysine 4 (H3K4me1, H3K4me2, H3K4me3) is associated with transcriptional activation; H3K4me3 is established by the histone methyltransferase

Meisetz/PRDM9 (Hayashi et al., 2005). In contrast to the H3K9 and H3K27 methyl marks, H3K4me1 and H3K4me2/3 are abundant in CIS (Almstrup et al., 2010). Interestingly, the marks show opposing patterns in seminomas and non-seminomas (embryonal carcinomas), with H3K4me1 abundant in the former and weakly distributed in the latter, and H3K4me2/3 exhibiting the reverse. The significance of this observation is not clear. H3K4me2/3 appears more broadly distributed than H3K4me1 in normal human adult spermatogenic cells (Almstrup et al., 2010). As with H3K9 methyl marks, Godmann and colleagues observe H3K4me3 in both non-seminomas and seminomas (Godmann et al., 2009).

2.4 H4/H2AR3 methylation

Symmetrical dimethylation of histones H4 and H2A at arginine 3 (H4R3me2 and H2AR3me2) is associated with transcriptional repression, and is established by the complex of BLIMP1 and PRMT5 (Ancelin et al., 2006). BLIMP1 is a transcriptional repressor with an N-terminal PR-SET domain, a C-terminal acidic domain, and five zinc-finger domains; PRMT5 is a member of the protein arginine methyltransferase family. In normal fetal male germ cells of both mice and humans, H4R3me2 and H2AR3me2 marks are abundant in migratory primordial germ cells and gradually lost upon the transition to post-migratory gonocytes (Ancelin et al., 2006; Eckert, et al., 2008). In normal human adult testis, both marks are present in Type A spermatogonia, as well as round spermatids (Eckert et al., 2008). In CIS and seminomas, H4R3me2 and H2AR3me2 marks are abundant, with stronger signals detected in the seminomas. Heterogeneous, weak distribution of both marks is observed in some nonseminomatous TGCTs (embryonal carcinomas and teratomas). Dimethylation of H4R3 and H2AR3 is absent from choriocarcinomas.

2.5 EZH2

Enhancer of Zeste homolog 2 (EZH2) is a member of the Polycomb group protein family, functioning as a histone methyltransferase and adding methyl groups to H3K27 to generate H3K27me3. It is also considered to be an oncogene, shown to play a critical role in the development of breast cancer, prostate cancer, and malignant melanomas (Varambally et al., 2002; Bachmann et al., 2006). When examined in four cases of CIS, 64 seminomas, and 36 non-seminomas (9 embryonal carcinomas, 3 teratocarcinomas, and 24 mixed/combined TGCTs), *EZH2* expression showed significant downregulation when compared to normal testicular tissue (~80 vs. ~18 in CIS/TGCTs, relative normalized units), as measured by quantitative real-time RT-PCR (Hinz et al., 2009). Surprisingly, this assessment of *EZH2* mRNA conflicts with several observations of Almstrup and colleagues, who found a strong cytoplasmic distribution of EZH2 protein in CIS and abundant H3K27me3 marks in seminomas (Almstrup et al., 2010). These discrepancies could be due to the different methodologies used by the two groups (RT-PCR with oligo primers vs. immunohistochemistry with antibodies), including the processing of the specimens. As EZH2 has previously been shown to be frequently expressed in TGCTs (Bracken et al., 2003), additional studies combining both quantitative RNA and protein analysis are warranted to clarify the role of EZH2 in CIS and seminomatous/nonseminomatous TGCTs.

2.6 PRMT5

Protein arginine methyltransferase 5 forms a complex with the BLIMP1 transcriptional repressor to symmetrically dimethylate H4R3 and H2AR3. As with the histone methyl marks it generates, PRMT5 is abundant in migratory primordial germ cells and is downregulated after the cells transition to post-migratory gonocytes (Ancelin et al., 2006; Eckert, et al., 2008). In normal human adult testis, PRMT5 is present in pachytene spermatocytes and round spermatids (Eckert et al., 2008). In CIS and seminomas, PRMT5 is strongly detected in the cytoplasm and nucleus, respectively. This enzyme is also observed in the cytoplasm of embryonal carcinomas and teratomas, but is absent from choriocarcinomas. The cytoplasmic localization of PRMT5 in many of the CIS and TGCT samples, compared to the nuclear distribution seen in normal fetal germ cells, might indicate misregulation occurring as a result of the oncogenic transformation events.

2.7 JMJD3 and UTX

Jumonji domain containing 3 (JMJD3; KDM6B) and ubiquitously transcribed tetratricopeptide repeat, X chromosome (UTX; KDM6A) are histone demethylase enzymes that remove methyl groups from H3K27me2 and H3K27me3. These proteins therefore functionally oppose EZH2. In normal human adult testis, JMJD3 is weakly detected in spermatogonia and round spermatids, with stronger distribution observed in spermatocytes (Almstrup et al., 2010). UTX exhibits a similar overall pattern, with less intensity seen at each germ cell stage. In CIS, neither JMJD3 nor UTX are observed, which is concordant with high levels of EZH2 detected in these samples (Almstrup et al., 2010). In seminomatous and nonseminomatous TGCTs, JMJD3 and UTX both exhibit weak distribution at levels similar to EZH2.

2.8 HDAC1

Histone deacetylase 1 removes acetyl groups from lysine residues that are localized at the N-terminal end of histones. HDAC1, as with other members of the HDAC family, induces transcriptional repression (De Ruijter et al., 2003). In normal human adult testis, HDAC1 protein is observed in the nuclei of spermatogonia and Sertoli cells (Omisano et al., 2007). In both seminomatous and nonseminomatous TGCTs (embryonal carcinomas and teratomas), HDAC1 is consistently detected.

