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Role of epigenetics in the etiology of germ cell cancer

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ABSTRACT Embryonic development is strictly controlled by functionality of genes in which the existing networks can act both on transcription and translation regulation. Germ cell cancers (GCC) are unique because of a number of characteristics. In spite of their clinical presentation, *i.e.*, predominantly after puberty, they arise from primordial germ cells/gonocytes that have failed appropriate maturation to either pre-spermatogonia or oogonia. GCC mimic embryonal development to a certain extent, including capacity for totipotency. This knowledge has allowed the identification of informative diagnostic markers, including OCT3/4 (POU5F1), SOX2 and SOX17. An additional marker is the overall demethylated status of the genome. Genetic mutations in GCC are rare, which is exceptional for solid cancers. Our hypothesis is that a disturbed epigenetic regulation (through combined interaction of genetic or environmental parameters; referred to as genvironment) affect embryonic germ cell development, resulting in delayed or blocked maturation, and potentially progression to GCC. In this respect, studies of patients with Disorders of Sex Development (DSD) have increased our knowledge significantly. Genvironmental influences can lead to retention of existence of embryonic germ cells, the first step in the pathogenesis of GCC, resulting into the precursor lesions gonadoblastoma or carcinoma *in situ*. Identification of epigenetic alterations could lead to better understanding these processes and development of specific markers for early detection, eventually leading to development of targeted treatment. This review describes an interactive model related to the role of epigenetics in GCC pathogenesis, focusing on DNA methylation, histone modifications, epigenetic memory and inheritance, as well as environmental factors.

KEY WORDS: *germ cell cancer, epigenetics, methylation, histone modification, environment*

Introduction

Type II (testicular) germ cell tumors, referred to as Germ Cell Cancers (GCC), are the most common malignancy in Caucasian adolescents and young adults and their incidence is still rising (Huyghe *et al*., 2003, Huyghe *et al*., 2007). GCC arise from primordial germ cells (PGC) or gonocytes and are subdivided into seminomas/dysgerminomas and non-seminomas with carcinoma *in situ* (CIS) or gonadoblastoma (GB) as precursor lesions (Looijenga *et al*., 2011). Non-seminomas can be further categorized into embryonal carcinoma, which can differentiate into somatic lineages and extra – embryonic tissues (teratoma vs yolk sac tumor and choriocarcinoma respectively) (Looijenga *et al*., 2011). PGC have the intrinsic capacity for pluri/totipotency, reflected in GCC, in which even the germ line can be formed in non–seminomatous tumors (Honecker *et al*., 2006).

Regulation of pluripotency

Embryonic development is controlled by highly orchestrated patterns of gene expression (both temporal and tissue specific). It

Abbreviations used in this paper: AIS, androgen insensitivity syndrome; CIS, carcinoma *in situ*; EC, embryonal carcinoma; ES cell, embryonic stem cell; GA, gestational age; GB, gonadoblastoma; GCC, cerm cell cancer; H3K4Me3, H3 lysine 4 trimethylation; H3K4me1, H3 lysine 4 monomethylation; H3K27Me3, H3 lysine 27 trimethylation; H3K27Ac, H3 lysine 27 acetylase; LINE, long interspersed nucleotide element; PGC, primordial germ cell; SINE, short interspersed nucleotide element; 5mC, 5-methylcytosine; 5hmC, 5-hydroxymethylcytosine.

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is essential to understand these networks in order to gain insight into disturbed development, including cancer. A number of genes are known to play critical roles in establishing and maintaining pluripotency, including OCT3/4, NANOG, SOX2 (Chambers *et al.*, 2003, Chan *et al.*, 2011, Scholer *et al.*, 1990, Young, 2011). As such they are candidates for involvement in GCC development. Indeed, OCT3/4 has been shown to be an important diagnostic marker for the different histological elements of GCCs, including the precursor lesions (Looijenga *et al.*, 2003). Expression of SOX2 is seen in non-seminomas, especially and consistently in embryonal carcinomas, and more heterogeneously in the other components, whereas expression of a related gene, SOX17, is present in normal germ cells, CIS/GB and seminomas (de Jong *et al.*, 2008, Korkola *et al.*, 2005).

Genetics and epigenetics in germ cell cancer

Although much knowledge has been gained over the last decades, the exact role of the various risk factors involved in development of GCC is still unclear. Several genetic loci have been linked to increased risk of GCC (Rapley *et al.*, 2009), and the familial predisposition (i.e., increased risk of brothers and sons) (Coffey *et al.*, 2007) supports a significant genetic component in the pathogenesis of this type of cancer. So far, somatic mutations are rare in GCC (Bignell *et al.*, 2006), with a few notable exceptions such as *KIT* and *KRAS2* (Biermann *et al.*, 2007, Hersmus *et al.*, 2012, McIntyre *et al.*, 2008). It is therefore likely that epigenetic factors are involved in GCC development as well. This is not unexpected as a clear role for epigenetic regulatory processes exist in both the mechanisms of initiating and protecting pluripotency of embryonic stem cells as well as in maintaining the identity of differentiated cell types (Hawkins *et al.*, 2011). Deregulation of this process may alter chromosomal stability, specifying properties of stem cells, self-renewal and the potential to differentiate, leading to initiation and/or progression of cancer. Disrupted epigenetic regulation might therefore be one of the underlying factors in the origin and biology of GCC. As such, epigenetic alterations could be candidates for specific diagnostic and prognostic markers. This review gives an overview of the rapidly growing field of understanding the impact of epigenetics in normal and disrupted development, specifically in the pathogenesis of GCC.

