DNA Methylation in Paediatric Germ Cell Tumours

UNITED KINGDOM • CHINA • MALAYSIA

Dzul Azri Mohamed Noor, BPharm, MPharm

Thesis submitted to the University of Nottingham

for the degree of Doctor of Philosophy

July 2013

PAGE MISSING IN ORIGINAL

Abstract

Germ cell tumours (GCTs) affect both paediatric and adult populations, and can occur either in gonadal or extragonadal regions along the body's ventral midline. These tumours can be broadly categorized into two subgroups, seminomatous (SEM) or non-seminomatous (N-SEM). The latter can be further subcategorized into embryonal carcinoma (EC), teratoma, yolk sac tumour (YST) and choriocarcinoma (CC) according to their differentiation. As in many other tumours, DNA methylation has been proposed to be involved in GCT development. However to date, most studies were performed using adult testicular GCTs. Furthermore, these studies only include a handful of genes in their analysis. Thus, the roles of DNA methylation in paediatric and extragonadal GCTs have not been explored. Therefore, this project attempted to fill this gap in knowledge by performing methylation analysis in a cohort of paediatric GCT samples and GCT cell lines.

Although paediatric GCTs mostly consist of teratomas, seminomas or YSTs, only the latter two were included in the methylation analysis as they were the only samples in the available tumour bank. Using the methylation level of LINE-1 repeat elements as a measurement of global genome methylation, we found that both paediatric seminoma and YST samples displayed global hypomethylation as compared to somatic controls. However, when methylation at gene promoter regions was investigated using Illumina Golden Gate methylation arrays, seminoma and YST exhibited very different methylation features. YSTs were found to be highly methylated at many of the sites investigated. Surprisingly, we found that the methylation features in seminoma were similar to the somatic controls. From this analysis, we identified 85 genes that were differentially methylated in the YSTs. However, by correlating our methylation data with the expression array data performed by our collaborators on the same samples, only eight of these genes (*PYCARD, CASP8, CD2, HDAC9, TFAP2C, ETV1, EVI2A, HLA-F*) were differentially expressed.

As in previous GCT studies, our analysis was focused on the methylation at CpG islands. During the course of this project technological advancement led to the

creation of new methylation arrays that offer wider genome coverage. One example is the Infinium Methylation 450K array that covers more than 450,000 CpG sites and includes regions flanking the CpG islands such as the CpG shores and CpG shelves. Since no previous GCT studies have attempted to investigate methylation in those regions, we utilized this methylation array on four GCT cell lines; TCAM2 (seminoma), NT2D1 (teratocarcinoma), GCT27 (embryonal carcinoma) and GCT44 (yolk sac tumour). Similar to previous GCT studies, we found that nonseminomatous GCT cell lines displayed higher methylation at the CpG islands as compared to the seminoma cell lines. Strikingly, expanding our analysis to other regions (CpG shores and shelves etc.) revealed that each GCT subtype exhibited distinct methylation features. Both EC and teratoma cell lines displayed higher methylation than the seminoma and YST cell lines at all regions. Interestingly, the YST cell line only showed higher methylation than the seminoma cell line at the CpG islands and to a lesser extent at the CpG shores while the seminoma cell line exhibited higher methylation at the CpG shelves as compared to the YST cell line. This is the first time such features have been reported for GCTs. From this Infinium methylation data, we have also identified a high number of hypermethylated genes including those that are uniquely methylated for each cell line. By correlating this methylation data with Affymetrix gene expression data, 98 genes that were differentially methylated and differentially expressed in the YST cell line have been identified. However, further analysis needs to be performed to understand the role of these genes in YST development.

As in other types of tumour, the hypermethylation observed in the YST cell line might be caused by many epigenetic modifiers. Using real-time RT-PCR on three epigenetic modifiers (*DNMT3B*, *EZH2*, *SUZ12*), we found that *DNMT3B* was highly expressed in the YST samples and cell line as compared to the seminoma samples and cell line. This suggests that *DNMT3B* might contribute to YST hypermethylation and resulting differences in their biology. However, knockdown of DNA methyltransferases (DNMTs) and *DNMT3B* using 5-azadeoxycytidine and microRNA-29b respectively, did not seem to have any effect on the response of all four GCT cell lines towards cisplatin. On the other hand, both knockdowns only caused little effect on cell migration;

affecting only the seminoma and YST cell lines. Nonetheless, further analysis is still needed to fully assess the role of DNA methylation in regulating cell behaviour.

In summary, paediatric YSTs displayed hypermethylation at many promoter regions as compared to seminomas. Meanwhile, methylation analysis at regions outside of CpG islands in GCT cell lines revealed unique methylation features for each GCT subtype which might indicate different underlying mechanisms in their development. Further analysis on genes found to be differentially methylated and differentially expressed in both paediatric and GCT cell lines are now needed to fully establish their role in GCT development.

Acknowledgement

First of all, I would like to praise Allah S.W.T. for all His help and guidance throughout my journey as a PhD student. Indeed, all this may not be possible without His help.

I would also like to extend my gratitude to my supervisor, Dr. Paul Scotting, for having me in his lab and for his faith in me to take this project. In addition, my great thanks as well for all his relentless effort in helping and guiding me throughout this process. My great thanks also go to Dr. Jennie Jeyapalan, for her help and patience in answering all my questions during her stay in the lab.

I would also like to take this opportunity to thank my family for all their support. To my parents and siblings, thank you for all your prayer and support. To my wife, thank you for all your effort in taking care of our family. To my lovely daughter, thank you for always be there to cheer up my day.

My thanks also go to all my current and previous lab members especially Peggy, Chris, Wallis, Dylan, Safiah and Michelle for all their input in improving my experiments. Last but not least, thank you to Magda, our lab technician, for putting up with my orders almost every day for the last 4 years.

Finally, I would like to thank the Ministry of Higher Education, Malaysia and University Science of Malaysia for sponsoring my study.

Table of Contents

List of Figures

List of Tables

Abbreviations

2-HG	2-hydroxyglutarate
5,6-dihydroAza	5,6-dihydroazacytidine
5-aza	5-azacytidine
5-azadC	5-azadeoxycytidine
5-hmC	5-hydroxymethylcytosine
5-mC	5-methylcytosine
Ado-Met	S-adenosyl-L-methionine
AID	activation-induced cytidine deaminase
ALL	Acute lymphoblastic leukemia
AML	acute myeloid leukemia
BASH	Beadarray Subversion of Highlight
BER	base excision repair
BLIMP 1	B-lymphocyte-induced maturation protein 1
BMP	bone morphogenetic signaling
CC	Choriocarcinoma
CCLG	Children Cancer Leukemia's Group
CDA	cytidine deaminase
CDH13	E-cadherin
CDX1	Caudal-related homeobox protein 1
CIMP	CpG island methylator phenotype
CIS	carcinoma in situ
COBRA	combination of bisulfite and restriction enzyme analysis
CRC	colorectal cancer
CTA	cancer testis antigen
DCK	deoxycytidine kinase
DMD	differentially methylated domain
DMEM	Dulbecco's Modified Eagle Medium
DNMT	DNA methyltransferase
EC	Embryonal Carcinoma
ES	embryonic stem
ETV1	ETS variant 1
EVI2A	ecotropic viral integration site 2a
EZH2	Enhancer of Zeste homolog 2
FBS	fetal bovine serum
FDA	Food and Drug Administration
GCT	Germ Cell Tumour
GNAT	GCN5-related N-acetyltransferase
H3K27	lysine 27 on histone H3
H3K27me3	trimethylation of lysine 27 on histone H3

H3K4	lysine 4 on histone H3
H3K4me3	trimethylation of lysine 4 on histone H3
H3K79	lysine 79 on histone H3
H3K9me	methylation of lysine 9 on histone H3
H3K9me3	trimethylation of lysine 9 on histone H3
H4K20	lysine 20 on histone H4
HDAC	Histone deacetylases
HDAC9	Histone deacetylase 9
HDM	histone demethylase
HERV	human endogenous retrotransposon
HLA-F	Major histocompatibility complex class 1 F
HMT	histone methyltransferases
HNSCC	head and neck squamous cell carcinoma
HPRT	hypoxanthine phosphoribosyltransferase
IAP	intracisternal A particle
ICF	Immunodeficiency, centromeric instability and facial anomaly syndrome
ICR	imprinting control region
IDH	isocitrate dehydydrogenase
IFITM1	interferon-induced transmembrane protein 1
ITGCNU	intratubular germ cell neoplasia, unclassified
JmjC	Jumonji C domains
L1	LINE-1
L1-ASP	L1 antisense promoter
LINE	long interspersed nuclear element
LOH	loss of heterozygosity
LTR	long terminal repeat
MBD	methyl-CpG binding domain
MBP	methyl-binding proteins
MDS	myelodysplastic syndrome
MeDIP	methylated DNA immunoprecipitation
mir-29b	microRNA-29b
MMR	mismatch repair
MSP	methylation specific PCR
ncRNA	non-coding RNA
non-LTR	non-long terminal repeat
NSC	neural stem cells
NSCLC	non-small cell lung carcinoma
N-SEM	Non-seminomatous
O/E	observed/expected ratio
ORF	open reading frames
PARP12	poly (ADP-ribose) polymerase family, member 12
PCA	principal component analysis

PCAF	p300/CBP-associated factor
PCNA	proliferating cell nuclear antigen
PFA	paraformalaldehyde
PGC	Primordial germ cells
PGK	phosphoglycerate kinase
PYCARD	PYD (Pyrin) and CARD containing domain
RB	retinoblastoma gene
RIN	RNA integrity number
RLGS	restriction landmark genomic screening
RT	Reverse-Transcriptase
SCC	squamus cell lung carcinoma
SCT	saccrococcygeal tumours
SINE	short interspersed nuclear element
STAMP	Sequence Tag Analysis of Methylation Patterns
SUZ12	Suppressor of Zeste 12 homolog
TDS	testicular dysgenesis syndromes
TED	Trypsin-EDTA
TET	ten-eleven translocation
TFAP2C	Transcription factor AP-2 gamma
TGCT	testicular germ cell tumour
TPRT	target-primed reverse transcription
TSG	tumour suppressor genes
TSS	transcription start site
UCSC	University of California, Santa Cruz
UTR	untranslated region
X_a	active X allele
XCi	X-chromosome inactivation
X_i	inactive X allele
XIC	X-inactivation center
YST	Yolk sac tumour
α-KG	α-ketoglutarate

1. Introduction

DNA methylation is an epigenetic mechanism known to play a major role in normal development such as in establishing genomic imprinting, X-chromosome inactivation and retrotransposon silencing. However, many cancer studies have revealed simultaneous presence of global hypomethylation and gene promoter hypermethylation. This suggests that DNA methylation might also have a role in tumour development.

Germ cell tumours (GCTs) are a rare tumour that can affect both children and adults, and occur in both gonadal and extragonadal regions. Histologically they can be categorized into two large groups; seminomatous and non-seminomatous GCTs. In addition to that, non-seminomatous GCTs could be further subdivided into four subtypes according to their differentiation; teratoma, embryonal carcinoma, yolk sac tumour and choriocarcinoma. Despite many studies conducted, the aetiology of GCTs is still not fully understood.

As in other tumours, methylation studies performed in GCTs also revealed similar global hypomethylation and gene promoter hypermethylation, with non-seminomatous GCTs exhibited higher degree of hypermethylation than the seminomatous GCTs. This implies DNA methylation involvement in GCT development. However, these studies were quite restricted in terms of samples, which consisted only of adult testicular GCTs, and the few genes that have been analysed. Therefore, the role of DNA methylation in the paediatric population and extragonadal GCTs remains to be determined. In this thesis I attempt to begin to fill in this gap in our knowledge.

1.1. Epigenetics

There is still no clear definition of the term 'epigenetics', but most researchers would agree it involves heritable changes in gene expression and chromatin organization without altering the DNA sequence itself (Jiang et al., 2008, Sawan et al., 2008). The two most well studied epigenetic processes are DNA methylation and histone modification. At the moment only DNA methylation appears to fulfil the epigenetic definition in terms of being heritable. The inclusion of histone modification as an epigenetic process is still questionable as at the moment only methylation of

lysine at histone H3 has been found to be heritable (Hansen and Helin, 2009). For the purpose of this thesis, most of the content will revolve around DNA methylation. However, since the histone modifications have also been linked with DNA methylation in regulating gene expression, it will also be briefly discussed here.

1.1.1. Histone Modifications

Histone modifications refer to modifications at the N-terminal tail of the core histones (H1, H2, H3 and H4) in the nucleosome. These modifications could be methylation, acetylation, phosphorylation, ubiquitylation and many others (Margueron and Reinberg, 2010) (Figure 1.1). These modifications will then either cause 'loosening' or 'tightening' of the chromatin by modulating nucleosome contacts to each other or the modifications could also recruit other proteins which have enzymatic activities that will further modify the chromatin. Two of the best studied histone modifications are acetylation and methylation, both of which will be briefly discussed here.

Acetylation of a histone's lysine residues is believed to neutralize the residue's positive charge, prevent chromatin compaction and reduce the interaction between histone tails and the DNA (Shahbazian and Grunstein, 2007). This leads to more open chromatin and promotes gene transcription. Indeed, histone acetylation can often be found at the transcribed region of active genes and has been associated with gene activation (Wang et al., 2008). This dynamic process is governed by two type of enzymes; histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs act by transferring an acetyl group from acetyl-CoA onto the ε-amino group of the lysine residues of the histone tail. Based on sequence similarities, there are three large families of HATs. These are GCN5-related N-acetyltransferase (GNAT), p300/CBP-associated factor (PCAF) and the MYST family. Wang *et al.*(2008) in their histone profiling in human $CD4^+$ T cells identified three patterns of histone acetylation. They could be highly elevated at the transcription start site (TSS) but low at the transcribed regions. The second pattern showed similar elevation at the TSS but gradually decreasing across the transcribed regions and lastly both TSS and the transcribed regions showed elevation of acetylation. The authors suggested that the first pattern was involved in transcription initiation while the last two were involved in transcription

elongation, since the last two patterns depended upon PCAF that is usually associated with the elongating form of RNA PolII.