2.9 Histone modifications in Sertoli cells

HDAC1 and H3K9me3, as well as other histone modifying enzymes and modified tail residues, are present in normal human and mouse Sertoli cells (Omisano et al., 2007; Payne and Braun, 2006). Because Sertoli cells must maintain constant physical contact with germ cells at all stages inside each seminiferous tubule, the influence they have upon spermatogenesis is profound. Two essential growth factors that ensure the survival and maintenance of spermatogenic cells, and which are expressed by Sertoli cells, are Steel (KITL) and glial cell line-derived neurotrophic factor (GDNF). Steel activates the kit receptor (KIT) on differentiating spermatogonia in the postnatal testis, while GDNF activates associated receptors in undifferentiated spermatogonia: GDNF family receptor alpha 1 (GFRA1) and ret tyrosine kinase (RET; Sette et al., 2000; Jing et al., 1996; Viglietto et al.,

2000). Loss-of-function mutations of *Steel* and *Gdnf* in Sertoli cells deleteriously affect germ cell fate (Tajima et al., 1994; Meng et al., 2000). The loss of spermatogenic cells due to infertility or TGCTs also alters the epigenetic state of Sertoli cells, resulting in an increase in global histone H4 acetylation within the Sertoli cell nuclei of these human testes (Faure et al., 2003). In patients with seminomas and Sertoli cell-only syndrome, hyperacetylated histone H4 is observed in 70% of Sertoli cells. When Sertoli cell-specific gene targeting in mice inactivates *Sin3a*, which normally encodes an HDAC1-interacting protein, mutant testes exhibit the following: a significant reduction in spermatogonial stem cells, a progressive loss of differentiating spermatogonia, and a block in spermatid elongation, followed by extensive germ cell degeneration (Payne et al., 2010). Additionally, fewer teratomas are formed in a mouse tumor induction assay when donor germ cells from fetal *Sin3a*-mutant testes are transplanted into recipient adult testes (Payne et al., 2010). These results suggest that the epigenome in normal Sertoli cells supports a niche for spermatogonial stem cell activity, and provides a permissive microenvironment for the formation of adult TGCTs from fetal CIS. Further investigation on how Sertoli cells contribute to the development of TGCTs should increase our understanding of extrinsic factors that influence tumorigenesis.

3. Genes exhibiting promoter DNA hypermethylation

Numerous studies have shown that repetitive DNA within the human genome, consisting of Small Interspersed Nuclear Elements (SINEs), Long Interspersed Nuclear Elements (LINEs), and Long Terminal Repeats (LTRs), is globally hypomethylated in tumors (Ehrlich and Wang, 1981; Gama-Sosa et al., 1983; Dunn, 2003; Rodriguez et al., 2006). These repetitive elements are enriched in m⁵C relative to the whole genome within non-tumorigenic cells, but exhibit reduced methylation relative to the genome in many cancers, including those of the breast and ovary (Narayan et al., 1998; Qu et al., 1999). One interpretation of these findings is that the loss of methylation in repetitive DNA promotes genomic instability, resulting in deletions, translocations, and chromosomal rearrangements (Eden et al., 2003; Esteller, 2008). Hypomethylated centromeric regions, for example, can contribute to aneuploidy, and the reduced methylation of satellite sequences can lead to inappropriate recombination (Hatzia Apostolou and Iliopoulos, 2011). Active LINEs and microsatellite DNA instability have both been discovered in TGCTs (Bratthauer and Fanning, 1992; Huddart et al., 1995). Interestingly, a recent study found that while seminomatous tumors contain both LINE1 and Alu repeats (SINE family members) in a hypomethylated state, nonseminomatous tumors exhibit hypomethylated LINE1, but methylated Alu repeats (Ushida et al., 2011).

In contrast, single-copy DNA regulatory elements of specific categories of genes undergo hypermethylation in many tumors (Estcio and Issa, 2011). CpG islands within promoter regions are usually unmethylated in non-tumorigenic cells, permitting gene transcription under the appropriate conditions (Suzuki and Bird, 2008). *De novo* methylation of CpG islands induces transcriptional silencing, often repressing tumor suppressor gene activity that creates optimal conditions for the onset and progression of most cancers. One of the first genes discovered to exhibit promoter DNA hypermethylation in tumor cells was *RB*, retinoblastoma (Sakai et al., 1991). To date, many genes identified as being susceptible to hypermethylation are known to suppress epithelial cancers, including those of the testis,

prostate, lung, and colon, as well as leukemias and lymphomas (Lind et al., 2007; Wali, 2010). It is important to note that in the majority of TGCTs examined for hypermethylated gene promoters, CpG islands of most selected genes are methylated in non-seminomas, but are not appreciably methylated in seminomatous tumors (Koul et al., 2002; Honorio et al., 2003; Lind et al., 2006). In support of this finding, global detection of m⁵C in CpG islands using a monoclonal antibody and immunohistochemistry revealed that the more differentiated TGCT histologies of non-seminomas (yolk sac tumors, teratomas, choriocarcinomas) exhibit hypermethylation, while the undifferentiated histology of seminomas do not show methylation (Wermann et al., 2010). Thus, it appears that in seminomas, both the repetitive DNA elements and the single-copy regulatory elements of genes are in an unmethylated state, highlighting a unique characteristic of these specific tumors. While many of the genes silenced by methylation are involved in DNA repair and the cell cycle checkpoint, others encode transcription factors, proteolytic enzymes, cell adhesion molecules, and proteins involved in various processes. Key genes that fall into each of these categories, identified to exhibit promoter DNA hypermethylation in TGCTs, will now be discussed in detail.