Gonadal development: an introduction

PGC are the progenitor cells of gametogenesis in later life, first recognized at day E6.5 in mice and 5-6 weeks gestational age (GA) in humans (McLaren, 2003). These PGC are characterized by their alkaline phosphatase reactivity, which is used as a specific marker (Millan and Fishman, 1995). Immediately prior to this, pre-PGC begin to express *BLIMP1* mRNA and protein, which maintains the pluripotent state (Ohinata *et al.*, 2005). During migration PGC proliferate extensively. Once they reach the genital ridge (around E9-10 in mice and 6-8 weeks GA in human, then called gonocytes), they are under the influence of the sex determination process of the bipotential gonad, under control of SOX9 and FOXL2, amongst others (Morais da Silva *et al.*, 1996, Uhlenhaut and Treier, 2006), into either testis or ovary. This determines germ cell fate towards either the male or female direction. Male germ cells continue to proliferate until they differentiate to pre-spermatogonia, that then enter mitotic arrest.

Epigenetics and (germ cell) development

Epigenetics is commonly defined as inheritable changes affecting gene regulation that are not due to alterations in primary DNA sequence. The epigenome is highly dynamic, and can change depending on cell type and developmental stage within a single organism. The epigenetic processes work together to establish and maintain both global as local chromatin states e.g. open or condensed which determines gene expression (see Fig. 1 for an overview (Baylin and Jones, 2011)). Epigenetic modifications are relatively stable in somatic cells. In germ cells, however, the epigenome is reprogrammed on a genome-wide level. By E12.5/10 weeks GA most DNA methylation is lost (Reik and Walter, 2001) and *de novo* methylation is initiated in males at E14.5, leading to highly methylated mature gametes. This allows re-establishment of parental imprints in germ cells, the erasure of epimutations, and the generation of toti- or multipotent cells (Godmann *et al.*,

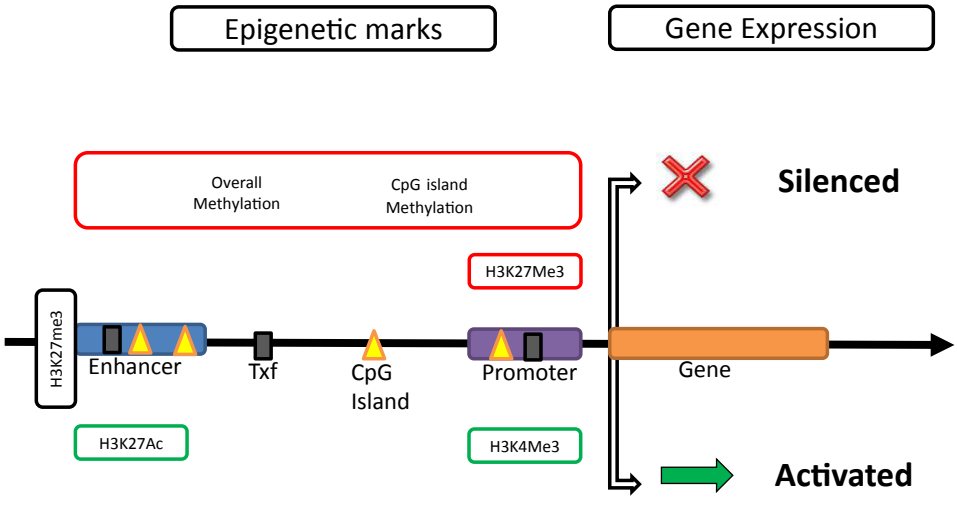

Fig. 1. Schematic representation of the role of epigenetic changes in regulating gene expression, controlled by DNA methylation and histone modification. *This will determine whether a gene is susceptible for transcription or not, related to the presence of appropriate transcription factors. In general, DNA methylation (indicated in the red box) is associated with gene silencing. It can occur at enhancer and promoter sites, as well as at CpG islands/shores. In this scheme the enhancer site is located in front of the gene, but can be located several Mb away (up- or downstream) from the gene. Histone modifications occur at enhancer or promoter sites and can be either activating or silencing (indicated in red and green box, respectively). In general, H3K4Me3 is active, whereas H3K27Me3 in the promoter region is inactive or silenced. The presence of both represents a bivalent state. A high H3K27Ac mark at an enhancer site is indicative for an active enhancer state. The presence of H3K27me3 together with the absence of H3K27ac will poise the enhancer for activation or repression at a later stage in development.*

2009, Hajkova *et al.*, 2002, Seki *et al.*, 2007). Genomic imprinting results in the silencing of one of the parental alleles in a subset of genes, and is different between different tissues and cell types (Prickett and Oakey, 2012). In addition, epigenetic modifications play essential roles in transmitting transcriptional memory; *i.e.* the phenomenon that epigenetic marks can be inherited across more than one generation (Jones and Liang, 2009). Our knowledge of the epigenome has increased enormously due to next – generation sequencing techniques for mapping DNA methylation and chromatin modifications. A number of relevant issues in this context will be presented in the next paragraph.