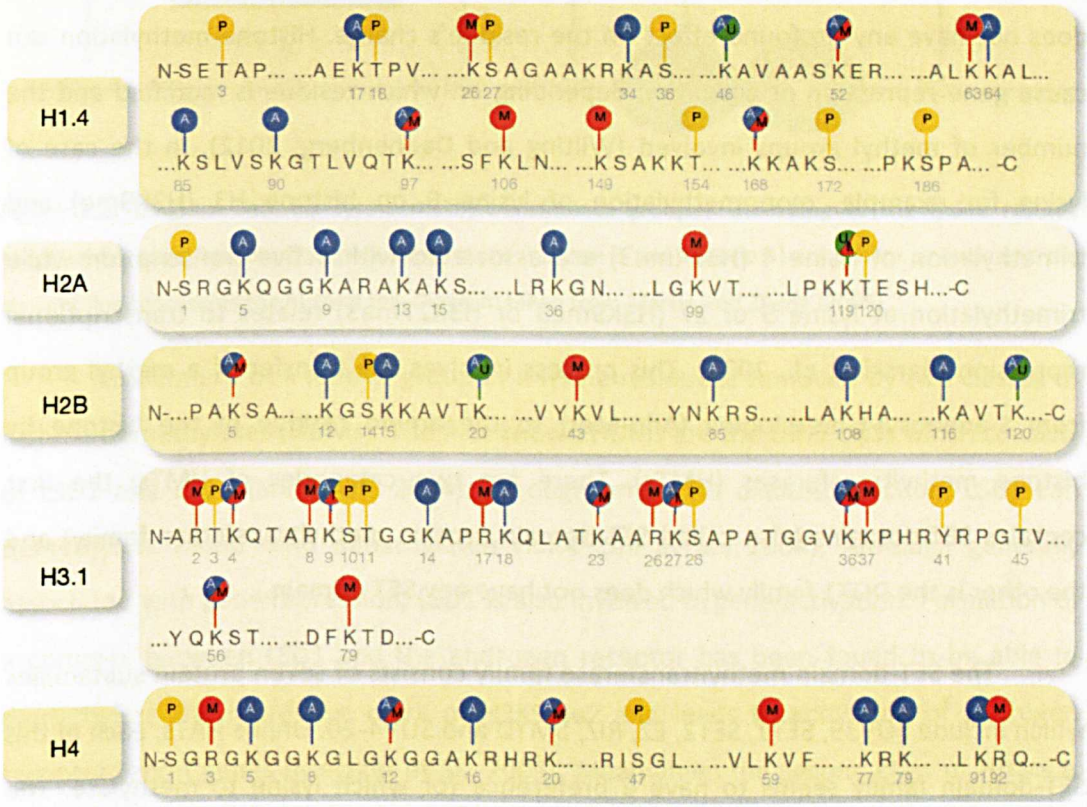


Figure 1.1: Diagram showing the modification at the histone cores. These modifications could be methylation (M), acetylation (A), phosphorylation (P), ubiquitination (U). Diagrams was taken from Portela and Esteller (2010).

On the other hand, HDACs remove the acetyl marks from histones and restore lysine's positive charge causing condensation of the chromation which leads to transcriptional inactivation. Again based on the sequence homology, HDACs can be categorized into four classes; HDAC class I (HDAC 1, 2, 3 and 8), HDAC class II (HDAC 4, 5, 6, 7, 9, 10), HDAC class III (also referred to as sirtuins) and HDAC class IV (HDAC 11). It is still not clear if each HDAC has specific substrates to bind to as most of the time they act in the form of complexes. For example HDAC1 and HDAC2 form a complex with NuRD, SIN3A (Peinado et al., 2004) or Co-REST (You et al., 2001) while HDAC3 and HDAC4 form a complex with SMRT/N-CoR for gene repression (Fischle et al., 2002).

Histone methylation can occur either at lysine or arginine residues. For the purpose of this thesis only methylation at the lysine will be covered since they are among the most widely studied modification. Unlike acetylation, histone methylation does not have any profound effect on the residue's charge. Histone methylation can cause gene repression or activation depending on which residue is modified and the number of methyl groups involved (Wilting and Dannenberg, 2012). In the case of lysine for example, monomethylation of lysine 9 on histone H3 (H3K9me) and trimethylation of lysine 4 (H3K4me3) are associated with active transcription while trimethylation at lysine 9 or 27 (H3K9me3 or H3K27me3) relates to transcriptional repression (Barski et al., 2007). This process involves the transfer of a methyl group from S-adenosyl-L-methionine (Ado-Met) to the amino residue of the histone by histone methyltransferases (HMTs). There are two categories of HMTs; the first contains SET-domains (also called SET-domain protein methyltransferase family) and the other is the DOT1 family which does not have any SET domain.

The SET-domain methyltransferase family consists of seven protein subfamilies which include SUV39, SET1, SET2, EZ, RIZ, SMYD and SUV4-20. Unlike HATs, each of this SET-domain family seems to have a preference for which lysine to methylate. The human SUV39h1 from SUV39 family for instance was found to be H3K9 specific (Rea et al., 2000) while the SET1 family methylates lysine 4 on histone H3 (H3K4). On the other hand, EZH2 from the EZ family methylates lysine 27 on histone 3 (H3K27) and the SUV4-20 family specifically methylates lysine 20 on histone 4 (H4K20). In contrast, the DOT1L family, which does not have any SET domain, was found to selectively methylate lysine 79 on histone 3 (H3K79). Other histone methyltransferase family members and their target residues are illustrated in Figure 1.2.

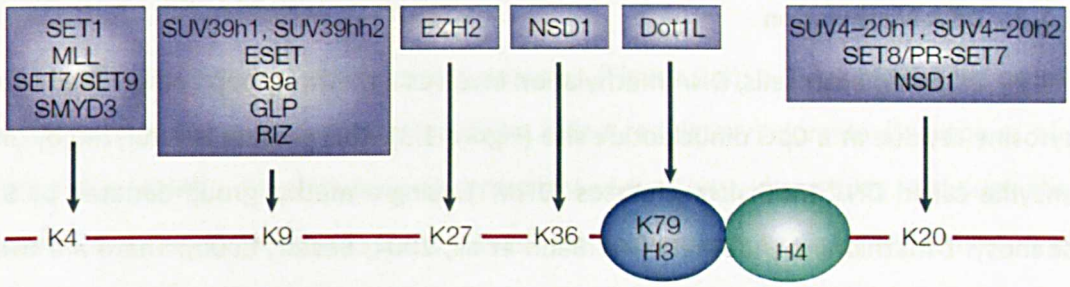


Figure 1.2: Diagram showing lysine methyltransferases grouped according to the lysine residue targeted for modification. The globular domains (histone 3 and histone 4) are shown as ovals and the straight line represents the histone tail. Diagram taken from Martin and Zhang (2005)

Attachment of a methyl group at a lysine residue is removed by two classes of histone demethylases (HDMs). The first known HDMs are the LSD1 class which consists of LSD1 and LSD2 (Shi et al., 2004). Through an amine oxidase reaction, LSD1 can demethylate mono- and dimethylated H3K4 (Shi et al., 2004). Although generally associated with gene repression, LSD1 is also involved in gene activation. Formation of a complex between LSD1 and the androgen receptor has been found to be able to demethylate the repressive mark of H3K9me2 and leads to activation of androgen receptor target genes (Metzger et al., 2005). Meanwhile, the second class of HDMs is proteins that contain Jumonji C domains (JmjC). Currently, 27 different JmjC domain proteins have been identified in the human genome (Agger et al., 2008). Unlike LSD1, they can also demethylate trimethylated H3K4 in addition to mono- and dimethylated H3K4 (Christensen et al., 2007). Methylation at other lysine positions has also been demonstrated; for example the UTX member of JmjC was observed to be able to demethylate di- and trimethylated H3K27 (Lee et al., 2007).

1.1.2. DNA Methylation

In mammalian cells, DNA methylation involves a methyl group being added to a cytosine residue at a CpG dinucleotide site (Figure 1.3). This process is catalyzed by an enzyme called DNA Methyltransferases (DNMT) using a methyl group donated by S-adenosyl-L-methionine (AdoMet) (Hermann et al., 2004, Bestor, 2000). There are five known types of DNMTs, namely DNMT1, 2, 3a, 3b and 3L, which are briefly discussed below.

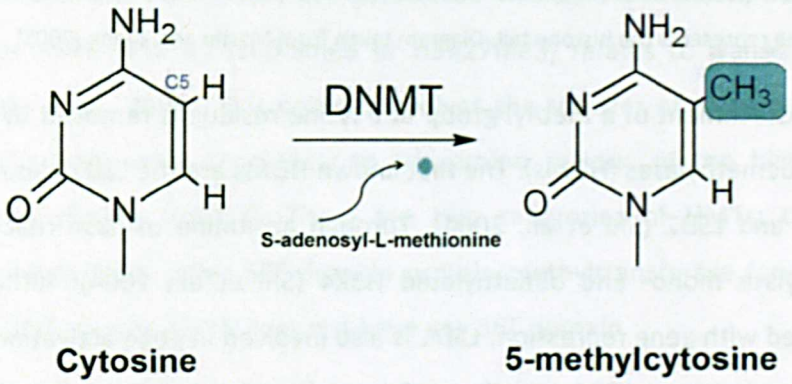


Figure 1.3: Diagram showing the process of adding a methyl-group to a cytosine base by a DNA methyltransferase (DNMT). S-adenosyl-L-methionine acts as the methyl-donor in this process. Diagram was taken from Daniel *et al.*(2011).

In normal genomes, CpG methylation is found to be distributed in a unique pattern whereby some sites display high CpG methylation while others show low CpG methylation. Sites with high CpG methylation correspond to pericentromeric regions, imprinted genes and genes on the inactive X chromosome while gene promoter regions are usually hypomethylated (Daniel et al., 2011). The function of CpG methylation on these sites will be discussed further in this chapter.Recently, methylation at sites other than a CpG has been reported in mouse and human embryonic stem cells, but this non-CpG methylation level was found to be low and variable (Ziller et al., 2011, Dodge et al., 2002, Ramsahoye et al., 2000).

1.1.2.1. *DNA Methyltransferases (DNMTs)*

DNMTs contain two important domains (Figure 1.4); a C-terminal (also called catalytic) domain and an N-terminal (also called regulatory) domain (Margot et al., 2003). For DNMT1, these two domains are linked by GlyLys dipeptides. Although the DNMT1 C-terminal domain contains a motif for active methyltransferase activity, it is not capable of any enzymatic activity on its own (Margot et al., 2000) but needs to be activated by the N-terminal domain (Margot et al., 2003). Abundant DNMT1 is targeted to replication foci (Daniel et al., 2011) and this is believed to be due to the presence of a DNA binding region in its N-terminal domain (Leonhardt et al., 1992). In addition to this, a preference of DNMT1 towards hemimethylated CpGs suggests its involvement in maintaining the DNA methylation pattern (Bacolla et al., 2001). Furthermore, the presence of a proliferating cell nuclear antigen (PCNA) binding domain (Leonhardt et al., 1992) and polybromo homology domain in DNMT1 (Liu et al., 1998) (Figure1.4) implies that it has the ability to bind to newly replicated DNA, thus supporting its role in maintaining methylation patterns. Mutation of *Dnmt1* in ES cells produced cells with a severely demethylated genome (Li et al., 1992) with high frequencies of deletions and rearrangements (Chen et al., 1998). This mutation also proved to be lethal in mouse embryos which showed biallelic expression of imprinted genes and activation of Xist gene on X chromosomes (Beard et al., 1995, Li et al., 1992).

Unlike DNMT1, both DNMT3A and DNMT3B show a lack of interaction between the C-terminal and the N-terminal domains with the C-terminal domains being catalytically active alone (Gowher and Jeltsch, 2002, Margot et al., 2003). Meanwhile, a PWWP domain in the N-terminal domain has been shown to be essential for DNMT3A and DNMT3B localization and directing methylation at major satellite repeats in the pericentromeric region (Chen et al., 2004). Both DNMT3A and DNMT3B also showed no preference towards hemimethylated CpGs and are scattered throughout the nucleus (Daniel et al., 2011, Margot et al., 2001). This has earned them the title of 'de novo methyltransferases' believed to function in setting up methylation patterns in previously unmethylated DNA. However, such titles are not exclusive as *in vitro*analysis

shows that DNMT1 can also act as a de novo methyltransferase (Hsieh, 2005).Homozygous mutation of either *Dnmt3a* or *Dnmt3b* proved to be fatal in mice (Okano et al., 1999) while heterozygous mutation of the C-terminal domain of DNMT3B resulted in demethylation of satellite sequences and has been linked to the development of immunodeficiency, centromeric instability and facial anomaly (ICF) syndromes (Xu et al., 1999).

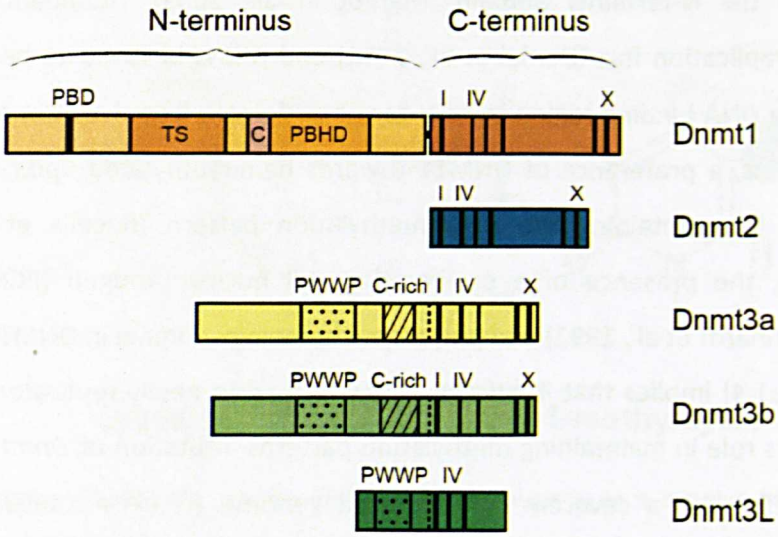


Figure1.4: Structure of DNA methyltransferases. Among the domains and motifs found at the N-terminus of DNMT1 are PCNA binding domain (PBD), a targeting sequence (TS), a cysteine-rich region (C), and a polybromo homology domain (PBHD). Among the shared motifs in DNMT1 and DNMT3 are tryptophan-rich region (PWWP) and another cysteine-rich region (C-rich). At the C-terminus, all the highly conserved motifs are represented by a vertical bars although here only motifs I, IV and X are labelled. Diagram was taken from Margot *et al.*(2003).

Unlike the DNMTs described above, DNMT2 does not show any enzymatic activity even though it has an apparent active methyltransferase motif in its C-terminal domain (Margot et al., 2003). In line with this, genome methylation was found to be unaffected in *Dnmt2* mutant mice (Okano et al., 1998). DNMT3L was also found to be catalytically inactive believed to result from the lack of a required domain for DNA methyltransferase activity (Cheng and Blumenthal, 2008). However unlike DNMT2,

Dnmt3l has been found to aid Dnmt3a in establishing methylation of maternally imprinted genes in mice (Hata et al., 2002, Kaneda et al., 2004).