3.1 DNA repair/cell cycle checkpoint genes

RASSF1A

This gene encodes the Ras association domain family 1 isoform A (*RASSF1A*). It is one of the most prevalent hypermethylated genes across all human cancers studied (Gordon and Baksh, 2011). In addition to TGCTs, *RASSF1A* is hypermethylated in the cancers of the breast, cervix, esophagus, brain, kidney, bladder, ovary, lung and skin (Pfeifer et al., 2002; Lusher et al., 2002; Koul et al., 2002; Honorio et al., 2003; Kuroki et al., 2003; Spugnardi et al., 2003; Lind et al., 2006). *RASSF1A* tumor suppressor activity is important for DNA damage repair of double strand breaks and for pro-apoptotic mechanisms (Hamilton et al., 2009; Avruch et al., 2009). Four major studies detecting hypermethylated *RASSF1A* in untreated TGCTs have been published to date, with conflicting results for seminomas. Koul and colleagues reported that of the seminomatous and nonseminomatous TGCTs they examined, nearly all of the hypermethylated CpG islands were observed in non-seminomas (frequencies of ~100% in yolk sac tumors, ~45% in embryonal carcinomas, ~22% in teratomas, and ~9% in mixed/combined tumors); only 1 of 29 seminomas exhibited hypermethylated *RASSF1A* (Koul et al., 2002). In their examination of TGCTs, Honorio et al. found hypermethylated CpG islands in 15 of 18 (83%) non-seminomas, with frequencies of 80% (4/5) in yolk sac tumors, 60% (3/5) in embryonal carcinomas, 100% (5/5) in teratomas, and 100% in two choriocarcinomas and one mixed tumor; 4 of 10 (40%) seminomas exhibited *RASSF1A* hypermethylation, distinguishing these results from the previous study (Honorio et al., 2003). Lind and colleagues, meanwhile, observed elevated methylation in 10 of 35 (28.6%) nonseminomatous TGCTs and in 0 of 19 seminomas (Lind et al., 2006). The frequencies in the non-seminomas were: 50% (3/6) in yolk sac tumors, 37.5% (6/16) in embryonal carcinomas, 77.8% (7/9) in teratomas, and 100% in one choriocarcinoma. In contrast, Tian et al. recently reported that both seminomatous and nonseminomatous tumors contained equivalent frequencies of hypermethylated *RASSF1A* CpG islands (~78.6%), with an average of 13/16 CpG sites in a methylated state as measured by sodium bisulfite sequencing (Tian et al., 2011). In an earlier study, Kawakami and colleagues did not

detect the hypermethylation of *RASSF1A*, or any other gene they examined, in any of the TGCTs under investigation (Kawakami et al., 2003). Thus, from all of these studies it appears that *RASSF1A* is hypermethylated at a frequency of up to 100% in yolk sac tumors, teratomas, choriocarcinomas, and up to 60% in embryonal carcinomas, but exhibits divergent methylation patterns in seminomas. Further characterization of *RASSF1A* in seminomas is needed to address these discrepancies. Interestingly, when Koul and colleagues examined 39 cisplatin-resistant and 31 cisplatin-sensitive non-seminomas, *RASSF1A* exhibited a much higher frequency of methylation in the cisplatin-resistant group (52%) than in the cisplatin-sensitive group (28%), though the sample size was too small for statistical significance (Koul et al., 2004). The methylation status of the *RASSF1A* promoter might therefore be a useful prognostic indicator of nonseminomatous tumor sensitivity to cisplatin, and could allow for the adjustment of chemotherapy treatment strategy accordingly. Finally, it has been shown that hypermethylated *RASSF1A* CpG islands are detected in the cell-free circulating serum DNA of TGCT patients at a frequency of ~47% (Ellinger et al., 2009). This method of detection could provide an additional benefit to existing parameters in the clinic.

MGMT

This gene encodes O-6-methylguanine-DNA methyltransferase (MGMT), which provides defense against the mutagenic effects of O-6-methylguanine (alkylation adduct) in DNA and potential G-to-A transition mutations. MGMT repairs the O-6 position of guanine in DNA by transferring the alkyl group from this position to a cysteine residue within the structure of the protein (Mittra, 2007). The *MGMT* promoter is hypermethylated in glioblastomas, hepatocellular carcinomas, colorectal, esophageal, and lung cancers, as well as in TGCTs (Cao et al., 2009; Zhang et al., 2003; Ahlquist et al., 2008; Baumann et al., 2006; Wolf et al., 2001; Smith-Sørensen et al., 2002; Koul et al., 2002; Honorio et al., 2003; Koul et al., 2004). Smith-Sørensen and colleagues identified hypermethylated CpG islands in both seminomatous tumors (24%, 8/33) and nonseminomatous tumors (69%, 24/35), while the groups of Koul and Honorio found *MGMT* hypermethylation only in non-seminomas (frequencies of ~25% in yolk sac tumors, ~45% in embryonal carcinomas, ~30% in teratomas, and ~9% in mixed/combined tumors for Koul et al.); 44% of non-seminomas exhibited hypermethylated *MGMT* for Honorio and colleagues (Smith-Sørensen et al., 2002; Koul et al., 2002; Honorio et al., 2003). In contrast to *RASSF1A*, Koul et al. found that *MGMT* was hypermethylated more often in cisplatin-sensitive non-seminomas (31%) than in cisplatin-resistant tumors (13%), though the P-value was not significant (Koul et al., 2004). Compared to the hypermethylation of *RASSF1A* and all other genes examined in TGCTs, *MGMT* exhibited the highest frequency of hypermethylation in seminomas (11%, 8 of 72 combined from all three studies). Given the overall small sample sizes and discrepancies reported among the groups, it would be worth conducting a larger study examining *RASSF1A* and *MGMT* hypermethylation in seminomas to more accurately conclude whether the CpG islands of these genes might be the exception to the overall trend of unmethylated cytosine in seminoma DNA.

BRCA1

Breast cancer 1 encodes a multi-faceted protein involved in numerous cellular functions: DNA damage repair, cell cycle checkpoint, centrosome duplication, transcriptional

regulation, and many others (Deng and Brodie, 2000). Hypermethylated *BRCA1* is observed in a variety of cancers, including breast, ovary, bladder, lung, and testis (Rice et al., 2000; Bianco et al., 2000; Cabello et al., 2011; Wang et al., 2008; Koul et al., 2002). Koul and colleagues reported that of the seminomatous and nonseminomatous TGCTs they examined, nearly all of the hypermethylated CpG islands were observed in non-seminomas (frequencies of ~49% in yolk sac tumors, and equivalent percentages in embryonal carcinomas, teratomas, and mixed/combined tumors, 20-24%); only 1 of 29 seminomas exhibited hypermethylated *BRCA1* (Koul et al., 2002). No differences were observed between cisplatin-sensitive (31%) and -resistant (30%) non-seminomas with respect to CpG island methylation (Koul et al., 2004).

FANCF

Fanconi anemia, complementation group F encodes a DNA repair protein that supports post-replication repair, cell cycle checkpoint function, and maintenance of normal chromosome stability (Léveillé et al., 2004). *FANCF* is hypermethylated in cervical and ovarian cancers, as well as TGCTs (Narayan et al., 2004; Taniguchi et al., 2003; Koul et al., 2004). Koul and colleagues examined CpG island methylation only in nonseminomatous tumors, and found the overall frequency to be 5.7%. No differences were observed between cisplatin-sensitive (7%) and -resistant (6%) non-seminomas with respect to hypermethylated *FANCF* (Koul et al., 2004).