Epigenetic memory and inheritance

Epigenetic modifications play essential roles in transmitting transcriptional memory to daughter cells following mitosis (Jones and Liang, 2009). In rare cases GCC development depends on known genetic alterations, however, abnormal cellular memory or epigenetic changes that lead to aberrant gene expression patterns are also critical for tumor initiation and progression (Esteller, 2008).

In general, epigenetic marks are cleared and re-established with each generation *i.e.* reprogramming in PGC to ensure the totipotency of cells of the early embryo. However, the environment can stably influence epigenetic marks, which suggest that transgenerational epigenetic inheritance exists. The first evidence that epigenetic marks could indeed be inherited across more than one generation involved transgenes (Allen *et al.*, 1990, Sapienza *et al.*, 1987, Swain *et al.*, 1987). More recently, Lang-Mladek *et al.,* showed in plants that, after extreme temperature or UV-B stress, a silent transgene and several transposable elements were activated, and these changes were heritable for two generations (Lang-Mladek *et al.*, 2010). This suggests that some epigenetic marks may avoid erasure during early development, including the germ line. Some repetitive elements show incomplete erasure, which may be essential for chromosome stability and for preventing activation of transposons to reduce the risk of germline mutations. It was shown in mice, that partial deficiency of Apobec1 cytidine deaminase in the maternal germ-line led to suppression of teratomas in both partially and fully deficient males, and significantly reduced teratoma risk in a transgenerational manner among wild-type offspring. These heritable epigenetic changes persisted for multiple generations, and could be fully reversed (Nelson *et al.*, 2012). Aberrant epigenetic reprogramming in the germ line would cause the inheritance of epimutations, that may have consequences for human diseases (Hajkova *et al.*, 2002, Popp *et al.*, 2010). The same is true for histone modifications. It was initially thought that all histones were cleared and replaced by protamines (Oliva *et al.*, 1987), however in sperm, part of the haploid genome remains packed into nucleosomes. These histones are enriched at gene promoters important for development, as well as imprinted genes (Hammoud *et al.*, 2009). This provides these genomic loci with the ability to convey instructive epigenetic information to the zygote. Indeed, genome-wide analysis in fertile and non-fertile men showed that there are moderate differences at these loci, which may have a cumulatively detrimental effect on fecundity (Hammoud *et al.*, 2011).

Recently the focus of epigenetic inheritance research has moved to the role of small RNAs e.g. miRNAs, endo-siRNAs and piRNAs. These are present in both sperm and oocytes, and are known to be involved in gene silencing (Ashe *et al.*, 2012, Grandjean and Rassoulzadegan, 2009, Lee *et al.*, 2012, Shirayama *et al.*, 2012, Watanabe *et al.*, 2006). Interestingly, small RNAs are associated with the production of a mobile signal that can travel between cells and over long distances in plants and nematodes (Melnyk *et al.*, 2011). Three recent papers suggest that piRNAs can trigger a multigenerational epigenetic memory in the germline of *C. elegans,* and that it might be involved in scanning the germline transcriptome for foreign sequences, while endogenous germ-line-expressed genes are actively protected from piRNA-induced silencing (Ashe *et al.*, 2012, Lee *et al.*, 2012, Shirayama *et al.*, 2012). Although there could be a role in GCC development and inheritance via aberrant piRNA regulation, the mechanisms and biology remain poorly understood. The role of RNAs in transgenerational inheritance is extensively reviewed elsewhere (Daxinger and Whitelaw, 2012, Jones and Liang, 2009, Rassoulzadegan and Cuzin, 2010).

DNA methylation development and cancer

Epigenetics includes DNA methylation. This refers to the fact that DNA can be modified by the addition of a methyl group to cytosine residues, generating 5-methylcytosine (5mC). Three genes are known to be involved in the methylation of cytosine residues. *DNMT3A* and *DNMT3B* are *de novo* DNA methyltransferases, and together with the maintenance methyltransferase *DNMT1* are necessary for DNA methylation essential for embryonic development (Li *et al.*, 1992, Okano *et al.*, 1999). Other functions for these enzymes have been reported recently, but will not be discussed here (Chen *et al.*, 2012). In germ cells, DNMT3L is expressed in testes at the stage of *de novo* methylation, and interacts with DNMT3A and B (Bourc'his and Bestor, 2004). Recently, DNMT3L was described as a novel Embryonal Carcinoma (EC) marker, shown to be essential for growth of these cells (Minami *et al.*, 2010). DNA methylation was primarily thought to occur at CpG islands (Sasaki and Matsui, 2008), but recent studies based on whole genome analyses have shown the existence of so-called CpG "shores". This refers to methylation areas located adjacent to CpG islands in regions of less dense CpG dinucleotides (Doi *et al.*, 2009). In addition, almost 25% of methylation in Embryonic Stem (ES) cells is found outside of CG motifs, being lost upon differentiation (Lister *et al.*, 2009). Hypermethylation of CpG islands (strongly associated with gene promoters), and associated gene silencing by transcriptional repression due to inhibition of transcription factor binding is the most extensively studied epigenetic mechanism in cancers (Baylin and Jones, 2011, Jones, 2012, Park *et al.*, 2011) including GCC (Ellinger *et al.*, 2009, Honorio *et al.*, 2003, Manton *et al.*, 2005). DNA hypomethylation is generally associated with gene activation. Therefore, DNA methylation is a critical part of the control of gene expression, and as such regulatory of differentiation. It is a key part of embryonic development, chromosome stability and genomic imprinting (Jones, 2012). A complete lack of methylation can only occur in ES cells, but has not been seen in cancer cells (Chen *et al.*, 2007, Tsumura *et al.*, 2006). Of specific interest is the fact that imprinting-free ES cells can result in malignant transformation, by conferring cellular immortality (Holm *et al.*, 2005), including a seminomatous-like cancer.