1.1.3. Links between Histone modifications and DNA methylation

DNA methylation and histone repressive marks are commonly observed in compacted chromation while chromatin with unmethylated DNA usually displays a high level of acetylated histone, an active histone mark. This suggests an intricate link between these two epigenetic modifiers in gene regulation. However, which one acts as an initiator in this process is still unresolved.

DNA methylation can directly regulate gene transcription by resisting the binding of transcription factors (Mulero-Navarro et al., 2006, Kulak et al., 2012). However, links between DNA methylation and histone modification can occur mediated by methyl-binding proteins (MBPs). MBPs are characterized by a methyl-CpG binding domain (MBD) and all MBP members (MBD1-4, MeCP2) except MBD3 show preferential binding to methylated CpGs (Fraga et al., 2003). In contrast, another MBP called Kaiso does not have the same methyl-CpG binding domain but still preferentially binds to methylated CpGs via zinc finger domain recognition (Prokhortchouk et al., 2001).

MBD1 has been suggested to participate in dissemination of the H3K9 methylation histone modification in a new DNA strand by recruiting the H3K9 methyltransferase, SETDB1 (Sarraf and Stancheva, 2004). Only during DNA replication, they form a complex with the chromatin assembly factor, CAF_{p150}. This complex was further found to interact with PCNA, which is known to bind to DNMT1 during replication (Figure 1.5). It is believed that this process leads to heritably maintained H3K9 methylation marks on the new strand since knockdown of MBD1 using siRNA resulted in loss of H3K9 methylation at multiple loci.

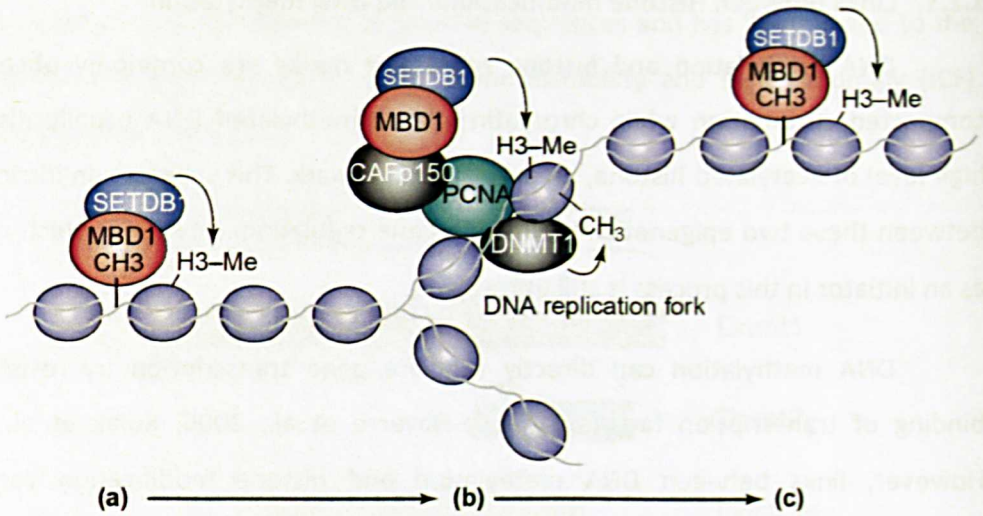


Figure 1.5: Maintenance of histone methylation mark by MBD. a) MBD1 recruits SETDB1 and established H3K9 methylation marks, b) during replication phase, DNMT1 forms a complex with proliferating cell nuclear antigen (PCNA) at the replication fork. At the same time, MBD1 and SETDB1 form a complex with CAF_{p150} and facilitates the establishment of H3K9 marks at the newly replicated methylated DNA, c) newly establish H3K9 methylation marks on the new DNA strand. Figures taken from Klose and Bird (2006)

Another MBP protein, MeCP2, has been associated with the histone methyltransferase that is specific to lysine 9 (H3K9) (Fuks et al., 2003). The differentially methylated domain (DMD) of the H19 gene, which is a MeCP2 regulated gene, was found to have a high level of H3K9 methylation when the MeCP2 level was high. However, in a Tet-Off MeCP2 cell line where the MeCP2 level was decreased by the addition of tetracycline, this reduction correlated with a low level of H3K9 methylation at H19 DMD. This suggests that H3K9 methylation at H19 DMD is regulated by MeCP2. Beside this, MeCP2 has also been found to associate with the mSin3A-HDAC co-repressor complex to repress *Bdnf* gene transcription (Chen et al., 2003).

On the other hand, Okamoto *et al.* (2004) observed that during the mouse embryo X-inactivation process, H3K27 and H3K9 methylation occurred at very early

stage even before de novo DNA methylation occured. Furthermore, in an experiment performed in *Neurospora crassa*, mutation of H3K9 by changing the lysine residue to leucine or arginine caused an abolishment of almost all DNA methylation (Tamaru and Selker, 2001). This suggests that histone modification might have some influence over DNA methylation.

Based on the finding that DNMT3L can interact with unmethylated H3K4 (Ooi et al., 2007), a model has been proposed for how H3K4 methylation might be involved in establishing DNA methylation in early development (Cedar and Bergman, 2009)(Figure 1.6). During this period, H3K4 methyltransferase is believed to be recruited by RNA Polymerase II, which is abundantly found at CpG islands. Therefore at this region, H3K4 residues are methylated while at other regions they are unmethylated. Meanwhile, *de novo* methylation was carried out by DNMT3A and DNMT3B which formed a complex with DNMT3L. Since DNMT3L can only bind to unmethylated H3K4, DNA methylation could not be established at the CpG islands where H3K4 is methylated. This could explain why CpG islands in normal tissues are generally unmethylated.

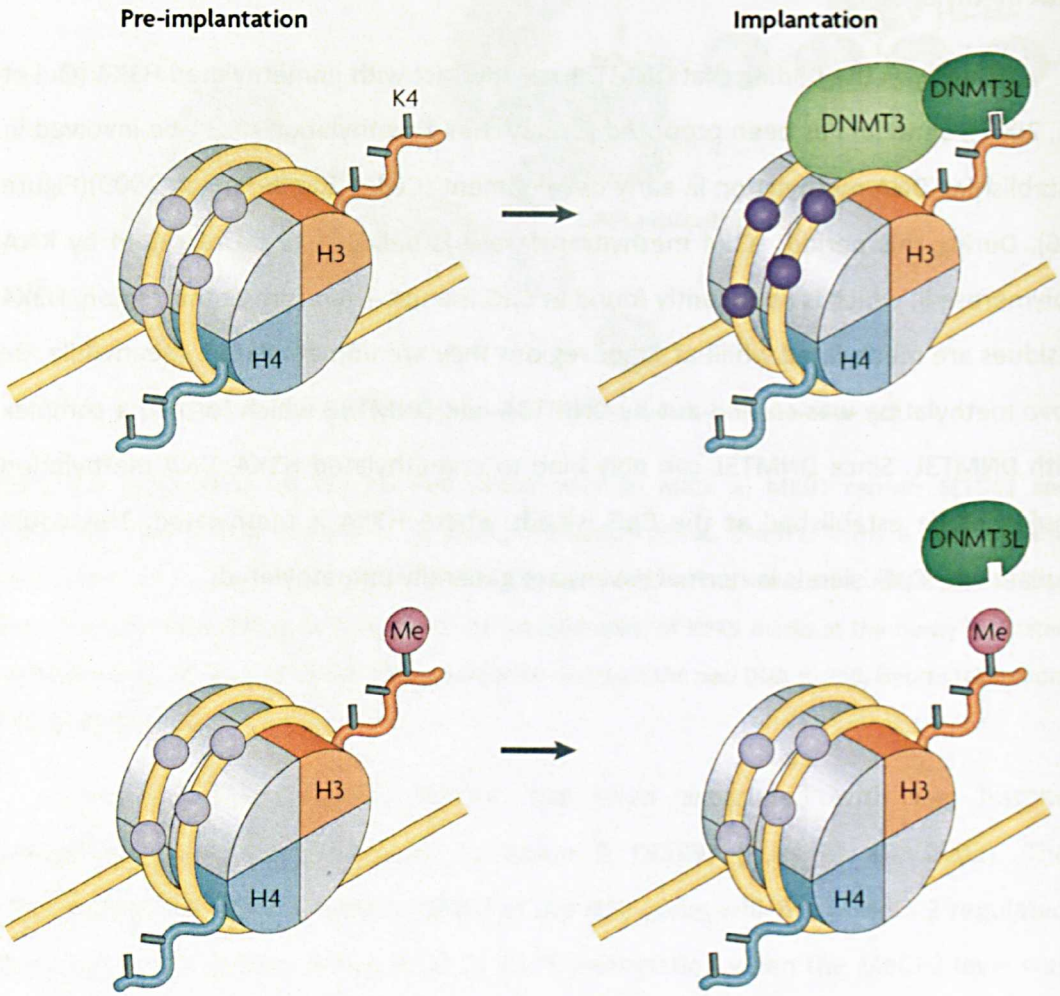


Figure 1.6: DNA methylation establishment through histone methylation during embryonic development. During pre-implantation, most CpGs are unmethylated (light purple circles). However, some regions are in the nucleosome that contains methylated lysine 4 at histone H3 (Me). During implantation, *de novo* methyltransferase (DNMT3) facilitated by its binding partner, DNMT3L can only bind to unmethylated lysine 4 resulting in methylated CpGs (dark purple circles). Meanwhile a DNMT3-DNMT3L complex could not bind to methylated lysine 4, thus leaving these CpGs as unmethylated. Diagram taken from Cedar and Bergman (2009)

1.1.4. DNA methylation in normal development

DNA methylation plays major roles during human development,among which genomic imprinting, X chromosome inactivation and repressing repetitive element transcription (McCabe et al., 2009) which will be briefly discuss here.

1.1.4.1. *Genomic Imprinting*

Genomic imprinting refers to a mechanism of gene regulation that results in parental-specific expression of these genes (Ferguson-Smith, 2011, Bartolomei, 2009). In other words, the affected genes will only be expressed from one allele (either maternal or paternal) while the other allele is silenced. Disruption of this process can lead to male infertility (Poplinski et al., 2010), neurodevelopmental disorders such as Prader-Willi and Angelman syndrome (Horsthemke and Wagstaff, 2008), growth disorders such as Beckwith-Wiedermann and Silver-Russell syndrome (Gicquel et al., 2005, Reik et al., 1995) and also has been implicated in tumour development (Kaneda and Feinberg, 2005).

Imprinted genes are usually located in a cluster that contains one non-coding RNA (ncRNA) and also an imprinting control region (ICR) (O'Neill, 2005). ICRs control the imprinting of genes in these clusters and these ICRs are usually methylated (Lucifero et al., 2002). Of all ICRs tested, all but one revealed a role for DNMT3A and DNMT3L in establishing methylation at these ICRs (Hata et al., 2002, Kaneda et al., 2004). Using crystallography, Jia *et al.* (2007) showed that the tetramer complex of DNMT3A and DNMT3L contains two active sites. These active sites were able to methylate two CG dinucleotides at once with a preference towards CG pairs that were eight to ten base pair apart. Since this feature is only observed in the maternally methylated imprinted loci but not in the paternally methylated imprinted loci, it is believed that both DNMT3A and DNMT3L are involved in establishing the imprinting at the maternal allele. Another mechanism that has been suggested for maternally methylated imprinted loci was by interaction between DNMT3L and the unmethylated lysine 4 of histone H3 which leads to recruitment of DNMT3A2 (Ooi et al., 2007). On the other hand, methylation of the paternally methylated *Rasgrf1* ICR involved *Dnmt3b*. This is believed to be due to the abundance of repetitive sequence

surrounding *Rasgrf1* (Kato et al., 2007). While the DNMT3 family has been recognized to establish imprinting, DNMT1 has been established to function in maintaining the imprinting (Li et al., 1993). However in preimplantation embryos, imprinting is believed to be maintained by a combination of oocyte-specific Dnmt1 (Dnmt1o) and Dnmt1 due to the low level of the latter during this period of time (Cirio et al., 2008).

1.1.4.2. *X-chromosome inactivation (XCi)*

X-chromosome inactivation (XCi) represents a gene dosage compensation to equilibrate sex-linked genes between males (XY) and females (XX). This inactivation will render one female X allele silenced and inactive (X_i) while the other X allele remains active (X_a). This process is regulated by Xist, which is transcribed from a specific site on the X-chromosome called the X-inactivation center (XIC). Xist appear to be involved in counting and selecting X chromosomes for inactivation (Lyon, 2002). This is especially true in the cases of supernumerical X chromosomes (XXY, XXX) where there could otherwise be two active X chromosomes. Usually for embryonic lineages, either the maternal or paternal X chromosome will be inactivated, but for extra-embryonic lineages, it is the paternal X chromosome that is preferentially inactivated (Figure 1.7) (Lyon, 2002). This is also referred to as imprinted type X-inactivation. To inactivate an X-chromosome, it was found that Xist is required in cis (Penny et al., 1996). In addition to that another gene was found called Tsix that overlaps with Xist in the opposite direction (Lee et al., 1999) and overexpression of Tsix was found to inactivate Xist (Stavropoulos et al., 2001). Therefore, Tsix appears to regulate Xist and this is particularly true for the imprinted type X-inactivation. In this particular type of inactivation, Tsix is imprinted itself resulting in expression only of the maternal allele. Therefore, Xist is only expressed from the paternal X-chromosome which leads to inactivation of that chromosome (Sado et al., 2001). Once X-inactivation occurs, Xist on the X_a chromosome is repressed by methylation in order to maintain the chromosome's activity (Lyon, 1999)