XPA

Xeroderma pigmentosum, complementation group A encodes a zinc finger protein that participates in the initial step of DNA excision repair (de Vries and van Steeg, 1996). *XPA* guards against the harmful effects of UV-B radiation and chemical compounds that would otherwise impair DNA replication and RNA transcription. While *XPA* expression is diminished in some colorectal cancers, CpG methylation has only been observed in one nonseminomatous TGCT (Yu et al., 2006; Lind et al., 2006). Lind and colleagues found that one of 6 yolk sac tumors exhibited hypermethylated *XPA*; no methylation was detected in any of 16 embryonal carcinomas, 9 teratomas, 1 choriocarcinoma, 3 mixed/combined tumors, and 20 seminomas (Lind et al., 2006). Thus, it appears that *XPA* is not appreciably methylated in either seminomas or non-seminomas.

MLH1

mutL homolog 1 (a homolog of the *E. coli* DNA mismatch repair gene *mutL*) encodes a protein that forms a component of the post-replicative DNA mismatch repair system. *MLH1* is involved in DNA damage signaling, inducing cell cycle arrest and apoptosis under certain conditions (Buermeier et al., 1999). The *MLH1* promoter is hypermethylated in numerous cancers, including colorectal, gastric, and endometrial carcinomas, and tumors of the breast, ovary, and testis (Herman et al., 1998; Leung et al., 1999; Esteller et al., 1998; Murata et al., 2002; Strathdee et al., 1999; Koul et al., 2002; Olsz et al., 2005). Koul and colleagues reported that of the seminomatous and nonseminomatous TGCTs they examined, nearly all of the hypermethylated CpG islands were observed in non-seminomas (no methylation detected in yolk sac tumors or teratomas, and frequencies of <10% in embryonal carcinomas and mixed/combined tumors); only 1 of 29 seminomas exhibited hypermethylated *MLH1* (Koul et al., 2002). Olsz et al. found an overall methylation frequency of 21.6% in the TGCTs they

examined, yet only 1 in 15 samples from refractory tumors showed CpG hypermethylation (Olasz et al., 2005). Unlike what has been observed in familial colorectal cancer, no correlation exists between *MLH1* methylation status and microsatellite instability in TGCTs (Herman et al., 1998; Olasz et al., 2005). No relationship is seen between the clinical outcome of TGCTs and *MLH1* hypermethylation, dampening its prospect as a potential biomarker.

APC

adenomatous polyposis coli encodes a tumor suppressor protein that antagonizes Wnt signaling. APC is also critical for cell migration and adhesion, transcriptional activation, DNA repair, and apoptosis (Senda et al., 2007). Hypermethylated APC is observed in many cancers, including colorectal, esophageal, breast, lung, prostate, and testis (Hiltunen et al., 1997; Kawakami et al., 2000; Virmani et al., 2001; Yegnasubramanian et al., 2004; Koul et al., 2002; Honorio et al., 2003). Koul and colleagues found an overall CpG methylation frequency of 9.8% in the nonseminomatous TGCTs they examined (~100% in yolk sac tumors, ~12% in teratomas, and ~3% in mixed/combined tumors; no methylation seen in embryonal carcinomas or seminomas), while Honorio et al. detected APC hypermethylation in 29% of non-seminomas (Koul et al., 2002; Honorio et al., 2003). No differences were observed between cisplatin-sensitive (24%) and -resistant (29%) non-seminomas with respect to hypermethylated APC (Koul et al., 2004). It was also recently shown that hypermethylated CpG islands of APC are detected in the cell-free circulating serum DNA of TGCT patients at a frequency of ~58% (Ellinger et al., 2009).

FHIT

fragile histidine triad gene encodes an enzyme, diadenosine 5',5'''-P1,P3-triphosphate hydrolase, that is involved in purine metabolism. It also promotes genomic integrity through DNA damage response and DNA repair mechanisms (Saldivar et al., 2010). *FHIT* is one of the most frequent targets of allele deletion in tumors, and is hypermethylated in cancers of the lung, cervix, liver, and testis (Hsu et al., 2007; Ki et al., 2008; Iyer et al., 2010; Koul et al., 2002; Honorio et al., 2003). Koul and colleagues observed *FHIT* hypermethylation at an overall frequency of 6.5% in non-seminomas (~49% in yolk sac tumors, ~8% in embryonal carcinomas, ~2% in teratomas, and ~3% in mixed/combined tumors), while Honorio et al. detected CpG methylation in 29% of nonseminomatous tumors (Koul et al., 2002; Honorio et al., 2003). No differences were observed between cisplatin-sensitive (7%) and -resistant (6%) non-seminomas with respect to hypermethylated *FHIT* (Koul et al., 2004).

CDKN2A

cyclin-dependent kinase inhibitor 2A generates multiple transcript variants that differ in their first exons. One of the transcripts contains an alternate open reading frame (ARF), yielding a protein that stabilizes tumor suppressor p53 through the sequestration of MDM2 (Gjerset, 2006). *CDKN2A* can also induce cell cycle arrest in G1 and G2, and promote apoptosis. *CDKN2A* hypermethylation in the forms of *p14(ARF)* and *p16(INK)* has been detected in numerous cancers, including those of the colon, stomach, kidney, uterus, ovary, and testis (Esteller et al., 2001; Chaubert et al., 1997). Variable results have been shown for TGCTs. Chaubert and colleagues reported the presence of hypermethylated *CDKN2A* in 13 of 26 (50%) TGCTs using the method of PCR amplification after *HpaII* enzyme digestion

(Chaubert et al., 1997). The groups of Smith-Sørensen, Honorio, and Kawakami, in contrast, were not able to detect methylated CpG islands of *CDKN2A* when examined by methylation-specific PCR (Smith-Sørensen et al., 2002; Honorio et al., 2003; Kawakami et al., 2003). Differing experimental strategies might explain this discrepancy. Hypermethylation of *p14(ARF)* and *p16(INK)* was recently observed in the cell-free circulating serum DNA of TGCT patients at a frequency of ~53% (Ellinger et al., 2009).