During epigenetic reprogramming of germ cells, the genome becomes demethylated, and reprogramming by *de novo* methylation will be initiated during later stages of development. Here, TET1 was found to have an important role, as it catalyzes the oxidation

of 5mC into 5-hydroxymethylcytosine (5hmC) and therefore might play a role in the removal of 5mC. Indeed, repression of TET1 correlated with a reduction in 5hmC levels (Tahiliani *et al.*, 2009), and high level of Tet1 expression is detected in PGC (Hajkova *et al.*, 2010). The role of TET1 and 5hmC in the epigenome is extensively reviewed elsewhere (Branco *et al.*, 2012).

Origin of GCC and link to methylation

All GCC originate from a common precursor, the PGC/gonocyte (Oosterhuis and Looijenga, 2005), this is in line with findings on Alkaline phosphatase (Stoop *et al.*, 2011). It has been shown that global methylation status of GCC subtypes differ according to the time point of their developmental arrest; more differentiated cells showed a higher degree of methylation (Okamoto and Kawakami, 2007, Smiraglia *et al.*, 2002, Wermann *et al.*, 2010). It is also known that CIS/GB cells show very little DNA methylation(Netto *et al.*, 2008, Smiraglia *et al.*, 2002, Wermann *et al.*, 2010). Seminomatous GCC subtypes showed more global hypomethylation and almost no CpG island methylation, whereas non– seminomas showed more methylated DNA, both globally and at CpG islands. A targeted analysis of 15 promoter regions confirmed that there are differences in methylation profiles between the different GCC (Brait *et al.*, 2012). Moreover, Ellinger *et al.,* showed that detection of hypermethylated cell – free circulating DNA is feasible in most patients with GCC who undergo orchiectomy (Ellinger *et al.*, 2009). The diagnostic information received from cell – free methylated DNA by testing multiple gene sites seemed to be superior to that of conventional markers. Fig. 2 shows the assessment of global 5mC methylation, DNMT3L mediated methylation and maintenance methyl transferase DNMT1 by immunohistochemistry for embryonic testis, different histological elements of GCC and their precursors. This confirms the presence of different methylation profiles in the GCC subtypes, with the more differentiated being more methylated, associated with different methyltransferases.

X inactivation and XIST expression in GCC

Acquired numerical chromosomal changes in X chromosomes are commonly observed in GCC (Oosterhuis *et al.*, 1997). Inactivation of one of the X chromosomes in female mammalian cells is necessary to balance the increased dosage of X-linked genes compared with male cells (Ballabio and Willard, 1992). This process is initiated by the RNA gene *XIST*, expressed exclusively by the inactive form of the X chromosome, and results in hypermethylation of specific sites of genes to be silenced. In males, *XIST* is only detectable in germ cells of the normal male testis (Salido *et al.*, 1992). In GCC XIST is expressed only in tumors derived from the germ cell lineage with supernumerical X chromosomes: seminomas, nonseminomas, and spermatocytic seminomas. Although low *XIST* expression is present in testicular parenchyma with spermatogenesis, it is expressed at a higher level in parenchyma with CIS (Looijenga *et al.*, 1997).

Kawakami *et al.,* showed that *AR*, *FMR1* and *GPC3* (all X-linked genes) remained unmethylated in both seminomas and non-seminomas with *XIST* expression (Kawakami *et al.*, 2004), normally methylated in inactive X chromosomes. Identification of unmethylated XIST DNA fragments in male plasma might serve as a diagnostic marker for GCC, although these findings need to be confirmed in independent studies.

Repetitive elements and germ cell cancers

A significant fraction of the genome (approximately 42%) is composed of repetitive elements (Lander *et al.*, 2001). Two major classes can be identified; long interspersed nucleotide elements (LINEs) and short interspersed nucleotide elements (SINEs). LINE1 and SINEs of the Alu family are the most prominent elements, and both are highly methylated in normal tissues (Kazazian, 2004, Lander *et al.*, 2001). Ushida *et al.,* showed that both LINE1 and Alu repeats are unmethylated in seminomas, whereas only LINE1 is unmethylated in non-seminomas and EC (Ushida *et al.*, 2011). Thus the degree of demethylation of the repetitive elements is more pronounced in seminomas compared to non-seminomas, and GCC were more demethylated compared to cancers originating from somatic tissues (Ushida *et al.*, 2011).