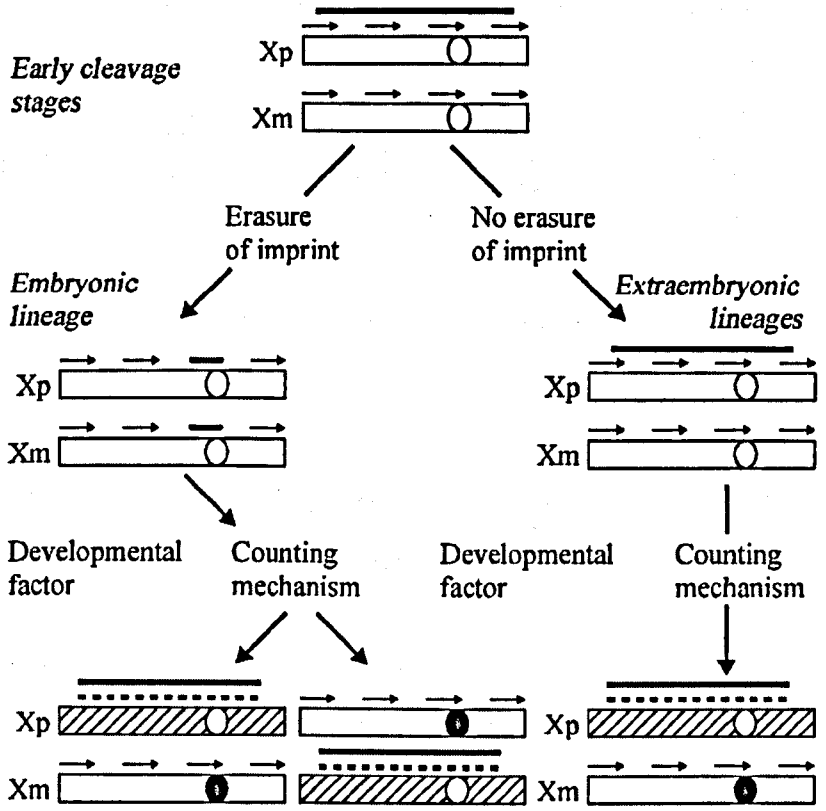


Figure 1.7: Diagram showing the X-inactivation process that occurs in the mouse embryo. Xp and Xm represent X-chromosomes of paternal and maternal origin respectively. Thick line represents the Xist RNA while short arrows represent active transcription. X inactivation center is represented by oval shape. In the early stage, Xist RNA is only expressed from Xp and not Xm due to paternal imprint. This imprint is retained in the extraembryonic lineages and with further counting mechanism and developmental factor (dotted line), Xp is inactivated. In the embryonic lineages, this imprint is lost and Xist RNA is expressed in both Xp and Xm. This is followed by random inactivation where one allele is inactivated (hatched line) and in the active allele, Xist is switched off (black oval). Diagram taken from Lyon (2002)

DNA methylation with DNMT activity is believed to play a role in X-inactivation since *Dnmt1* mutant mice could not maintain the repression on the X_i (Sado et al., 2000). Using the methylated DNA immunoprecipitation (MeDIP) assay, it was found that inactive X (X_i) display higher methylation at both promoter and the intergenic regions (Sharp et al., 2011). Interestingly, these authors found that significant numbers of CpG Islands (CGI) exhibited reduced methylation at the X_i and the majority of these CGI positions correlated with those genes that escape XCI. This supports the

involvement of DNA methylation in XCI. Furthermore, demethylating agents have been shown to reactivate genes on the X_i(Mohandas et al., 1981) which further implies a role played by DNA methylation in X-inactivation.

1.1.4.3. *Retrotransposon silencing*

DNA methylation in normal cells also represses transposable elements such as retrotransposons. These retrotransposons originated from provirus insertion into the human genome millions of years ago, and represents almost 40% of the human genome(Goodier and Kazazian, 2008).

Retrotransposons can be subcategorized into two groups; autonomous which have the necessary apparatus for mobility, and non-autonomous which do not. Autonomous retrotransposons consist of two groups; the long terminal repeat (LTR) form and non-long terminal repeat (non-LTR) form. An example of an LTR is the human endogenous retroviral (HERV), however they have lost their ability to move because they no longer encode functional proteins (Goodier and Kazazian, 2008, Prak and Kazazian, 2000). The only HERV family believed to be functional in human is HERV-K since antibodies have detected them in germ cell tumour cell lines and testicular cancer patients (Goedert et al., 1999). Nonetheless, to date, no mobilization of HERV retrotransposons has been demonstrated. On the other hand, another autonomous retrotransposon, LINE-1 (L1), which belongs to the long interspersed nuclear element (LINE) family, is believed to be the most active retrotransposon in the human genome. It occupies almost 17% of the human genome. Full length L1 is 6 kilobases in length and consists of a 5' untranslated region (UTR), two open reading frames (ORF1 and ORF2) and a 3'UTR that ends with a poly (A) signal and tail (Goodier and Kazazian, 2008) (Figure 1.8). ORF2 encodes a protein with endonuclease and reverse transcriptase activities while ORF1 is believed to be essential for retrotransposition (Alisch et al., 2006). Although only a few thousand of these full length L1s' are left in the genome, there are still a handful of them considered to be active (Sassaman et al., 1997). Other well known transposons are non-autonomous retrotransposon such as Alu in human and B1 in mice. Alu belongs to the short interspersed nuclear elements (SINE) family and unlike L1, they do not have any ORF in their structure. Due to this, it

is believed that they require help from other mobile element such as L1 for their mobilization (Deininger and Batzer, 1999).

Although not yet proven, retrotransposition of L1 and Alu is believed to involved mechanism called target-primed reverse transcription (TPRT). Through this retrotransposition, these repeat elements could cause deletions, duplication or gene sequence rearrangements. It has also been postulated that repeat element insertion could have an impact on nearby gene expression (Cruickshanks and Tufarelli, 2009, Weber et al., 2010a, Wolff et al., 2010). Therefore by repressing these repeated sequences, DNA methylation helps to maintain the integrity of the human genome. Indeed a knockout study in mouse has shown that *Dnmt3a*, *Dnmt3b* and *Dnmt3l* are required for SINEB1 and LINE1 methylation (Kato et al., 2007).

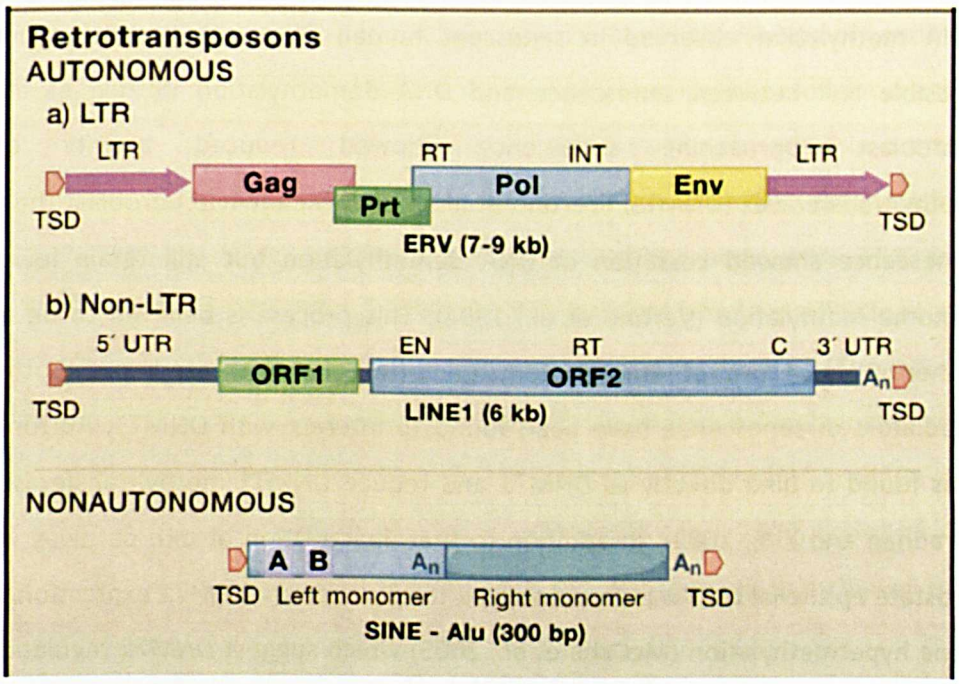


Figure 1.8: Diagram showing the domains of both autonomous and non-autonomous retrotransposons. Autonomous retrotransposons can be categorized into long terminal repeat (LTR) and non-long terminal repeat (Non-LTR). Both have the necessary accessory for mobilization but only LINE1 known to be actively mobile. Meanwhile Non-autonomous retrotransposon such as Alu relied upon L1 for their mobility. Abbreviations: Gag: group-specific antigen, Prt: potease, Pol: polymerase, Env: envelope, RT: reverse trascriptase domain, INT: integrase domain, TSD: target site duplication, LTR: long terminal repeat, EN: endonuclease domain, C: zinc knuckle domain, A_n: poly(A), A/B: A- and B- box Pol III promoter. Diagram taken from Goodier and Kazazian (2008).

1.1.5. DNA methylation in cancer development

1.1.5.1. *Global hypomethylation*

Genome-wide hypomethylation has been identified in many types of cancer. Numerous studies have shown the presence of global hypomethylation not only in the early stage of cancer but also have been linked to the progression of cancer (Jackson et al., 2004, Park et al., 2009b, Roman-Gomez et al., 2008, Yegnasubramanian et al., 2008).

The mechanism behind global hypomethylation in cancer is not yet fully understood. However, many possible factors have been postulated. A hypothesis that genome hypomethylation in tumours may be due to the presence of precancerous cell that have undergone senescence. Studies to support this theory have shown reduced DNA methylation observed in senescent human fibroblast genome prompting a possible link between senescence and DNA demethylation (Suzuki et al., 2002). Fibroblast approaching senescence showed reduced activity of DNA methyltransferases (DNMTs) (Vertino et al., 1994). Meanwhile fibroblast that escapes senescence showed cessation of DNA demethylation but still retain low level of genome methylation (Vertino et al., 1994). This process is believed to be mediated either by active form of retinoblastoma gene (RB) or an increase in p21. Both of these mediators of senescence have been found to interact with DNMT1. RB for instance was found to bind directly to DNMT1 and reduce DNMT1 methyltransferase activity (Pradhan and Kim, 2002). In addition to that, inactivation of pRb pathway in murine prostate epithelial cells was found to lead to elevation of *DNMT1* expression level and gene hypermethylation (McCabe et al., 2005) which suggest *DNMT1* regulation by *RB*. Meanwhile, p21(WAF1), a cell cycle regulator was found to disrupt the interaction between DNMT1 and proliferating cell nuclear antigen (PCNA) needed during DNA replication and repair (Chuang et al., 1997). This might suggest the regulation of methylation by p21.

Another factor that may contribute to genome hypomethylation is cell hypoxia. In tumours, it has been shown that necrotic tissue occurs due to lack of blood and oxygen supplies. Experiments performed in glioblastoma, colorectal and melanoma cell lines that were cultured under hypoxic condition were found to show low level of short interspersed nuclear elements (SINE) and 5-methylcytosine, respectively, indicating loss of methylation (Pal et al., 2009, Shahrzad et al., 2007). It was postulated that hypoxia caused the reduction in S-adenosylmethionine from inactivation of methionine adenosyltransferase (Shahrzad et al., 2007). Since S-adenosyltransferase act as a methyl donor during cytosine methylation, its reduced level may affect genome methylation.

Even though there is evidence towards genome hypomethylation it is not clear how it contributes to hypomethylation. Among the postulated contribution of genomic hypomethylation to tumour development is by causing overexpression of oncogenes and expression of cancer testis antigen (CTA) that were usually repressed in normal cells (De Smet and Loriot, 2010). This is based on studies that showed overexpression of oncogenes associated with hypomethylation. Among those genes were *MYC* and *HRAS* in gastric carcinoma (Fang et al., 1996), *TCL1* in Burkitt's lymphoma (Yuille et al., 2001), and *TKTL1* in head and neck squamous cell carcinoma (HNSCC) (Sun et al., 2010). Meanwhile methylation studies performed in leukemia, melanoma and colorectal cancer samples revealed activation of cancer testis antigen *HAGE*, *MAGE-A1* and *MAGE-A3* respectively (Roman-Gomez et al., 2007, Kim et al., 2006, Loriot et al., 2006). This activation was found to be correlated with the hypomethylation of the gene promoter region.

Beside activation of normally repressed genes, hypomethylation was also thought to cause genomic instability. In an experiment, global hypomethylation was observed in DNMT1 hypomorphic mice which have only 10% of the wild type DNMT1 expression (Gaudet et al., 2003). Furthermore, these *Dnmt1* hypomorphic mice developed aggressive T-cell lymphomas which feature high level of chromosome 15 trisomy. In subsequent study by the same group, insertion of *Dnmt11* hypomorphic allele in mice carrying mutation of neurofibromatosis 1 and *p53* caused the

development of sarcomas at an earlier age as compared to the same group of mice with normal methylation level (Eden et al., 2003). They also found that the hypomethylated cells showed significant increase in loss of heterozygosity (LOH) rate as compared to the methylated cells. Thus, they suggest that DNA hypomethylation promotes tumour development through chromosomal instability.

DNA hypomethylation has also been suggested to cause activation of repeat elements and leading to tumourigenesis. Hypomethylation is believed to enable mobilization of the repeat elements and creates genome instability. Indeed, studies in colorectal, lung cancer and HNSCC have associated hypomethylation of repeat elements with genomic instability detected through the use of microsatellite markers and microarray (Daskalos et al., 2009, Martinez et al., 2012, Ogino et al., 2008). However, to date no study has yet shown a direct link between activation of repeat elements and genome instability. In fact, Howard *et al.*(2008) in their experiment found that the expression of the intracisternal A particle (IAP), an endogenous retroviral-like element found in mice, was only elevated in the induced-tumour of the *Dnmt1* hypomorphic mice but no significant increase was observed prior to tumour development. Since *Dnmt1* hypomorphic mice have previously displayed genomic instability(Eden et al., 2003), there is a question of whetheractivation of the repeat elements are just a consequences of further demethylation events and therefore have no effect on genomic instability. On the other hand, another avenue where activation of repeat elements might contribute to tumourigenesis is by activating transcription of adjacent genes. LINE-1 repeat elements for instance have a bidirectional promoter in its sequence; a sense promoter that transcribe its two open reading frame and an antisense promoter that can trancscribe adjacent genes (Speek, 2001). Indeed, many studies in multiple type of cancer have shown the presence of genes that were transcribed by L1 antisense promoter (L1-ASP) (Lin et al., 2006, Roman-Gomez et al., 2005, Weber et al., 2010a, Wolff et al., 2010). In fact in one of these studies, truncated isoform of MET proto-oncogenes transcribe by L1-ASP in acute myeloid leukemia patients has been associated with increased risk of progression into blast crisis (Roman-Gomez et al., 2005).

Despite all these studies, none have shown conclusive evidence that can directly link global hypomethylation and tumourigenesis. Thus this area might be worth investigating in the future.

1.1.5.2. *Promoter hypermethylation*

Another common feature of cancer is the hypermethylation at gene promoter region. Although both genome-wide hypomethylation and promoter hypermethylation often observed together in many cancers, studies suggest the two events may not be linked (Ehrlich et al., 2002, Ehrlich et al., 2006, Lee et al., 2009).

Unlike genome-wide hypomethylation, contribution of the promoter hypermethylation to tumour development has been pin-pointed to its effect in causing gene silencing. As mentioned before, this could be achieved by direct inhibition of transcription factor binding or through mediation of methyl-binding protein (MBP). However, the mechanism behind the selection of genes to be methylated is still unclear. One hypothesis proposed that specific genes were preferentially inactivated in order to provide selective clonal growth advantage. In one study, mice with overexpressed *Myc* which developed immature T cell lymphoma, were crossbred with mice deleted for tumour suppressor gene (either *p53*, *Pten* or *E2f2*) (Opavsky et al., 2007). Strikingly, each genotype showed different CpG island methylation pattern and using unsupervised hierarchical clustering, each genotype segregated perfectly. This suggests that the loss of tumour suppressor function led to selective pressure on the cells and caused specificity of CpG island methylation.