3.2 Transcription factor genes

NANOG

This gene encodes an important regulator of pluripotency maintenance in the inner cell mass of blastocyst-stage embryos and in undifferentiated embryonic stem cells. *NANOG* prevents differentiation into mesoderm by interacting with SMAD1 and blocking the recruitment of co-activators in that signaling pathway (Suzuki et al., 2006). Migratory primordial germ cells express *NANOG*, but upon the differentiation of male germ cells into fetal gonocytes and neonatal spermatogonia, *NANOG* is downregulated (Gaskell et al., 2004). Thus, normal juvenile and adult male germ cells do not express *NANOG*. Interestingly, Nettersheim et al. detected hypomethylated *NANOG*, and corresponding protein, in all seminomas and embryonal carcinomas (undifferentiated non-seminomas) they obtained from TGCT patients (Nettersheim et al., 2011). In contrast, all differentiated non-seminomas (yolk sac tumors, teratomas, and choriocarcinomas) exhibited CpG hypermethylation and an absence of *NANOG* protein. Similarly, normal adult spermatogonia contain hypermethylated *NANOG*, supporting the prevailing hypothesis that seminomas are derived from gonocyte precursors in the fetal gonad (Nettersheim et al., 2011). Thus, differences within the state of TGCT differentiation correlate with the extent of *NANOG* hypermethylation.

HIC1

hypermethylated in cancer 1 encodes a transcriptional repressor protein that functionally interacts with p53 to suppress the development of cancer throughout aging (Chen et al., 2005). The *HIC1* promoter is hypermethylated in many cancers, including those of the breast, cervix, and testis (Fujii et al., 1998; Dong et al., 2001; Koul et al., 2002). Koul and colleagues observed *HIC1* hypermethylation only in non-seminomas (frequencies of ~100% in yolk sac tumors, ~35% in embryonal carcinomas, ~21% in teratomas, and ~9% in mixed/combined tumors). None of 29 seminomas exhibited hypermethylated *HIC1* (Koul et al., 2002). *HIC1* also exhibited a much higher frequency of methylation in the cisplatin-resistant group (47%) than in the cisplatin-sensitive group (24%), though the sample size was too small for statistical significance (Koul et al., 2004). Like *RASSF1A*, the methylation status of the *HIC1* promoter could be a useful prognostic indicator of nonseminomatous tumor sensitivity to cisplatin.

HOXA9

homeobox A9 encodes a transcription factor that regulates differentiation and morphogenesis, and is important for hematopoiesis (Lawrence et al., 1997). Hypermethylated *HOXA9* has been reported for ovarian carcinomas, oral squamous cell carcinomas, lung cancer, liver cancer, and TGCTs (Wu et al., 2007; Guerrero-Preston et al., 2011; Hwang et al., 2011; Feng et

al., 2010; Lind et al., 2006). Lind and colleagues observed *HOXA9* hypermethylation only in nonseminomatous tumors, in 9 of 35 samples (detected in yolk sac tumors, teratomas, and one choriocarcinoma; Lind et al., 2006).

HOXB5

homeobox B5 encodes a transcription factor involved in lung and gut development, regulating differentiation and morphogenesis, and providing specific positional identities on the anterior-posterior axis (Volpe et al., 1997; Fu et al., 2003). Hypermethylated *HOXB5* has been reported for ovarian carcinomas and TGCTs (Wu et al., 2007; Lind et al., 2006). Lind and colleagues observed *HOXB5* hypermethylation only in nonseminomatous tumors, in 5 of 35 samples (detected exclusively in teratomas; Lind et al., 2006).

RARB

retinoic acid receptor, beta encodes a nuclear receptor that binds to retinoic acid, heterodimerizing with the retinoid x receptor and regulating transcriptional activity through retinoic acid response elements located in target genes (Dollé, 2009). *RARB* hypermethylation has been detected in numerous cancers, including those of the cervix, prostate, lung, bladder, and testis (Narayan et al., 2003; Singal et al., 2004; Feng et al., 2008; Cabello et al., 2011; Koul et al., 2002; Honorio et al., 2003). The groups of Koul and Honorio found *RARB* hypermethylation only in non-seminomas (frequencies of ~49% in yolk sac tumors, ~15% in embryonal carcinomas, and ~5% in teratomas for Koul et al.); only 5% of non-seminomas exhibited hypermethylated *RARB* for Honorio and colleagues (Koul et al., 2002; Honorio et al., 2003). Koul et al. also found that *RARB* was hypermethylated more often in cisplatin-sensitive non-seminomas (14%) than in cisplatin-resistant tumors (0%), with a P-value of 0.05 (Koul et al., 2004).

EMX2

empty spiracles homeobox 2 encodes a transcription factor that regulates neuroblast proliferation, migration and differentiation, patterns the forebrain, and defines cortical territories (Cecchi, 2002). In the urogenital system, *EMX2* is expressed in epithelial cells and is negatively regulated by *HOXA10* (Troy et al., 2003). Hypermethylated *EMX2* has been reported for lung cancer and TGCTs (Okamoto et al., 2010; Lind et al., 2006). Lind and colleagues found that one of 6 yolk sac tumors exhibited hypermethylated *EMX2*; no methylation was detected in any of 16 embryonal carcinomas, 9 teratomas, 1 choriocarcinoma, 3 mixed/combined tumors, and 20 seminomas (Lind et al., 2006). Thus, it appears that *EMX2* is not appreciably methylated in either seminomas or non-seminomas.

MSX1

msh homeobox 1 encodes a member of the muscle segment homeobox gene family. Functioning as a transcriptional repressor, *MSX1* influences limb pattern formation and craniofacial development (Alappat et al., 2003). Hypermethylated *MSX1* has been reported for acute lymphoblastic leukemia, and cancers of the breast, lung, colon, prostate, and testis (Dunwell et al., 2009; Shames et al., 2006; Lind et al., 2006). Lind and colleagues found that one of 6 yolk sac tumors exhibited hypermethylated *MSX1*; no methylation was detected in any of 16 embryonal carcinomas, 9 teratomas, 1 choriocarcinoma, 3 mixed/combined tumors, and 20 seminomas (Lind et al., 2006). Thus, it appears that *MSX1* is not appreciably methylated in either seminomas or non-seminomas.