Cisplatin sensitivity of germ cell cancers

Another factor that distinguishes seminomas and non-seminomas compared to other solid cancers is their overall responsiveness to cisplatin, a chemotherapeutic agent which binds to DNA, inducing crosslinks, which ultimately triggers apoptosis (Rosenberg *et al.*, 1965). Seminomas are usually sensitive to chemotherapy with cisplatin, whereas the response of non-seminomas differ according to their histology. Teratomas, which are the most differentiated GCC, show the highest degree of methylation and are cisplatin resistant (Krege *et al.*, 2008, Mayer *et al.*, 2003). In addition, cisplatin-resistant GCC showed different overall methylation profiles than non – resistant forms (Koul *et al.*, 2004, Netto *et al.*, 2008, Wermann *et al.*, 2010). Hypermethylation might therefore be a diagnostic marker as well as a predictive marker of treatment response. Exposure of TCam-2, a highly cisplatin resistant seminoma cell line, to the demethylation agent 5-aza-cytidine resulted in an increased sensitivity to cisplatin. Subsequent analysis of CpG island methylation showed that there were different methylation profiles in the treated and non-treated cells. For example, the promoter region of the *CFLAR* (c-FLIP) gene was hypermethylated in the treated cell line (Wermann *et al.*, 2010). CFLAR has an important role in regulation of apoptosis via the caspase pathway and therefore could be a therapeutic target (Yang, 2008), something that needs further evaluation. In addition, it has been shown that CFLAR can identify GCC patients with cisplatin resistance, based on genome-wide expression analysis (Sugimura *et al.*, 2004). Induction of c-FLIP has been reported in EC cell lines, as expected this results in resistance to cisplatin (Spierings *et al.*, 2004).

Histone modifications

Histones and associated chromatin proteins control the accessibility of genes and genomic elements. DNA is folded into nucleosomes; histone octameres consisting of two copies of each of the four histone proteins H2A, H2B, H3 and H4, wrapped with approximately 147 bp of DNA. A wide range of histone modifications, including methylation, acetylation and phosphorylation of specific amino acid residues, have been identified (Ernst and Kellis, 2010). These are often associated with genomic regions with regulatory potential, and it has been proposed that different combinations of histone modifications can be linked to specific types of functional elements (Strahl and Allis, 2000).

Technological advances have allowed the mapping of diverse

histone modifications in a large number of cell types (Doege *et al.*, 2012, Ferguson *et al.*, 2012, Shahhoseini *et al.*, 2010). In this context a number of relevant DNA segments must be recognized, which will be discussed in more detail in the next paragraphs.

Promoters

Specific promoter-associated chromatin signatures involved in regulating proliferation have been identified. In general, H3 lysine 4 trimethylation (H3K4Me3) is associated with active regions, whereas H3 lysine 27 trimethylation (H3K27Me3) is associated with inactive or silenced loci. The presence of both represents a bivalent state, and has been identified in cells with pluripotent potential such as ES cells (Azuara *et al.*, 2006, Bernstein *et al.*, 2006). Hawkins *et al.,* showed that modifications at promoters remain largely invariant during differentiation, except at a small number of promoters where a dynamic switch between acetylation (H3K27Ac) and methylation at H3K27 marks the transition between activation and silencing of gene expression. This suggests a hierarchy in cell fate commitment over most differentially expressed genes (Hawkins *et al.*, 2011).

Enhancers

Enhancers are defined as sequences, often outside the gene body, that regulate when and where a gene is expressed. Several histone marks have been associated with enhancers, including H3K4me1/2 and H3K27ac/me3. The current model in human ES cells is that high H3K27Ac is indicative for an active enhancer state. Loss of H3K27Ac and presence of H3K27Me3 will poise the enhancer for activation or silencing during differentiation (Rada-Iglesias *et al.*, 2011).

Germ cell development and GCC

PGC actively suppress somatic differentiation programs by epigenetic modifications, a mechanism which might also account for CIS and seminoma (Hayashi *et al.*, 2007). In mice, PGC increase levels of H3K27me3 by E8.5-E9.5, with levels reduced at E10.5-11 (Hajkova *et al.*, 2008). Utx, an H3K27 demethylase, regulates efficient induction of pluripotency. In the absence of Utx, PGC showed aberrant, cell-autonomous germ cell development during their embryonic maturation *in vivo,* as well as aberrant epigenetic reprogramming (Mansour *et al.*, 2012). CIS cells showed low levels of the repressive histone modifications H3K9me2 and H3K27me3, but high levels of activating marks H3K9Ac, H3K4me and H2A.Z (Almstrup *et al.*, 2010). This permissive chromatin structure is in accordance with the high levels of RNA polymerase II activity and proliferation that were observed in CIS cells. Epigenetic patterns similar to that of CIS cells