Another hypothesis suggests that promoter methylation might have developed by targeting DNMTs to certain region in the genome and/or the regions possess a feature that made them a better substrate for methylation. This is based on the finding that PML-RAR, an oncogenic fusion protein is able to direct DNMTs to its target genes (Di Croce et al., 2002). In addition, DNA binding studies have shown that Kaiso, a methyl binding protein, required two adjacent methylated CpG for DNA binding. Any nucleotide in between these two methylated CpGs was found to disrupt Kaiso binding to the DNA (Prokhortchouk et al., 2001). Analysis of MeCP2 bound DNA fragment was

found to have four or more A/T bases adjacent to the methylated CpG and this feature was essential for MeCP2 binding to its target genes such as *Bdnf* and *Dlx6* (Klose et al., 2005).

Promoter hypermethylation is thought to deliver the 'second-hit' as proposed by Knudson in his theory of 'two-hit' model (Knudson, 2001). This model proposed that loss of tumour suppressor genes function could only occur if both of its allele is inactivated. Indeed, studies of familial cancer have shown that when one of the gene allele is mutated, promoter hypermethylation in the wild type allele will lead to gene inactivation (Kim et al., 2003, Ohtani-Fujita et al., 1997, Grady et al., 2000). In contrast, *BRCA1* germ-line mutation observed in familial breast and ovarian cancer was rarely found in the sporadic form of both cancers (Berchuck et al., 1998, Merajver et al., 1995, Futreal et al., 1994). Instead in this form, loss of function of *BRCA1* has been shown to be due to promoter hypermethylation in association with loss of heterozygosity (LOH) in the other allele (Dobrovic and Simpfendorfer, 1997, Esteller et al., 2000b). This suggests promoter hypermethylation is enough to cause loss of tumour suppressor gene function. Indeed, subsequent methylation studies revealed many tumour suppressor genes that are silenced only by promoter hypermethylation. Cheung *et al.*(2009) in their review of methylation studies have listed down 14 genes commonly found to be hypermethylated in multiple type of cancer. Among them are genes involved in cell cycle (*CDKN2A/p16-INK4*, *CDKN2B/p15-INK4B*), cell growth (*RARB*), DNA repair (*MGMT*, *MLH1*), apoptosis (*DAPK*, *TP73*), metastasis (*CDH1*), detoxification (*GSTP1*), hormone response (*ESR1*), Ras signaling (*RASSF1*) and Wnt signalling (*APC*). Clearly, silencing of these genes by methylation offers an advantage for tumour growth and development.

The recent introduction of genome-wide methylation technology revealed new information on promoter hypermethylation in cancer. From these genome-wide studies, it was found that promoter methylation in cancer were not restricted to the CpG islands but can also be found in regions adjacent to the CpG islands called CpG shores (Irizarry et al., 2009, Saied et al., 2012, Shen et al., 2013). Furthermore, methylation at the CpG shores has been found to be more correlated to gene

expression as compared to methylation at the CpG islands (Irizarry et al., 2009). This finding has shifted the paradigm on how methylation affects tumour development since previous studies were focusing only on CpG islands methylation. In addition to this, genome-wide methylation analysis also enable researcher to map tumour methylomes and studies revealed that each tumour displayed a distinct and unique methylation profile (Figueroa et al., 2010b, Houshdaran et al., 2010, Martinez et al., 2009, Wu et al., 2010a). Furthermore, genome-wide analysis could also distinguished a distinct phenotype in tumours which displayed hypermethylation of a subset of genes (Arai et al., 2012, Noushmehr et al., 2010, Shinjo et al., 2012, Weisenberger et al., 2006). This so-called CpG island methylator phenotype (CIMP) has also been found to be associated with poor prognosis in renal clear cell carcinoma and lung cancer (Arai et al., 2012, Shinjo et al., 2012, Takai and Jones, 2002)

Although a plethora of studies have linked promoter methylation to gene silencing, promoter hypermethylation has also been associated to higher frequency of mutation. Studies performed in colorectal cancer showed that *MGMT* hypermethylation was linked to significantly higher frequency of transition mutation of *TP53* and *KRAS* as compared to unmethylated *MGMT* (Esteller et al., 2001, Esteller et al., 2000c). The same finding was observed in low grade astrocytoma where majority (92%) of low grade astrocytoma with *MGMT* hypermethylation showed significantly higher G:C to A:T transition mutation of *TP53* as compared to those with unmethylated *MGMT* (39%). This suggests that promoter methylation might also contribute to tumourigenesis by causing genetic changes.

1.2. Germ Cell Tumours (GCTs)

A germ cell tumour is a tumour believed to derive from germ cell progenitors. Since most studies available were performed in adolescence and young adults with testicular GCTs, all discussion here will be about these unless otherwise stated. To have a better understanding of how a germ cell might transform into a tumour, it is essential to know how germ cell develop and function in normal conditions.

1.2.1. Germ cell development

Primordial germ cells (PGCs) arise from a population of pluripotent cells in the epiblast. In mice, they are visible at embryonic day 7.5 (E7.5) as a group of 20 cells at the base of the allantois (Donovan, 1998). Their fate has been earlier determined during gastrulation, around E6.5, via bone morphogenetic signalling (BMP) and other undetermined signal from surrounding tissue (Saga, 2008, Saitou et al., 2002). These epiblast cells are also regulated by B-lymphocyte-induced maturation protein 1 (*Blimp1* or the human homolog *PRDM1*) which is essential in PGC specification as it repressed expression of somatic genes such as the Hox family genes (Ohinata et al., 2005). Other than *Blimp1*, a recent study also identified another transcriptional regulator, *Prdm14* that displays a similar function in preserving PGCs as germ cell (Yamaji et al., 2008). Knockdown of either gene showed loss of PGC specific markers and an increase in Hox gene's expression (Kurimoto et al., 2008, Ohinata et al., 2005).

After the specification stage, PGCs start to initiate their migration from the primitive streak into the endoderm around E7.5 before entering the hindgut (E8.0-E9.5). From there, they migrate into the mesoderm before continuing to the genital ridge (E10.5-E11.5) (Anderson et al., 2000, Molyneaux et al., 2001). At the moment, the mechanisms involved in initiating PGC migration are still not well understood. Using RNA interference, Tanaka and his group found that silencing of interferon-induced transmembrane protein 1 (*Ifitm1*) in mouse embryos prevented PGCs from migrating into the endoderm, suggesting that this gene is involved in regulating PGC migration (Tanaka et al., 2005). However, a recent study in mice carrying a deletion of this gene showed normal germ cell migration (Lange et al., 2008) which makes *Ifitm1*'s role in initiating PGC migration questionable. Other genes that have been identified in

the PGC migration process are *SOX17* (Hara et al., 2009), *SDF-1* and its receptor CXCR4 (Ara et al., 2003, Takeuchi et al., 2010) and *c-kit* and its ligand*steel*(Gu et al., 2009, Runyan et al., 2006). Mutant *Sox17* mice were found to have failure of hindgut expansion which leads to immobilization of PGCs in the hindgut. The majority of these PGCs were observed to move into the visceral endoderm layer and relocate outside of embryonic gut, resulting in PGCs scattered in the extraembryonic yolk sac endoderm (Hara et al., 2009). While *SOX17* is crucial in early PGC migration, *SDF-1* and its receptor *CXCR4* was found to be important in the late migration phase (Ara et al., 2003, Takeuchi et al., 2010).*SDF-1* is a member of a family of chemoattractive chemokines and is expressed around the genital ridges while *CXCR4* is abundantly expressed on PGCs. It is believed that PGCs migration into the genital ridge depends upon this interaction (Figure 1.9) since knockdown of either one of these genes in mice will significantly reduces PGC numbers in the gonad (Ara et al., 2003). In addition to that, induced expression of *Sdf1* outside of the gonad region was found to attract PGCs resulting in PGCs accumulating at these ectopic sites (Takeuchi et al., 2010). On the other hand, PGCs continuously express c-kit and the somatic cells along the migration route expressed steel which implies the importance of this signalling throughout PGCs migration (Richardson and Lehmann, 2010, Keshet et al., 1991). A hypomorphic mutant of *KitL* (*steel*) in mice showed impairment in PGCs migration with the majority of them ended up in the hindgut region (Mahakali Zama et al., 2005) believed to be due to impairment in PGC motility (Gu et al., 2009). Once PGCs arrive at the genital ridge, PGCs become known as gonocytes. Depending on the micro-environment, they undergo different fate decisions. In the male micro-environment, they will enter mitotic arrest and become pre-spermatogonia while in the female environment they enter meiosis I arrest, during which they become oocytes.

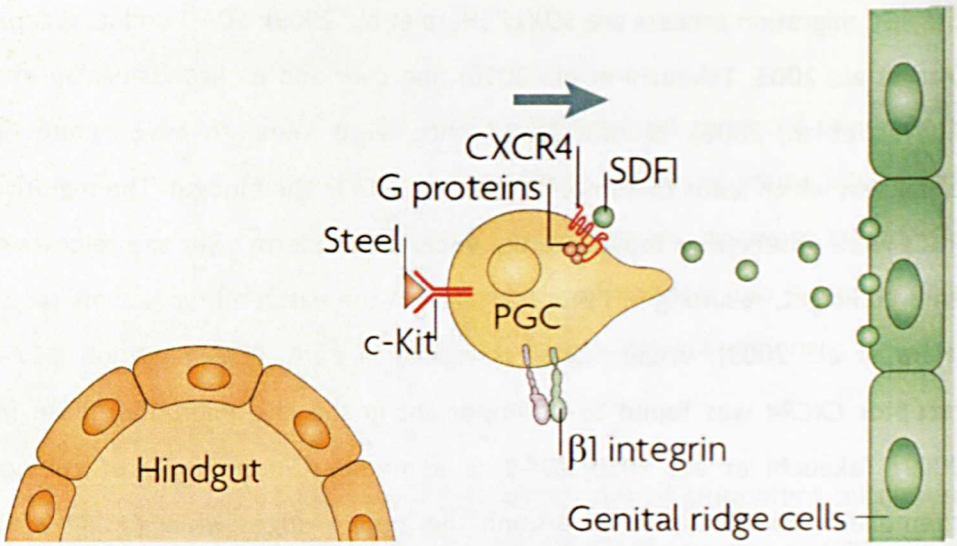


Figure 1.9: Mechanism behind PGCs migration towards the genital ridge. Interaction between CXCR4 and SDF-1 will guide PGCs towards the genital ridges. Interaction between steel and c-kit helps PGCs migration by regulating the PGCs motility. Diagram taken from Richardson and Lehmann (2010)

1.2.1.1. *Epigenetics in Germ Cell Development*

Around the time of PGC specification, epigenetic marks related to transcriptional repression, such as DNA methylation, dimethylation of lysine 9 of histone H3 (H3K9me2) and trimethylation of lysine 27 of histone H3 (H3K27me3) were observed and seem to be similar to those in somatic cells (Seki et al., 2005). During the migration period, extensive epigenetic modification occurs. Starting at E7.5 and E8.0, H3K9me2 and DNA methylation marks start to decrease while H3K27me3 mark is upregulated beginning at E8.25 and reaching a significant level at E9.5 (Seki et al., 2007). Decrease in H3K9me2 has been proposed to be due to downregulation of a methyltranferase called GLP that is required for H3K9 methylation (Tachibana et al., 2005, Hackett et al., 2012). It is also believed that upregulation of H3K27me3 is to compensate for the decrease in H3K9me2 and retaining the genome repressive state (Sasaki and Matsui, 2008).

By comparing the DNA methylation profile of E7.5 epiblast and E13.5 PGCs, Guibert and his group showed extensive demethylation occurred during this timeframe. In support of previous studies, they found high methylation at intergenic regions and gene bodies in E7.5 epiblast (Borgel et al., 2010, Smallwood et al.,

2011)but this was erased in E13.5 PGCs (Guibert et al., 2012). They identified 511 gene promoters that were heavily methylated at E7.5 epiblast and the majority of these promoters were demethylated in E13.5 PGCs. Interestingly, the affected genes were not restricted to germ line specific genes but also included genes with somatic function. Guibert and his group also showed that some genes start the demethylation process at much earlier stage, at E9.5 and complete this by E11.5. This is in contrast to previous studies of imprinted genes which showed demethylation between E10.5 and E12.5 (Hajkova et al., 2002, Li et al., 2004). They conclude that imprinted and non-imprinted genes underwent demethylation at different stage of PGC development. Interestingly, IAP and LTR-ERV1, repeat elements in mice were found to be able to resist both waves of demethylation, thus supporting the importance of repressing these elements. Despite this, Guibert and his group proposed that methylation erasure in PGCs is more complete as compared to those in pre-implantation embryos (Saitou et al., 2012) and suggested that PGCs are the main site of reprogramming (Guibert et al., 2012).

1.2.2. GCTs classifications

1.2.2.1. *Histology*

Germ cell tumours (GCTs) can be classified into two group, seminomatous and non-seminomatous, based on their histology. Seminomatous GCTs consist of undifferentiated cells that resemble germ cell progenitors. They are also known by a number of terms depending on the tissue where they arise. In the testis, seminomatous GCT are called seminomas while in the ovary, they are also known as dysgerminomas. For those that arise at extragonadal sites, the term germinoma has also been used. For the purpose of this discussion, the term seminoma will be used to represent all seminomatous GCTs.

On the other hand, non-seminomatous GCTs consist of more differentiated cells and their histology varies, resembling different stages and pattern of either embryonic or extra-embryonic differentiation. From this histology, non-germinomatous GCTs can be further categorized into embryonal carcinoma (EC),

teratoma, yolk sac tumour (YST) and choriocarcinoma (CC). Most EC consist of undifferentiated cells which are able to differentiate into many cell types thus suggesting them to be the stem cells of GCTs (van de Geijn et al., 2009, Kristensen et al., 2008). On the other hand, teratomas represent somatic differentiation while YSTs and CCs display extra-embryonic differentiation (van de Geijn et al., 2009).

1.2.2.2. *Multifactorial classification*

Another type of GCT classification is based on a combination of parameters. This includes cell of origin, histology, genomic imprinting status, age at diagnosis, and chromosomal constitution (van de Geijn et al., 2009, Oosterhuis and Looijenga, 2005). This type of classification is believed to be much better in helping clinical diagnosis and treatment choice and has been recognized by World Health Organization (WHO). Following this classification, GCTs can be categorized into five entities.