RUNX3

runt-related transcription factor 3 encodes a member of the runt domain-containing family of transcription factors that binds to core sequences found in a number of enhancers and promoters (Otto et al., 2003). The *RUNX3* promoter is hypermethylated in many cancers, including those of the lung, liver, colon, bladder, breast, and testis (Li et al., 2004; Xiao and Liu, 2004; Goel et al., 2004; Kim et al., 2005; Hwang et al., 2007; Kato et al., 2003; Lind et al., 2006). Interestingly, Kato and colleagues reported that while 8 of 10 infantile yolk sac tumors exhibited *RUNX3* hypermethylation (specimens obtained from pediatric patients), 0 of 12 adult nonseminomatous TGCTs contained methylated *RUNX3*, despite 7 of the 11 mixed/combined TGCTs containing yolk sac tumors (Kato et al., 2003). Similarly, Lind et al. found that only one of 6 adult yolk sac tumors exhibited hypermethylated *RUNX3*, and no methylation was detected in any of 49 other adult non-seminomas (16 embryonal carcinomas, 9 teratomas, 1 choriocarcinoma, 3 mixed/combined tumors, and 20 seminomas; Lind et al., 2006). These observations strongly suggest that distinct epigenetic dysregulatory mechanisms occur in pediatric, but not adult yolk sac tumors, and that *RUNX3* is not appreciably methylated in either seminomatous or nonseminomatous adult TGCTs.

3.3 Proteolysis genes

PRSS21

protease, serine, 21 encodes a member of the trypsin family of serine proteases that uses the hydroxyl group of serine to cleave target peptide bonds involving the carboxyl group of lysine or arginine (Rawlings and Barrett, 1994). *PRSS21* is thought to regulate proteolytic events associated with male germ cell maturation, as it is expressed highly only in normal testis, and its hypermethylation and transcriptional repression have been observed in TGCTs (Manton et al., 2005; Kempkensteffen et al., 2006). Manton and colleagues reported that the majority of CpG islands were methylated in both seminomatous (86%, 3 samples) and nonseminomatous (87%, 5 samples) tumors (Manton et al., 2005). While Kempkensteffen et al. found the overall level of hypermethylation lower in seminomas than in non-seminomas, the levels were still significantly higher when compared to normal testicular tissue (Kempkensteffen et al., 2006). Thus, *PRSS21* appears to show the most extensive hypermethylation of any gene examined to date in seminomas, and might therefore be a useful biomarker for early detection of these tumors in the clinic, especially if detected in the cell-free circulating serum DNA.

TIMP3

tissue inhibitor of metalloproteinase 3 encodes a protein that inhibits matrix metalloproteinases, enzymes that degrade components of the extracellular matrix (Apte et al., 1995). *TIMP3* hypermethylation has been detected in numerous cancers, including those of the kidney, colon, breast, brain, lung, and testis (Esteller et al., 2001; Koul et al., 2002). Koul and colleagues, however, observed an overall frequency of hypermethylation of just 3.3% in non-seminomas, with detection of methylated CpG islands limited to embryonal carcinomas and mixed/combined tumors (Koul et al., 2002). No hypermethylation was seen in seminomas. Therefore, compared to other genes, *TIMP3* does not appear to be as extensively hypermethylated in non-seminomas.

3.4 Cell adhesion genes

CDH1

cadherin 1 encodes a calcium dependent cell-cell adhesion glycoprotein highly expressed in epithelial tissues, E-cadherin, that is comprised of five extracellular cadherin repeats, a transmembrane region, and a highly conserved cytoplasmic tail. Mutations in *CDH1* that disrupt the adhesive properties of E-cadherin occur in some breast and gastric carcinomas (Hirohashi, 1998). *CDH1* hypermethylation is also detected in many cancers, including leukemia and those of the esophagus, breast, prostate, and testis (Esteller et al., 2001; Koul et al., 2002; Honorio et al., 2003). Koul and colleagues reported that of the seminomatous and nonseminomatous TGCTs they examined, the majority of hypermethylated CpG islands were observed in non-seminomas (frequencies of <10% in embryonal carcinomas, teratomas, and mixed/combined tumors); only 1 of 29 seminomas exhibited hypermethylated *CDH1* (Koul et al., 2002). Honorio et al. detected an overall methylation frequency of 11% in non-seminomas (Honorio et al., 2003). No differences were observed between cisplatin-sensitive (3%) and -resistant (6%) non-seminomas with respect to hypermethylated *CDH1* (Koul et al., 2004).

CDH13

cadherin 13 encodes a calcium dependent cell-cell adhesion glycoprotein highly expressed in heart tissues, H-cadherin, that is comprised of five extracellular cadherin repeats, a transmembrane region, but unlike other cadherins, lacks the highly conserved cytoplasmic tail. The *CDH13* promoter is hypermethylated in numerous cancers, including chronic myeloid leukemia and those of the breast, lung, bladder, and testis (Roman-Gomez et al., 2003; Toyooka et al., 2001; Maruyama et al., 2001; Honorio et al., 2003; Lind et al., 2006). Honorio and colleagues reported an overall frequency of hypermethylated *CDH13* in 12% of non-seminomas, while Lind et al. detected hypermethylation in 9% of nonseminomatous (2 of 16 embryonal carcinomas; 0 of 9 teratomas) and 6% (1/17) of seminomatous TGCTs (Honorio et al., 2003; Lind et al., 2006). Despite the small sample size, *CDH13* hypermethylation was observed in the more undifferentiated TGCTs and not in the more highly differentiated non-seminomas, distinguishing this gene from most others that have been described here.

3.5 Genes involved in other processes

SCGB3A1 (extracellular/cytokine)

secretoglobin, family 3A, member 1 encodes a secreted protein, formerly called *high in normal 1* (*HIN1*), which has tumor suppressor activity, although the exact function of the protein is unknown. *SCGB3A1* is highly expressed in the lung and trachea of humans and mice, with additional expression in a species-dependent manner (Tomita and Kimura, 2008). Hypermethylated *SCGB3A1* has been reported for cancers of the nasopharynx, breast, lung, prostate, pancreas, stomach, and testis, among others (Wong et al., 2003; Fackler et al., 2003; Krop et al., 2004; Gong et al., 2011; Lind et al., 2006). Lind and colleagues observed *SCGB3A1* hypermethylation in 19 of 35 non-seminomas (frequencies of ~100% in choriocarcinomas, ~75% in teratomas, ~47% in yolk sac tumors, and ~35% in embryonal carcinomas); no methylation was detected in seminomas (Lind et al., 2006).