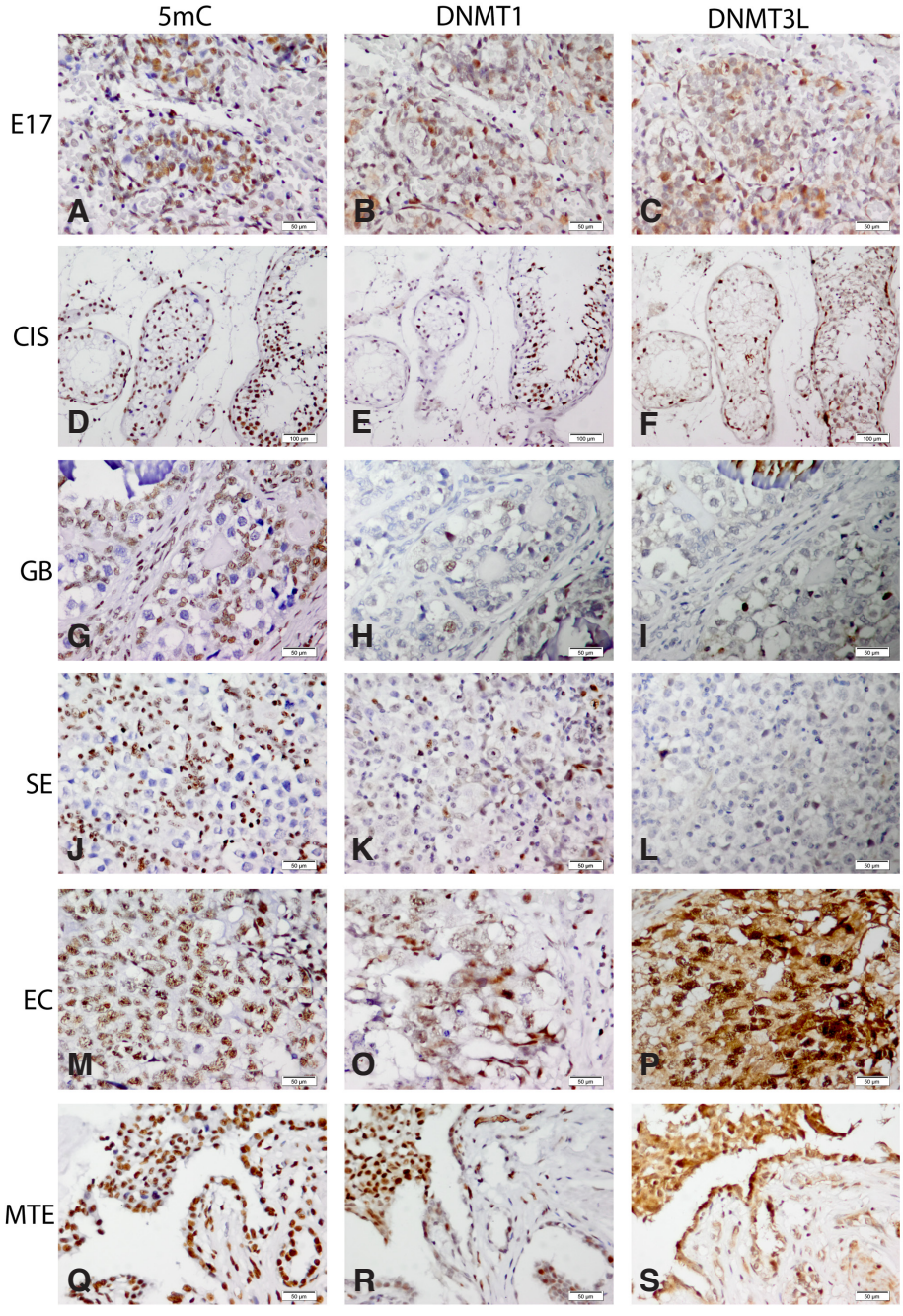

Fig. 2. Immunohistochemical staining for 5mC (left column), DNMT1 (middle column) and DNMT3L (right column) on an embryonic testis, at the 17th week of development (E17) (first lane), the different GCC and their precursor lesions (2nd-6th lane). *In the embryonic testis the germ cells are negative for 5mC* **(A)**, *negative to weak positive for DNMT1* **(B)** *and DNMT3L; Carcinoma* in situ *cells are negative for 5mC* **(D)**, *positive for DNMT1* **(E)** *and positive for DNMT3L* **(F)**; *Gonadoblastoma stains negative for 5mC* **(G)**, *DNMT3L* **(I)**, *and negative to weak positive for DNMT1* **(H)**; *Seminoma/Dysgerminoma (SE/DG) stain negative for 5mC* **(J)**, *DNMT1* **(K)** *and DNMT3L* **(L)**; *Embryonal Carcinoma (EC) shows a heterogeneous pattern for both 5mC* **(M)**, *DNMT1* **(O)** *and positive for DNMT3L* **(P)**; *Mature Teratoma (MTE) stains positive for all markers* **(Q,R,S)**. *All slides are counterstained with hematoxylin. Magnification 200x for all but 100x for CIS.*

were observed in human gonocytes present within sex cords in foetal testes and corresponds to migrating primordial germ cell in mice (Almstrup *et al.*, 2010). CIS cells therefore have a permissive and foetal-like chromatin structure, which is associated with high transcriptional and proliferative activity.