Type I GCTs represent teratomas and YSTs that occur in neonates and infants. Type II represents the most common GCT, which occurs in young adults. Similar with Type I, type II GCT could occur in the gonad and extragonadal sites. Both types were believed to arise from PGCs although in Type I the PGCs responsible are still at earlier stage than PGCs precursor in Type II. Type III GCT is testicular GCTs that are restricted to men over 50 years old. Rather than PGCs, precursor lesion for this group of GCTs is believed to be spermatogonium or spermatocyte since they showed paternal imprinting. Meanwhile, type IV GCTs occur only in females over 50 years old and are believed to arise from oogonia or oocytes. The last group, called type V consists of hydatiform moles that have developed from the fertilization of a non-nucleated egg, these usually occur in the placenta or uterus.

1.2.3. GCTs epidemiology

Germ Cell Tumours (GCTs) can arise in the gonads where germ cells are normally found, but also can be found in extragonadal sites. These extragonadal sites are usually along the body midline such as in the coccyx, thorax and the ventral midline of the brain.

1.2.3.1. *Adult GCTs*

In a recent epidemiological study carried out using 8 regional registries in the UK, the majority of GCT patients were male with an overall adjusted incidence rate of 26.44 per-million person-year (Arora et al., 2012). The majority of GCTs cases were testicular GCTs (92.5%) followed by ovarian GCTs (3.9%) and extragonadal GCTs (3.2%).

The most common extragonadal GCTs in the adult population are mediastinal and retroperitonium (Schmoll, 2002). In general, non-seminomatous GCT is more common than seminomatous GCT at extragonadal locations with non-seminomatous usually present in younger patients (Schmoll, 2002)

Another extragonadal GCT, intracranial GCT, represents a smaller proportion of adult GCTs in the West. However, the incidence in Japan and the Far East is five to eightfold greater. Most intracranial GCTs consist of germinoma and normally arise in the suprasellar and pineal region of the brain although other parts of the brain can be affected.

1.2.3.2. *Paediatric GCTs*

From the data available concerning the incidence amongst paediatric population, a bimodal distribution of age was observed only in male patient diagnosed with gonadal GCTs (Johnson et al., 2009, Murray and Nicholson, 2009). As illustrated in Figure 1.10, the incidence of gonadal GCTs in this sub-population usually peaks before 3-years old and after puberty. Meanwhile for female gonadal GCTs, the incidence only starts to increase from 6-years of age again peaking around puberty. Beside bimodal distribution, testicular GCTs in paediatric patients also present with distinct histology associated with these age groups. Testicular GCTs in infants and toddlers predominantly consist of teratoma (mature and immature) or yolk sac tumour while the adolescents showed similarity with the adult population which consist of

seminoma, embryonal carcinoma or choriocarcinoma (Horton et al., 2007, Ross et al., 2002, Schneider et al., 2004). On the other hand, ovarian GCTs consist of teratoma followed by seminoma, yolk sac tumour, embryonal carcinoma and mixed tumour regardless of age (Horton et al., 2007).

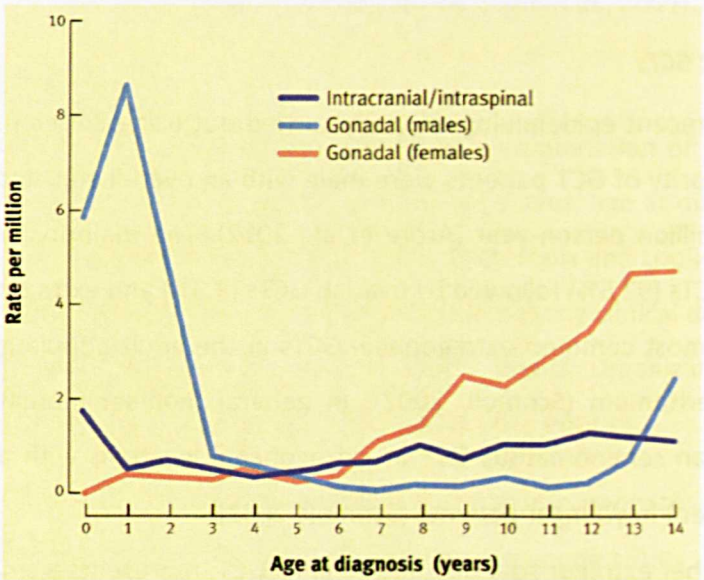


Figure 1.10: Graph showing the incidence rate of GCTs in Great Britain from 1962 to 1997. It clearly showed that the incidence of gonadal GCT in male are at highest either during infancy or during teen age while female GCTs usually arise during teen age years. On the other hands, intracranial GCTs incidence is at the highest before or right after birth. Diagram was taken from Murray and Nicholson (2009).

Extragonadal GCTs in children are commonly found in those aged less than 5 years old (Gobel et al., 2000, Veltman et al., 2003) although they can also arise in the adolescent. The most common extragonadal GCT in the paediatric population is the saccrococcygeal tumours (SCTs) (Horton et al., 2007). This could be categorized into two types; the benign form which usually arises in the neonatal period or the malignant form with intra-pelvic or intra-abdominal tumour involvement which usually presents between birth and 4 years old (Horton et al., 2007). Beside SCTs, mediastinal and retroperitoneum GCTs also arise in the paediatric population although they are quite rare representing only around 5% of all GCTs in this age group (Horton et al.,

2007, Billmire et al., 2001). Despite this, 15% of these type of GCT can be malignant and have a poor outcome (Billmire et al., 2003, Billmire et al., 2001).

The incidence of intracranial GCTs in the paediatric population varies geographically. In Western countries, they account for 0.4-3.4% of all paediatric brain tumours but in Japan and Asian countries, this incidence could be as high as 11% of all paediatric brain tumours (Echevarria et al., 2008). Males usually present with tumours in the pineal region while in females, tumours at the suprasellar regions are most common. Histologically, younger children usually present with non-seminomatous histology while seminomatous histology is more common in older children (Echevarria et al., 2008)

1.2.4. Risk factor to GCTs

Certain factors have been found to increase the chances of getting GCTs, in particular testicular GCTs. These include family history (Forman et al., 1992, Hemminki and Chen, 2006) and previous experience of GCTs (Wanderas et al., 1997). However, efforts to identify genes responsible for familial TGCT have been quite a challenge due to small sample sizes (Rapley et al., 2000, Lutke Holzik et al., 2004, Rapley et al., 2003). A genotyping study has identified that individuals with gr/gr deletion at Y chromosomes have an increased two-fold risk of TGCT and this increases to three-fold if the individual has a family history of TGCT (Nathanson et al., 2005). This might be linked to infertility as this gr/gr deletion removes AZFc regions on the Y chromosomes which have been associated with reduced fertility(Ferlin et al., 2005).An environmental cause has also been proposed to influence the chances of getting TGCT. This is based on observation of Finnish immigrants in Sweden who have lower risk for TGCT, but develop the same risk as Swedish population in their second generation (Hemminki and Li, 2002).

Men with infertility problems have also been identified as at a higher risk for TGCT (Doria-Rose et al., 2005, Baker et al., 2005). In addition to this, the risk of getting TGCT is even higher in this population if they showed a presence of minutes particles in the testis, also known as microlithiasis (de Gouveia Brazao et al., 2004). Beside this, low sperm count has also been suggested as a risk factor for testicular cancer. This was

based on association studies that showed Danish and Norwegian men have higher incidence of testicular cancer and low sperm count as compared to Finnish and Estonian men respectively (Jensen et al., 2000, Jorgensen et al., 2002). Other testicular dysgenesis syndromes (TDS) such as cryptorchidism and undescended testis were also recognized as TGCT risk factors (Looijenga et al., 2007). In fact, cryptorchidism is the only congenital abnormalities found to be associated with paediatric GCTs in a recent study conducted by Children's Oncology Group (Johnson et al., 2009). These reproductive abnormalities are postulated to occur due to high estrogen exposure during fetal life according to the estrogen hypothesis, and this definition has been expanded to other endocrine disruptors that can cause similar disruption in hormonal balance (Sharpe, 1993, Sharpe and Skakkebaek, 1993). Indeed, estrogen has been shown to suppress androgen production and insulin-like factor 3 that leads to cryptorchirdism and birth defects of the urethra (Sharpe, 2003). Other chemicals have also been suggested to be able to alter hormonal function such as certain phthalates and polychlorinated biphenyls (Fisher et al., 2003, Sharpe, 2003). However, until now no direct cause has been established between hormonal imbalance and GCTs.

Males with Klinefelter syndrome, characterized by multiple X chromosomes, has been associated with higher risk of extragonadal GCTs (Hasle et al., 1995, Aguirre et al., 2006) with majority of them were germinoma (Kaido et al., 2003, Inai et al., 2005). Meanwhile, individuals with skeletal defects, mental retardation and congenital heart disease have also been identified to have higher risk for extragonadal GCT (Johnson et al., 2009)

1.2.5. Origin of GCTs

1.2.5.1. *Gonadal GCTs*

Gonadal GCTs are believed to arise from carcinoma in situ (CIS), also known as intratubular germ cell neoplasia, unclassified (ITGCNU). CIS is a lesion characterized by enlarged, atypical germ cells (Reuter, 2005), believed to develop from gonocytes/PGCs that are arrested in their development.This is supported by the similarities in gene expression profile between CIS and gonocyte (Sonne et al., 2009) and the finding that

genes involved in testicular development are differentially expressed in CIS (Hoei-Hansen et al., 2004). This theory of CIS as the precursor lesion for gonadal GCTs is based on the observation that patients with CIS have a tendency to develop invasive GCTs (Skakkebaek, 1978). Furthermore using a mathematical modelling, it was predicted that 50% of patient with unilateral GCTs and positive with CIS will develop invasive GCTs within 5 years (von der Maase et al., 1986). Indeed, CIS are usually found adjacent to the invasive GCTs (Jacobsen et al., 1981, Reuter, 2005). Recent morphological analysis has further shown the presence of intratubular seminoma and intratubular embryonal carcinoma adjacent to invasive GCTs and, this was suggested to be the intermediate stage between CIS and invasive GCTs (Oosterhuis et al., 2003). This came as no surprise since CIS, seminoma and embryonal carcinoma do share some similarities in terms of gene expression and chromosomal constitution (Almstrup et al., 2004, Sperger et al., 2003, Summersgill et al., 2001, Looijenga et al., 2000). In fact, the only thing that sets CIS apart from the invasive GCTs is the absence in chromosomal gain of 12p (Rosenberg et al., 2000). Even more striking is that this gain of 12p is first detected at the preinvasive state (Ottesen et al., 2003). Therefore, it has been suggested that gain of 12p is important for the development of invasive GCT (Rosenberg et al., 2000, Summersgill et al., 2001). However, this model is only applicable to gonadal GCTs in young adults as the paediatric population does not shares similar aneuploidy, the full erasure of imprinting and 12p amplification seen in adults (Bussey et al., 2001b, Schneider et al., 2001). Instead, partial erasure of imprinting pattern in the paediatric GCTs suggests that they arise from early PGCs, possibly before they enter the gonad and become gonocytes (van de Geijn et al., 2009, Reuter, 2005).

1.2.5.2. *Extragonadal GCTs*

Unlike gonadal GCTs, the origin of extragonadal GCTs is still contentious. The current accepted theory is based on PGCs displacement during migration, however another theory that involves stem cells has more recently been proposed.

1.2.5.2.1. PGC migration theory

The germ cell migration theory states that extragonadal tumours arise from displaced PGCs during their migration towards the genital ridge. These PGCs are usually displaced along the migration route which is along the body midline. Later, due to local environmental cues, these displaced PGCs will undergo reactivation and proliferate to form tumours(Teilum, 1958, Teilum, 1965, Takeuchi et al., 2009)

This theory is supported by the finding that extragonadal and gonadal GCTs in young adults shares many similar characteristics suggesting they both arise from PGCs. Both of them share 12p amplifications (Looijenga et al., 2003), similar expression profiles (Palmer et al., 2008, Sperger et al., 2003) and epigenetic profiles (Netto et al., 2008, Furukawa et al., 2009).

This is further supported by previously mentioned studies which showed PGCs have a tendency to stay outside the gonad after deletion of SOX17, SDF1, CXCR4 or steel (Mahakali Zama et al., 2005, Ara et al., 2003, Hara et al., 2009, Takeuchi et al., 2010). Furthermore, *Bax* mutation has been shown to favour the accumulation of PGCs outside the gonad regions (Runyan et al., 2008). All of these imply many possibilities that extragonadal tumour may arise from PGCs that are displaced during migration.

1.2.5.2.2. Stem Cell theory

Unlike previous theories relating to PGC migration, this theory proposed that extragonadal GCTs arises from cells localized to the tissue where the tumour occurs. However at the moment, studies that support this idea came from studies conducted in neural progenitor (Tan and Scotting, 2012). Thus, further discussion will revolve around neural progenitor cells and intracranial GCTs. Teratoma, a subtype of GCTs, is able to give rise to all three germ layers and is thus regarded as pluripotent. Therefore, it has become a standard in reprogramming studies that cells need to be able to form a teratoma before they can be considered as pluripotent. Indeed it has been proven that this can be achieved by just overexpressing four key genes in the somatic cells such as fibroblast (Takahashi and Yamanaka, 2006). This study therefore suggests that not just PGCs, but any cells could have the ability to give rise to teratoma. In addition to this, a further study in mouse neural stem cells (mNSCs) showed that reactivation of only one

out of these four key genes, *Oct4*, was enough to induced mNSCs to become pluripotent and give rise to a teratoma (Kim et al., 2009). The reason behind this is the expression of all pluripotency genes except Oct4 in the brain. A study in mice has showed that *Oct4* expression in the brain starts to decline after embryonic day 8.5 (E8.5) and becomes undetectable after E15.5 and this decline was found to coincide with methylation of the *Oct4* promoter region (Lee et al., 2010). Interestingly, a recent animal study showed that demethylation using 5-azadeoxycytidine can reactivate *Oct4* expression (Lim et al., 2011). If this finding can be applied to human cells, it would mean that a disruption of methylationwould allow continuous expression of *OCT4* in neural stem cells and might allow them to be transformed into teratoma. Thus it has been proven that NSCs could give rise to teratomas, but could NSCs forms the other subtypes of GCTs? Given that GCTs often have a mixed histology (Taccagni et al., 1989) and several reports showed GCT recurrence as another subtype following resection (Wong et al., 2010, Sugimoto et al., 2002) suggests that all GCTs shares common cell lineage. Thus, the suggestion has been made that *OCT4* activation could initiate teratoma formation from NSCs which after some time could convert into any subtype of GCTs (Tan and Scotting, 2012).