SORBS1 (glucose transport)

sorbin and SH3 domain containing 1 encodes a protein involved in tyrosine phosphorylation of c-Abl, via an interaction with the insulin receptor, and is required for insulin-stimulated glucose transport (Lin et al., 2001). Lind and colleagues reported *SORBS1* hypermethylation in 1 of 20 seminomatous TGCTs examined, with no detection in non-seminomas (0/35; Lind et al., 2006). Given the sample size, additional studies will be necessary to conclusively determine whether this gene is a useful biomarker for TGCTs.

GSTP1 (metabolic process)

glutathione S-transferase, pi 1 encodes an enzyme important for detoxification by catalyzing the conjugation of hydrophobic and electrophilic compounds with reduced glutathione (Strange et al., 2001). The GST family is categorized into four classes: alpha, mu, pi, and theta. *GSTP1* hypermethylation is observed in many cancers, including those of the liver, kidney, breast, lung, esophagus, brain, and colon (Esteller et al., 2001). The groups of Kawakami and Honorio did not find hypermethylated *GSTP1* in any of their seminomatous or nonseminomatous TGCT samples, but Koul et al. reported *GSTP1* hypermethylation in 1 of 29 seminomas (none in non-seminomas) and Ellinger et al. detected CpG island methylation in the cell-free circulating serum DNA of TGCT patients at a frequency of ~25% (Koul et al., 2002; Kawakami et al., 2003; Honorio et al., 2003; Ellinger et al., 2009). The TGCT patients had seminomas (N=36) as well as non-seminomas (N=37), and the hypermethylation frequency observed by Ellinger and colleagues was equivalent between the two groups (Ellinger et al., 2009). Clearly, additional studies will be necessary to determine why discrepancies exist between not observing *GSTP1* hypermethylation in the majority of TGCT samples and frequently observing it in the cell-free serum DNA of TGCT patients.

PTGS2 (oxidation-reduction)

prostaglandin-endoperoxidase synthase 2 encodes a key enzyme in prostaglandin biosynthesis, also known as COX2, which acts as both a dioxygenase and a peroxidase. Two isozymes of PTGS exist, the constitutive PTGS1 and the inducible PTGS2, which differ in their regulation of expression and tissue distribution. This gene encodes the inducible isozyme, and is misregulated in many cancers (Rizzo, 2011). Hypermethylated *PTGS2* has been reported for gliomas, gastric carcinomas, prostate cancer, and breast cancer, among others (Uhlmann et al., 2003; Yu et al., 2003; Yegnasubramanian et al., 2004; Chow et al., 2005). Ellinger and colleagues detected *PTGS2* CpG island hypermethylation in the cell-free circulating serum DNA of TGCT patients at a frequency of ~45%; no differences were observed between patients with seminomas and those with non-seminomas (Ellinger et al., 2009). It remains to be seen whether a similar frequency of hypermethylation is observed directly in TGCT specimens. Currently, the cell-free serum-based detection of hypermethylated *PTGS2*, like *RASSF1A*, *APC*, *CDKN2A* and *GSTP1*, could provide additional benefits to existing parameters in the clinic.

NME2 (phosphorylation)

non-metastatic cells 2, protein (NM23B) expressed in (official gene name) encodes a protein that, together with the product of *NME1*, forms the hexamer nucleoside diphosphate kinase (Postel et al., 2002). Koul and colleagues reported *NME2* CpG hypermethylation in one embryonal carcinoma sample (Koul et al., 2002), but no other known observations in

TGCTs or other cancers have been published, raising the uncertainty over the significance of this discovery.

4. DNA methyltransferases

DNA methylation is established by the coordinated activity of enzymes known as DNA methyltransferases (DNMTs). The mammalian DNMT family consists of DNMT1, DNMT3A, DNMT3B, and DNMT3L, and is responsible for the generation, acquisition, and maintenance of methylated cytosines residing in CpG dinucleotides (Jurkowska et al., 2010). DNMT3A and DNMT3B are *de novo* methyltransferases, while DNMT1 is a maintenance methyltransferase, required to preserve the methylated status of the DNA. Levels of DNMTs are increased in numerous cancers, accounting for the hypermethylation of CpG islands observed in the promoters of many genes. Thus, DNMTs are promising targets for the design of new anti-cancer therapeutics.

4.1 DNMT1

DNA methyltransferase 1 is responsible for the maintenance of the DNA methylation status in CpG dinucleotides (Bestor et al., 1988). Hypermethylation of CpG islands in gene promoters, as Section 3 in this chapter extensively describes, is associated with the transcriptional silencing of tumor suppressors and other genes in many cancers, including TGCTs. Omisanjo and colleagues examined the expression of DNMT1 mRNA and protein in TGCTs to determine whether *DNMT1* is upregulated. In normal human adult testis, *DNMT1* mRNA is expressed in spermatogonia, pachytene spermatocytes, and round spermatids, as measured by *in situ* hybridization; the protein is nuclear in spermatogonia, cytoplasmic in round spermatids, and interestingly, is not detected in pachytene spermatocytes (Omisanjo et al., 2007). In non-seminomas (8 embryonal carcinomas and 8 teratomas), both mRNA and protein are detected, with a stronger signal observed in the embryonal carcinomas. Neither mRNA nor protein are detected in seminomas (0/16; Omisanjo et al., 2007). These differential TGCT expression patterns of *DNMT1* were also found using RNA microarray analysis and quantitative real-time RT-PCR, showing significantly upregulated transcript levels in embryonal carcinomas, but low levels in seminomas (Biermann et al., 2007). In contrast, semi-quantitative RT-PCR analysis performed by Ishii and colleagues showed no difference in *DNMT1* expression between control tissue and either seminomatous or nonseminomatous TGCTs (Ishii et al., 2007). DNA methyltransferase inhibitors, such as 5-aza-2'-deoxycytidine, have been shown to reactivate silenced tumor suppressor genes in treated cancer cells, implying that DNMTs are both necessary and sufficient to maintain hypermethylated CpG islands in many tumors.