Ohinata *et al.,* showed that Blimp1, a known transcriptional repressor, has a critical role in the development of the mouse germ cell lineage, as disruption of Blimp1 causes a block early in the process of PGC formation. Blimp1-deficient mutant embryos form a tight cluster of about 20 PGC-like cells, which fail to show the characteristic migration, proliferation and consistent repression of homeobox genes that normally accompany specification of PGC (Ohinata *et al.*, 2005). BLIMP1 and PRMT5 were expressed, and dimethylation of histones H2A and H4 was detected in human male gonocytes at weeks 12–19 of gestation, indicating a role of this mechanism in human fetal germ cell development (Eckert *et al.*, 2008). In addition, this study also showed that BLIMP1/PRMT5 and histone H2A and H4 arginine 3 dimethylation are present in CIS and most seminomas, and less in EC and other nonseminomas. Recently, Schuster-Böckler and Lehner showed that chromatin organization has a major influence on regional mutation rates (Schuster-Bockler and Lehner, 2012). Mutation rates were positively correlated with heterochromatin related marks, of which histone modification H3K9Me3 was most important, and accounted for more than 40% of the somatic mutation variants (Schuster-Bockler and Lehner, 2012). The open chromatin in regions involved in embryonic development showed a negative association, which is in line with the fact that mutations are rarely found in GCC and the idea that the germ line is protected for mutations (immortal strand). In fact it points towards a regulatory role for histone modifications and chromatin structure on germline mutation rates, as the chromatin organization in the germline is substantially different to that in somatic cells (Hajkova *et al.*, 2002).

Histone modification in GCC cell lines

Relatively little is known about histone modifications involved in GCC. We therefore initiated a study to explore the epigenetic differences between GCC subtypes, using the cell lines TCam-2 and NCCIT as representatives of seminomas and non-seminomas respectively (van der Zwan, in preparation). As depicted in Fig. 3, our initial analysis matched the classification of the cell-lines. SOX17 was strongly enriched for H3K4me1 and H3K27ac in TCam-2 compared to NCCIT cells, whereas the opposite pattern was observed for SOX2. Additional analysis of the epigenetic differences between the two cell lines, along with the extension of these studies to cancer tissues, will contribute to our understanding of the role of histone modifications in GCC.

Environmental factors in the pathogenesis of GCC

Environmental factors can influence epigenetic processes, and may therefore be related to cancer development (Brait *et al.*, 2009, Lang-Mladek *et al.*, 2010). There are indications that endogenous factors such as the hormonal balance between estrogens and androgens might play a role (Godmann *et al.*, 2009). It is known that diethylstilbestrol (DES), used widely to reduce the risk of abortions, led to a higher risk of hypospadias, cryptorchidism and poor semen quality in male offspring (Sharpe and Skakkebaek, 1993). The use of DES was abandoned after it was associated with cervical cancer (Herbst *et al.*, 1971). It was shown that DES exposure in the third generation still leads to an increased risk of hypospadias(Brouwers *et al.*, 2006). The risk of GCC development in this patient group is controversial, and a slightly increased risk has been reported (Strohsnitter *et al.*, 2001).The transgenerational effect of DES is

Fig. 3. Display of H3K4me1 and H3K27ac tracks for both NCCIT and TCam-2. *The red box indicates a repressive state and the green box an active state. Figure derived from the UCSC Genome Browser.* **(A)** *Genomic region around the SOX17 gene, showing strong enrichment for both H3K4me1 and H3K27ac in TCam-2 (representative of seminoma) vs NCCIT (representative of Non-seminoma) cells. Enrichment outside this region is likely indicating regulatory elements such as enhancers. SOX17 is used as a diagnostic marker for differentiating seminoma from non-seminoma.* **(B)** *Genomic region around the SOX2 gene, showing strong enrichment for both H3K4me1 and H3K27ac in TCam-2 vs NCCIT cells. SOX2 is used as a diagnostic marker for differentiating non-seminoma from seminoma.*

however suggestive for heritable epigenetic alterations. In addition, exogenous estrogen may interrupt the maturation of primitive germ cells by reducing the secretion of Anti- Müllerian hormone from the Sertoli cells (Longnecker *et al.*, 2002). Hormonal factors can also induce methylation of promoter regions of certain genes (Kutanzi *et al.*, 2010).

Major geographic differences in incidence are consistent with environmental influences. Increased use of endocrine disruptors has been suggested to be one of the environmental factors responsible for the increasing incidence of testicular GCC in testicular dysgenesis syndrome (TDS) (Skakkebaek *et al.*, 2001). A meta–analysis confirmed the link between estrogen exposure and GCC (Storgaard *et al.*, 2006). Developmental disturbances of the micro-environment could result in inadequate maturation of the germ cells. This may result in a foetal epigenetic profile which, upon hormone stimulation during puberty, leads to an aberrant induction of transcription and proliferation, ultimately leading to GCC later in life (Almstrup *et al.*, 2010).

In this context, patients with Disorders of Sex Development (DSD) form an intriguing model to study the impact of intrinsic and environmental factors on normal and abnormal gonadal development. Indeed the diagnosis of 46, XY and chromosomal DSD is a risk factor for the development of GCC, with higher risk associated with an earlier block in differentiation. Other risk factors include anatomical position of the gonad, the presence of Y-chromosomal material (GBY region), genetic and epigenetic anomalies (Cools *et al.*, 2006, Cools *et al.*, 2011).