Other evidence that links NSCs as the cell of origin for extragonadal GCTs come from studies looking into the profile of imprinted genes such as *SNRPN*. Study in mice showed that during gametogenesis, *Snrpn* is unmethylated and expressed at the paternal allele while in the maternal allele it is silenced by methylation(Shemer et al., 1997). Furthermore, lack of *SNRPN* methylation has not been associated with other type of tumours like other imprinted genes have (Zhan et al., 1995, Kohda et al., 2001). Thus, its restricted methylation pattern in GCTs and PGCs suggested that *SNRPN* imprinting pattern could be used to determine the PGC developmental stage at which the tumour arises (Schneider et al., 2001). Indeed, studies have shown that both gonadal and extragonadal GCTs exhibited PGC-like pattern of *SNRPN* methylation (Bussey et al., 2001a, Schneider et al., 2001) which ledthe authors of both papers to conclude that PGCs could be the cell of origin for extragonadal GCTs. However, recent methylation analysis using restriction enzyme and bisulfite sequencing on human NSCs

also showed hypomethylation of *SNRPN* that resembles PGCs and GCTs (Lee et al., 2011). This therefore also supports the possibility of NSCs as the cell of origin for intracranial GCTs.

1.2.6. Aberrant DNA methylation as potential mechanism in paediatric GCT development

Paediatric germ cell tumours (GCTs) like other childhood cancers, develop in quite a short time, with most of them occuring within the first fiveyears after birth or even while still in the womb. Thus, there is little time for cells in the paediatric population to accumulate a series of mutations. This was evident from a recent study that screened 70 paediatric GCT samples for *BRAF* and *KRAS* mutation (Masque-Soler et al., 2012). In this study, *BRAF* V600E point mutation and *KRAS* mutation at codon 12 and 13, both previously shown to be presence in adult GCTs (Honecker et al., 2009), were screened using Sanger sequencing and pyrosequencing. Comparing their data to previous study performed in adult GCTs (Honecker et al., 2009), this group found no evidence of mutation in all paediatric samples analysed. This finding was not restricted to paediatric GCTs, as genome wide screening studies performed in other paediatric cancers such as retinoblastoma (Zhang et al., 2012) and glioblastoma (Schwartzentruber et al., 2012) also revealed less frequent mutations as compared to the same tumour type in the adult population. In addition to that, paediatric GCTs such as teratoma subtypes have been shown to have little or no cytogenetic damage (Oosterhuis and Looijenga, 2005, Mostert et al., 2000, Rickert, 1999). This suggests that in this class of GCT, mutations and DNA damage may not be the main culprit in causing dysregulation of gene expression. Thus, other mechanisms such as DNA methylation could be responsible for such rapid development of GCTs in the paediatric population.

As mentioned before, DNA methylation plays a major part during PGC development. Therefore, any disruption to this regulation would result in abnormal PGCs development that could lead to tumour formation. Thus, methylation studies in these populations are warranted to further improve our understanding of these tumours.

1.2.7. Previous studies in GCTs

1.2.7.1. *DNA methylation study*

Among the first evidence of differences in methylation between seminomatous and non-seminomatous GCT came from a study that looked into the methylation of the 5' region of two X-chromosome genes, hypoxanthine phosphoribosyltransferase (*HPRT*) and phosphoglycerate kinase (*PGK*) (Peltomaki, 1991). Using methylation sensitive restriction enzyme digestion, they found that teratocarcinoma samples displayed hypermethylation in both genes. In contrast, seminoma samples featured a hypomethylated pattern resembling active X-chromosome genes. Similar findings were also found when they expanded their analysis to other chromosomal regions leading them to conclude that teratocarcinomas in general display hypermethylation characteristics while seminomas are usually hypomethylated.

In another study using restriction landmark genomic screening (RLGS) to measure the 5-methylcytosine (5-mC) level across the genome, it was also found that non-seminomas possessed much higher 5-mC levels (an average of 1.11%) as compared to the seminomas with an average level of 0.08% 5-mC (Smiraglia et al., 2002). The same finding was observed when using immunohistochemical staining of 5-mC as a measurement of the methylation level in GCT samples (Wermann et al., 2010). Despite having higher methylation than seminoma, both seminomas and non-seminoma were found to show significant hypomethylation of LINE-1 repeat elements when compared to other tumours of somatic tissue origin (Ushida et al., 2011). Since the LINE-1 methylation level has been shown to be a good surrogate for measuring global methylation level (Estecio et al., 2007), this suggests that GCTs in general are hypomethylated across the intergenic regions of the genome. Based on these studies, it can be concluded that most GCTs usually display global hypomethylation of LINE-1 repeat elements.

Besides global hypomethylation, all cancers analysed so far feature hypermethylation at gene promoter regions, and GCTs are no exception. Adopting a candidate gene approaches, among the genes found to be hypermethylated in

testicular GCTs are *MLH1* and *p16*^{INK4} (using a real-time PCR and methylation sensitive restriction enzymes respectively) (Chaubert et al., 1997, Olasz et al., 2005). In their study, Olasz *et al.* (2005) found that 21.6% of testicular GCTs showed methylation of hMLH1 while Chaubert and his group showed methylation of p16^{INK4} in half of their testicular GCT samples. Unfortunately, these studies did not provide any methylation data for the different types of GCTs samples analysed. However, looking at other methylation studies performed in testicular GCTs (listed in Table 1.1) it is clear that non-seminomatous GCTs are characterized by a higher number of hypermethylated promoters as compared to seminomatous GCTs. To date, only two genes, *SORBS1* and *GSTP1*, have been shown to be hypermethylated in seminomas (Koul et al., 2002, Lind et al., 2006). Even then, both genes are only methylated in 5% (1/20 samples) and 3% (1/29 samples) of the seminoma samples respectively. Clearly, more data is needed to confirm these findings.

One study comparing seminoma, teratoma and YST, showed that the latter were found to have a significantly higher number of methylated tumour suppressor genes (TSGs) as compared to the former two (Furukawa et al., 2009). In fact, YST comprise the majority of non-seminomatous GCTs that have methylated genes in most of the methylation studies performed in testicular GCTs. Furthermore, at the moment, five genes (*APC, RASSF1A, HOXA9, SCGB3A1* and *HIC1*) have been shown to be hypermethylated in 100% of the YST samples analysed (Koul et al., 2002, Lind et al., 2006). Interestingly, YSTs have also been shown to have a higher number of methylated tumour suppressor genes as compared to other childhood solid tumours such as neuroblastoma, hepatoblastoma and Wilm's tumour (Furukawa et al., 2009).

Other genes that were also found to be methylated in non-seminomatous testicular GCTs are *XPA1, EMX2, MSX1* (Lind et al., 2006) and *NME2*(Koul et al., 2002). However, the number of non-seminomatous GCT samples that showed methylation of these genes was too small.

Genes	Percentage of samples exhibiting methylation (%)		References
	Seminoma	Non-seminoma	
APC	0-11.6	24-85	(Honorio et al., 2003, Koul et al., 2002, Koul et al., 2004, Okpanyi et al., 2011, Brait et al., 2012)
RASSF1A	0-78	21-84	(Furukawa et al., 2009, Lind et al., 2006, Tian et al., 2011)
HOXA9	0-11	26-88	(Furukawa et al., 2009, Lind et al., 2006)
PRSS21	86	87	(Manton et al., 2005, Kempkensteffen et al., 2006)
RUNX3	0-11	3-80	(Furukawa et al., 2009, Lind et al., 2006, Kato et al., 2003)
MCAM	32.6	71.4	(Brait et al., 2012)
MLH1	27.9	71.4	(Brait et al., 2012)
MGMT	0-24	20-69	(Honorio et al., 2003, Koul et al., 2002, Koul et al., 2004, Smith-Sorensen et al., 2002, Brait et al., 2012)
ER-β	27.9	64.3	(Brait et al., 2012)
S100	0	57.1	(Brait et al., 2012)
SCGB3A1	0-11	52-54	(Furukawa et al., 2009, Lind et al., 2006)
VGF	16.3	50	(Brait et al., 2012)
DCR2	11	44	(Furukawa et al., 2009)
HIC1	0	41	(Koul et al., 2002, Koul et al., 2004)
ER-α	18.6	35.7	(Brait et al., 2012)

FKBP4	2.3	35.7	(Brait et al., 2012)
FHIT	0	6-29	(Honorio et al., 2003, Koul et al., 2002, Koul et al., 2004)
AIM1	0	21.4	(Brait et al., 2012)
RAR-β2	2.3	21.4	(Brait et al., 2012)
RASSF2A	0	20	(Furukawa et al., 2009)
BRCA1	3	18	(Koul et al., 2002, Koul et al., 2004)
CCNA1	4.6	14.3	(Brait et al., 2012)
PGP9	9.3	14.3	(Brait et al., 2012)
HOXB5	0	13	(Lind et al., 2006)
CDH13	0-6	9-12	(Honorio et al., 2003, Lind et al., 2006)
CDH1	0	4-11	(Honorio et al., 2003, Koul et al., 2002, Koul et al., 2004)
RARB	0	5-23	(Honorio et al., 2003, Koul et al., 2002, Koul et al., 2004)
FANCF	0	6	(Koul et al., 2002, Koul et al., 2004)
TIMP3	0	3	(Koul et al., 2002)

Table 1.1: List of genes previously found to be methylated and their percentage of methylation in seminomatous and non-seminomatous TGCT. Genes in bold were those found to be methylated in 100% of YST.

1.2.7.2. *Gene expression studies*

Based on the previous findings of amplification and overrepresentation of chromosome 12 and 17 in adult TGCTs (Kraggerud et al., 2002, Roelofs et al., 2000, Summersgill et al., 2001), two gene expression microarrays were performed to identify genes overexpressed in these two chromosome arms (Rodriguez et al., 2003, Skotheim et al., 2002). Rodriguez *et al.* conducted arrays using a chromosome 12 specific array in 18 adult TGCT samples (eight seminomas samples, three EC samples, four EC cell lines, two teratoma cell lines and one YST cell lines). They reported that many genes showed a correlation between their expression and amplification. Among the genes found to be overexpressed in most TGCTs were *MRPS35, LDHB, CMAS, EKI1, LRMP, KRAS2, ITPR2, TM7SF3, SURB7, CCND2, MLF2, PTMS, GLU3, HPH1 AND LRP6*(Rodriguez et al., 2003). However, they mentioned *BCAT1* as the only gene that was highly expressed specifically in the non-seminomas. Using a similar approach Skotheim et al. used a microarray mainly specific to chromosome 17 (Skotheim et al., 2002).Comparing to normal testis, they found that all eight seminomas and six non-seminomas in their analysis showed higher expression of *GRB7, JUP, LLGL2* and *PDE6G*(Skotheim et al., 2002). They also mentioned that *GRB7* was significantly more expressed in the non-seminoma while expression of *JUP* was higher in the seminoma samples compared to normal testis. Looking at locations other than chromosome 17, they found *CCND2, POV1* and *MYCL1* were among the genes overexpressed in all TGCT samples as compared to normal testis; with the latter two showing significantly higher expression in the seminoma samples (Skotheim et al., 2002).

Meanwhile, 23 overexpressed genes were identified when CIS were compared with normal testicular parenchyma (Hoei-Hansen et al., 2004). Among those genes were *CCND2, LDHB* and XIST, whichhad been previously shown to be overexpressed in CIS or seminoma (Houldsworth et al., 1997, Kawakami et al., 2003b, Looijenga et al., 1997, von Eyben et al., 2000). This study also identified the'overexpressed in CIS' (*OIC1*) gene as a novel gene that was highly expressed in CIS. Other genes found to be expressed only in the CIS were *CALD1, C7, CYBRD1* and *DCN*.

On the other hand, gene expression analysis performed in 26 adult GCT samples and cell lines and embryonic stem (ES) cells revealed the similarity between EC and ES cells (Sperger et al., 2003). This group found that EC and ES cells share a much higher number of overexpressed genes than EC or ES cells share with seminomas. They also found that almost half of the overexpressed genes shared by EC, ES and seminomas were identified only as accession numbers, EST or hypothetical protein which suggests there were many unexplored genes in this group of cells. Meanwhile, between EC and ES cells but not in seminoma, *DNMT3B, FOXD3* and *SOX2* were highly overexpressed. Interestingly, when they compared EC and ES cells, they found a significantly higher number of genes from chromosome 12 were overrepresented in ECs which was in line with chromosome 12 amplification in TGCT (Roelofs et al., 2000, Summersgill et al., 2001).

The only gene expression study that used CIS as a control was conducted by Almstrup (Almstrup et al., 2005). They only reported a small number of genes that showed differential expression between seminoma and non-seminoma which might be due to the experimental design that only included one sample for each tumour type. They did find some genes overlapped with the study done by Sperger (Sperger et al., 2003) which included *DNMT3B* in non-seminoma and KIT in seminoma samples.

As mentioned before, the presence of two or more histological components in GCTs is not unusual. In a bid to identify genes that can predict the major histological components of mixed non-seminomatous GCTs, Korkola *et al.* performed Affymetrix gene expression arrays on 84 non-seminomatous GCT samples, with 42 samples of mixed histology (Korkola et al., 2005). Using predictive analysis of microarray (PAM) on all pure GCT samples, this group found 146 transcripts unique for each histology. This finding was found to be valid when they did RT-PCR of the genes in their pure and mixed GCT samples. Unfortunately, they only did validation on two genes unique for each histology. Furthermore, only two mixed samples (choriocarcinoma with EC component) were used with one showing expression of CST1 that was found unique to YST. This suggests the presence of YST component in the mixed choriocarcinoma samples that was not detected before. While this might imply the gene specificity in

discriminating YST component from the mixed GCT samples, more samples might be needed to make this finding more credible. In addition, more validation needs to be done on all of the genes to determine its reliability as a marker for each GCT subtype.

All genes found to be overexpressed in previously mentioned studies are summarized in Table 1.2.