4.2 DNMT3A

DNA methyltransferase 3A is responsible for the *de novo* transfer of methyl groups to CpG dinucleotides. DNMT3A catalyzes the transfer of a methyl group from S-adenosyl-methionine to the 5' position of cytosine in DNA (Okano et al., 1999). Studies in mice have shown that DNMT3A is essential for imprinting, as well as for spermatogenesis (Kaneda et al., 2004). Ishii and colleagues recently examined the expression of DNMT3A mRNA and protein in TGCTs, known to exhibit hypermethylated CpG islands in numerous genes, to

determine whether *DNMT3A* is upregulated. Semi-quantitative RT-PCR analysis revealed that *DNMT3A* and its isoform, *DNMT3A2*, are significantly overexpressed in seminomas as well as non-seminomas (embryonal carcinomas, teratomas, choriocarcinomas, yolk sac tumors; Ishii et al., 2007). Protein analysis by immunoblot revealed that the *DNMT3A2* isoform is exhibited several times higher than control levels in both seminomatous and nonseminomatous TGCTs (Ishii et al., 2007). Thus, the upregulation of *DNMT3A2* appears to generate higher amounts of protein, and seems to be tumor cell-specific. Ishii et al. then examined the methylation levels of CpG-rich regions in the *DNMT3A* gene, and found more unmethylated *DNMT3A* CpG dinucleotides in seminomas than in adjacent non-tumor tissue (Ishii et al., 2007). Overexpression of *DNMT3A* in TGCTs therefore appears to result from the hypomethylation of CpG islands in key regulatory regions, allowing inappropriate transcription to occur.

4.3 DNMT3B

DNA methyltransferase 3B is responsible for the *de novo* transfer of methyl groups to CpG dinucleotides. DNMT3B catalyzes the transfer of a methyl group from S-adenosyl-methionine to the 5' position of cytosine in DNA (Okano et al., 1999). Beyrouthy and colleagues recently examined the protein expression of DNMT3B in embryonal carcinoma cell lines to determine whether they exhibit higher protein levels than in normal tissues or somatic tumors. Western blot analysis revealed that DNMT3B is significantly overexpressed in embryonal carcinoma cell lines when compared to all other cell types (Beyrouthy et al., 2009). Three earlier microarray studies reported upregulated *DNMT3B* mRNA in non-seminomas (primarily embryonal carcinomas), but not in seminomas when compared to non-cancerous controls (Almstrup et al., 2005; Korkola et al., 2005; Skotheim et al., 2005). In contrast, Ishii et al. found no significant difference in *DNMT3B* expression between control tissue and either seminomatous or nonseminomatous TGCTs, which were mostly teratomas, choriocarcinomas, and yolk sac tumors (Ishii et al., 2007). This difference in TGCT sample type could explain the discrepancy in the results; clearly *DNMT3B* appears to be overexpressed in embryonal carcinomas. Furthermore, knockdown of *DNMT3B* reverses 5-aza-2'-deoxycytidine hypersensitivity in both cisplatin-sensitive and cisplatin-resistant embryonal carcinoma cells (Beyrouthy et al., 2009). While cisplatin sensitivity itself is not affected by *DNMT3B* expression, 5-aza-2'-deoxycytidine hypersensitivity in embryonal carcinoma cells appears to be dependent on *DNMT3B* expression, regardless of whether the cells are sensitive or resistant to cisplatin.

5. Clinical applications

When compared to many other cancers, a remarkable aspect in the majority of TGCTs is the high curability rate using cisplatin-based chemotherapy in combination with radiation therapy. However, up to 30% of metastatic germ cell tumors of the testis are refractory to cisplatin treatment and can lead to mortality. As discussed earlier, hypermethylation of CpG islands in the promoters of two genes, *RASSF1A* and *HIC1*, has been observed in cisplatin-resistant tumors at a frequency of approximately 50%, while promoter hypermethylation for *MGMT* and *RARB* has been detected at higher frequencies in cisplatin-sensitive vs. chemoresistant tumors (Koul et al., 2004). Recent investigation into the use of DNA demethylating agent 5-azacytidine in TCam-2 cells as a method to decrease cisplatin

resistance has shown promising results (Wermann *et al.*, 2010). Drugs that target DNMTs are currently in use in clinical trials, such as 5-aza-2'-deoxycytidine for treating myelodysplastic syndromes (Müller-Thomas *et al.*, 2009), and newer drugs are under development.

6. Conclusion

A greater understanding of the role of histone modifications, histone modifying enzymes, genes exhibiting promoter DNA hypermethylation, and DNA methyltransferases in adult TGCTs should yield potentially useful biomarkers to aid in early detection, as well as new methods of treatment to overcome chemotherapy-resistant tumors. The presence of hypermethylated *RASSF1A*, *APC*, *CDKN2A*, *GSTP1*, and *PTGS2* in the cell-free serum DNA of TGCT patients raises the important question of how early in the tumorigenic process these markers could be detected at interpretable levels. Optimizing this strategy of identifying potential TGCTs before they develop to the extent of requiring aggressive chemotherapy and radiation treatment will be an important endeavor during the next decade. Because some metastatic TGCTs are refractory to cisplatin-based chemotherapy, searching for an alternative treatment regimen for these tumors is equally important. The potential use of 5-azacytidine, or similar DNMT inhibitors, in overcoming cisplatin resistance holds promise for such cases. Further investigation of epigenetic modifications in TGCTs at the bench and in the clinic will address these goals.

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8. References

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The book aims to provide an overview of current knowledge regarding germ cell tumors. It deals with the clinical presentations, treatment modalities, the biology and genetics of germ cell tumors in children and adults. Most chapters are focused on testicular germ cell tumors whose incidence has been increasing in young males. Included are reviews on the pathogenesis, risk factors, diagnosis and treatment regimens applied to precursor, pre-invasive lesions as well as to seminomatous and non-seminomatous germ cell tumors of the testes. In addition, a review is included on the diagnosis and current management options for intracranial germ cell tumors in children. Authors have also contributed articles on the genetics and epigenetics of germ cell tumor development in humans and in the mouse model system. This book will be of interest to scientists, physicians and lay readers wishing to review recent developments in the field of germ cell cancers.

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