It has been shown that seminomas are more often found in abdominal testes compared to scrotal testes (Ogunbiyi *et al.*, 1996). This might also explain the occurrence of dysgerminomas in the ovary and dysgenetic gonads, which are both located in the abdomen. Indeed, seminoma and dysgerminomas are similar in morphology and gene expression profile, and therefore might have the same epigenetic profiles (Dietl *et al.*, 1993, Looijenga *et al.*, 2006, Susnerwala *et al.*, 1991). Bens *et al.,* showed that *HOXA5* represents a candidate gene of androgen-mediated promoter methylation, by studying patients with Complete or Partial Androgen Insensitivity Syndrome (CAIS and PAIS respectively) (Bens *et al.*, 2011). *HOXA5* was significantly hypermethylated in CAIS patients compared to normal male controls, whereas PAIS patients could be both hyper- or hypo methylated. This suggests that *HOXA5* promoter methylation is at least partly controlled by androgen receptor activity and could possibly explain PAIS heterogeneity. In addition, TSPY was found to be a repressor for androgen signaling due to entrapping of the cytosolic androgen receptor, even in the presence of androgens. Androgen treatment stimulated cell proliferation and TSPY expression was found to be reduced in more malignant GCC (Akimoto *et al.*, 2010). Together, these results underline the theory that the androgen-estrogen balance is important in the etiology of GCC, in both AIS patients as well as 'healthy' men.

Perspectives and concluding remarks

Next generation sequencing has made it possible to study epigenetic processes in a genome-wide matter. Mapping and integration of these data with information from genetic and protein experiments will lead to a broader knowledge of cancer initiation and progression. Fig. 4 shows a pathogenetic model for GCC based on the different aspects discussed in this review. Genetic, epigenetic and environmental factors ('genviroment') play essential roles in normal gonadal development. After fertilization the PGC completely erase their biparental genomic imprint. Gonocytes undergo different epigenetic modifications (i.e. increased methylation and more condensed chromatin structure) during their differentiation along the male (testis) or female (ovary) pathway. The process of testis formation is referred as 'testicularization' of the gonad. The micro-environment of the PGC is essential for physiological maturation, and disruption of this process may lead to delayed maturation and possibly malignant transformation. In this respect, studies of DSD patients have increased our knowledge. Genetic factors, epigenetic aberrant reprogramming end/or environmental factors, referred to genvironmental parameters, can block the PGC in a fetal-like state, allowing proliferation. This block in maturation of the PGCs or gonocytes can therefore initiate a pathogenetic pathway, leading to the precursor lesions GB or CIS that can eventually progress to

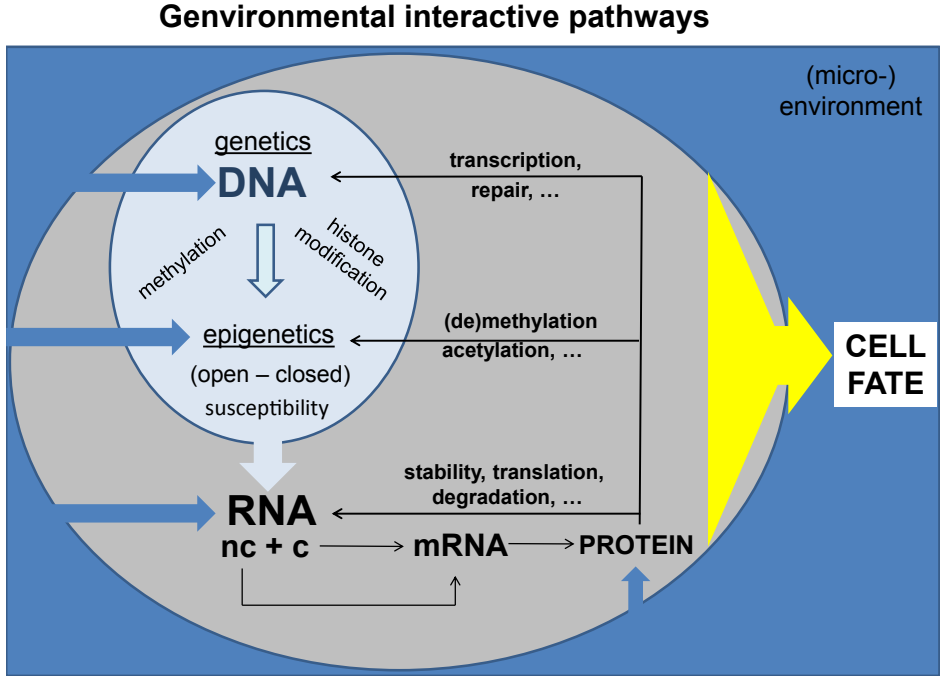

Fig. 4. Final cell fate depends on interaction of a large number of parameters, including genetics, epigenetics, transcription and translation, in interaction with the (micro)environment. *This is referred to as Genvironment. At the genetic level, DNA functionality can be influenced by various mechanisms, for instance transcription or repair. Epigenetics (*i.e. *DNA methylation and histone modifications) will determine chromatin structure, and as such susceptibility of the genome for transcription. The process of gene transcription and translation is dependent on these parameters, the presence of appropriate transcription factors, as well as stability, translation and degradation of enzymes, RNA and protein. In fact, (micro)environmental factors can impact on all these levels.*

invasive GCC. Additional research may allow epigenetic profiles to identify risk groups, predict clinical outcomes and allow the development of targeted therapies in patients with high risk for development of GCC.

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