Phenotype	Highly expressed genes	References
CIS	*XIST, SFRP1, IGFBP6, TXNIP, OIC1, CALD1, CD74, C7, CXCL16, CYBRD1, IFITM1, CCND2, SALL1, TRA1, COL1A2, HDAC3, HLA-DRA, OPA1, BPAG1, DCN, LDHB*	(Hoei-Hansen et al., 2004)
Seminoma	*POU5F1, KIT, TFCP2L1, UTP14C, HLXB9, LBP9, TCL1A, FRCP2, MMP9, JUP, XIST, CD74, CXCL16, IFITM1, CCND2, SALL1, TRA1, HDAC3, HLA-DRA, BPAG1, LDHB*	(Sperger et al., 2003, Almstrup et al., 2005, Skotheim et al., 2002, Hoei-Hansen et al., 2004)
EC	*DNMT3B, FOXD3, SOX2, KRT19, DNMT3L, AGR2, FGB, STELLA, CDX2, TDGF1, EBAF, CBS, GPC4, TERF1, GABARAPL1, IMP1, SEMA6A, GGTLA1, GABRB3, GAL, AK3, NALP2, SCNN1A, CXCL16, IFITM1, CCND2, SALL1, TRA1, OPA1, LDHB, GRB7,* ***NANOG, MICB, POU5F1, UPP1, DPPA4, NALP7, SEPT6***	(Almstrup et al., 2005, Sperger et al., 2003, Hoei-Hansen et al., 2004, Skotheim et al., 2002, Korkola et al., 2005)
Teratoma	*SFRP1, IGFBP6, TXNIP,SALL1, TRA1, COL1A2, HDAC3, HLA-DRA, OPA1,* ***MFAP4, EMP1, MAIL,TM4SF3, EMP1***	(Hoei-Hansen et al., 2004, Korkola et al., 2005)
YST	***CST1, APOA2, BMP2, FOXA2, VTN, C5, EOMES, CYP26A1, CCKBR***	(Korkola et al., 2005)
Choriocarcinoma	***CGB, CGA, PLAC6, HSD3B1, LEP, TFP1***	(Korkola et al., 2005)

Table 1.2: Genes previously found to be overexpressed in CIS and each type of TGCT. All genes that were found to be uniquely expressed in each TGCT subtype are highlighted in bold.

Expression analysis of GCTs in paediatric populations is still scarce. Among the earliest studies using Northern hybridization and immunohistochemistry, it was found that paediatric YSTs and their cell lines showed expression of *GATA-4*(Siltanen et al., 1999). This gene was previously shown to be a marker for yolk sac endoderm in mouse (Arceci et al., 1993). However, since immature teratoma was also found to express *GATA-4*, this marker should not be regarded as specific to YSTs. This was further supported by a recent study using immunohistochemistry (Salonen et al., 2010). They found that GATA-4 was also expressed in gonocytes until 17th gestational week, after which it became undetectable. They also found that variable expression of *GATA-4* could be observed in CIS, although this could be due to contamination as the CIS samples were heterogenous and mixed with either seminoma or non-seminoma component. By testing the GATA-4 antibody in their adult GCTs samples; which include seminoma, embryonal carcinoma, YST, choriocarcinoma and immature teratoma, Salonen *et al.* (2010) also found that all samples except embryonal carcinoma and choriocarcinoma showed expression of GATA-4.

One limitation of GCTs studies in paediatric populations is the small number of samples available especially those taken from extragonadal sites. This was agreed upon by Palmer and his group when they analysed 27 samples of paediatric GCTs, where only five were from extragonadal samples (Palmer et al., 2008). Out of the 27 samples, nine were seminomas while the rest were YSTs. Only ten samples were from patients less than 5 years old and (all of which were YSTs) while 17 samples (including all seminoma samples) were from patients more than 5 years of age. Interestingly, when they compared their YST testis samples to adult YST testis samples from a previous study (Korkola et al., 2006), they observed a separation between these two cohorts from hierarchical clustering analysis. From this observation, they concluded that such a difference was not due to the site of the tumour. Although not mentioned, it is possible that this observation could have been due to a batch effect that can arise from comparing data between independent experiments. In this case, different techniques were used for sample preparation and different array panels were used by each group (one used U133A panel while the other used U133 A+B

panel).Unfortunately, due to unavailability of seminoma testis samples, they could not make similar comparisonto their paediatric seminoma testis samples. Seminomas and YSTs were also found to have different chromosomal enrichment. Transcripts from 15q, 12p, 8q and 1p were highly expressed in seminomas while YSTs showed higher expression at 11q, 11p, 3p, 5q, 10p and 13q. Interestingly, they also found two separate enrichments when they compared YST samples according to age.

Samples from patient age between 5 to 16 years old have higher transcript expression at chromosome 19, Xp, Xq, 2q, 7q and 22q while samples from patient less than 5 years old showed enrichment at 12q, 2p and 3p. Looking into differentially expressed genes, they found seminomas to display higher expression of pluripotency genes such as *NANOG*, *POU5F1* (*OCT4*) and *UTF*. Other genes that were highly expressed in seminoma were *TFAP2C*, *ASH1L* and *PDPN*. On the other hand, YSTs exhibited higher expression of differentiation associated genes such as *KRT19* and *KRT8*. Beside these two genes, *AFP*, *APOA1*, *APOA2*, *CCKBR* and *PDZK1* were also found to be highly expressed by YSTs.

In light with the findings that suggest the importance of TGF-β and BMP signalling in germ cell development and PGCs specification respectively (Itman et al., 2006, Saitou et al., 2002, Surani et al., 2004), Fustino *et al.* evaluated these pathways in eleven paediatric samples (seven YSTs and four seminomas) using a custom tissue microarray and immunohistochemistry (Fustino et al., 2011). They only observed a significant difference in BMP signalling with YSTs showed higher activity than seminomas. Using quantitative RT-PCR of genes involved in TGF-β/BMP signalling, they found that 29 genes were highly expressed in YSTs but only 5 were highly expressed in seminomas (Table 1.3). Interestingly, among the genes highly expressed in YSTs were *NOG* and *FST*, which are known to be negative regulators of the pathways (Bartholin et al., 2002).

TGF-β/BMP signalling pathway genes found highly expressed in YST and seminoma	
Higher expression in YST	Higher expression in seminoma
NODAL, AMH, TGFB2, LEFTY1, BMP7, BMP4, GDF3, GDF6, BMP5, BMP2, COL3A1, COL1A1, LTBP1, BMP1, NOG, FST, CER1, ID1, ID2, JUNB, IGFBP3, TSC22D1, JUN, GSC, EVI1, TGFB1/1, AMHR2, BMPER, ENG	GDF5, PLAU, BMPR1B, ACVR1, STAT1

Table 1.3: List of TGF-B/BMP signalling pathways genes found to be highly expressed in YST and seminomas from study conducted by Fustino et al (2011)

1.2.7.3. *Limitations of previous studies*

From all previous studies involving GCTs, it is clear that most focus has been on adolescence and young adult populations. In addition, the majority of these studies revolved around testicular GCTs. Thus, there is still lack of study involving paediatric populations and also insufficient knowledge of extragonadal GCTs.

It is also notable that the majority of the studies looking at promoter methylation used a technique called methylation specific PCR (MSP). This technique involves the use of bisulfite-treated DNA that will convert unmethylated cytosine into thymine but leaves methylated cytosine unaffected. By using a specific primer pair for each methylated and unmethylated CpG site, the level of promoter methylation can be determined by the intensity of the amplified PCR products. Since it is quite laborious to design primers for all the genes, most studies have pre-selected their genes of interest. These genes of interest are usually those known to be a tumour suppressor or gene previously shown to be methylated in other types of cancer. Therefore, previous analysis on promoter methylation was restricted to a few genes and did not represent the global genome-wide characteristics of GCTs. In addition, only a few of these studies were analysed for both their methylation and their expression using germ cell tumour cell lines (Koul et al., 2002, Koul et al., 2004, Lind et al., 2006). These studies also looked at reactivation of silenced genes using the demethylating agent, 5-azadeoxycytidine (Koul et al., 2002, Koul et al., 2004). Although it proved that these

genes were repressed by methylation, the notion that CpG islands methylation at the gene promoter region was responsible for such repression needs to be treated with caution. This is because in recent genome-wide scale methylation analysis in colorectal cancer, it was shown that CpG shores (a region 2kb away from the CpG islands) instead of CpG islands were the most differentially methylated regions (Irizarry et al., 2009). Furthermore, they also suggest that methylation at CpG shores were the most correlated with the gene expression. Since no genome-wide methylation study has been conducted in GCTs, it is still unknown whether the methylation at CpG islands or shores will have any effect on gene regulation in GCTs.

Similar to the methylation studies, gene expression analysis conducted in GCTs was mainly using samples taken from young adults testicular GCTs. Only a handful of studies were conducted in the paediatric population to date (Palmer et al., 2008, Fustino et al., 2011). Despite revealing quite a number of uniquely expressed genes in most of the GCTs subtype, none of these studies have any methylation data linked to it. Therefore, there is still a knowledge gap in determining the extent of DNA methylation in regulating the expression of these genes.

1.3. Aims & Objectives

1) To investigate the methylation profile of paediatric GCTs (gonadal and extragonadal) using global approaches. A global methylation profile performed by measuring methylation of LINE-1 (L1) repeat elements using the combination of bisulfite and restriction enzyme analysis (COBRA) technique will be discussed. On the other hand, Golden Gate methylation arrays will be used to assess the methylation status of gene promoter regions.

2) To gain a more genome-wide methylation profile and a wider coverage of genes, InfiniumHumanMethylation 450K array will be utilized on representative GCT cell lines for all histological cell type.

3) Genes repressed by methylation in the paediatric samples and the GCT cell lines will be identified by correlating methylation array data with their corresponding gene expression array data.

4) To identify factor(s) behind the methylation profile of paediatric samples and GCT cell lines by measuring expression levels of *DNMT3B*, *EZH2* and *SUZ12* using real-time quantitative PCR.

5) To investigate the function of methylation in GCT by knocking down the contributing factor(s) and observing the effect on GCT cell line behaviour.

2. MATERIALS& METHODS

2.1. Materials

2.1.1. 2.1.1: DNA samples & Cell lines

2.1.1.1. *Paediatric germ cell tumour cohort*

The cohort of tumour samples used in this study consisted of seminomas, yolk sac tumours (YSTs) and controls. All tumour samples were obtained from the Children Cancer Leukemia's Group (CCLG) in the form of genomic DNA and RNA. All samples obtained from CCLG were from both gonadal and extragonadal site. In addition, twelve paediatric control samples were also acquired from the same group. However, no prior history was obtained for these samples. The other 3 paediatric controls (infant's blood controls) were kindly given by Dr Beth Coyle (University of Nottingham).

2.1.1.2. *: Human Neural Stem Cells (NSCs)*

The human NSCs were obtained from Milipore under the name of ReNcell VM. These NSCs had been immortalized by retroviral transduction with the v-myc oncogene. They were derived from the ventral mesencephalon region of a 10-week old human fetal brain tissue. Cells were grown on Laminin (Sigma) as adherent cells in 'NB medium' (DMEM/F12 (1:1) (1X)(Invitrogen) and Neurobasal Medium (1X) (Invitrogen)). Medium was supplemented with 20ng/ml FGF (Invitrogen), EGF (Sigma) 5X N2 (Invitrogen) and 10X B27 (Invitrogen) supplements. 0.5% Penicillin/Streptomycinwas also added to these medium.

2.1.1.3. *: Germ Cell Tumour (GCTs) Cell Lines*

Four GCTs cell lines were used in this study. They are NT2D1 (teratocarcinoma), GCT27 (embryonal carcinoma), TCAM2 (seminoma) and GCT44 (yolk sac tumour) cell lines. All except the teratocarcinoma were kindly given by Dr Janet Shipley (Institute of Cancer Research UK), while the teratocarcinoma cell line was obtained from Dr Nicola Royle (University of Leicester).

All except the seminoma cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum (FBS) (Sigma) and 1% penicillin/streptomycin (Sigma).The seminoma cell line was grown in RPMI-1640 medium (Sigma) supplemented with 10% FBS and 1% penicillin/streptomycin

2.2. : Standard Reagents

2.2.1. : Agarose Gel

2.2.1.1. *: Preparation of 0.5M EDTA solution*

93.05 grams of EDTA (Sigma) was dissolved in 400ml sterile deionized water using a magnetic stirrer. Sodium hydroxide (Sigma) was added carefully until the pH reached 8.0. Additional deionized water was added to the solution to make up to 500ml.

2.2.1.2. *: Preparation of 50X TAE buffer*

242 grams of Tris base (Invitrogen) was dissolved in 750ml sterile deionized water. To this solution, 57.1ml glacial acetic acid (Sigma) and 100 ml of EDTA (pH 8.0) were added. Additional deionized water was added to the solution to make up to 1 litre.

2.2.1.3. *: Preparation of working 1X TAE buffer*

20ml of 50X TAE buffer was added to a 1 litre bottle. Sterile deionized water was then added to make up to 1 litre.

2.2.1.4. *: Preparation of agarose gel*

For 1.0% agarose gel, 0.5 grams of Tris base (Invitrogen) was added to 50ml 1X TAE buffer. The solution was then heated in a microwave for 5 minutes at high temperature. Once the solution cooled enough to be held, 5µl of 10mg/5ml Ethidium bromide (Sigma) was added to the solution. The solution was then poured into a gel rack with a comb and was left to solidify at room temperature. Once solidify, the gel was then submerged into a gel tank containing 1X TAE buffer.

2.2.2. : Chemicals

2.2.2.1. : *Decitabine (5-azadeoxycytidine, 5-azadC)*

5 mg Decitabine (Sigma) was diluted with 438 µl of PBS to make up 50 mM stock solution. Working solution of 5 mM was made by mixing 100 µl of 50 mM stock solution into 900 µl of medium (DMEM or RPMI-1640).

2.2.2.2. : *Cisplatin*

Cisplatin solution (Accord) was received at a concentration of 3.3 mM. For working solution of 0.33 mM, 150 µl of 3.3 mM Cisplatin were diluted with 1,350 µl of 0.9% NaCl.

2.3. : Standard Methods

2.3.1. : DNA Extraction

300 µl of lysis solution (Tris 1M at ph 8.5, EDTA 0.5M, NaCl 1M, SDS 10% and water) and ProteinaseK 10µg/µl were added to each DNA pellet and incubated at 37°C over 1 hour or until all tissue were dissolved. Samples were then subjected to centrifugation at 11,714 X G. The supernatants were transferred into 1.5ml tube. 300 µl isopropanol was then added to each sample to precipitate DNA. Visible DNA precipitates were then transferred into new tubes and washed with 70% ethanol. Samples were then air-dry before water was added to resuspend the DNA.

2.3.2. : BisulfiteConversion

Bisulfite treatment was performed according to the protocol supplied by EZ DNA Methylation Kit (Zymo Research). Briefly, each sample consisting of 500ng of DNA was mixed with M-Dilution Buffer and water was then added to a total volume of 50 µl. Samples were then incubated at 37°C for 15 minutes. CT Conversion Reagent was then added to each sample and the samples were incubated overnight in the dark at 50°C. The next day, samples were incubated on ice for 10 minute. Samples were then loaded into the designated column that had been already filled with a binding buffer and were centrifuged at 13,000 X G for 30 seconds. Samples were then washed with the wash buffer supplied before subjected to centrifugation at 13,000 X G for 30

seconds. Then, desulphonation buffer was added to each samples and were left to stand at room temperature for 20 minutes, after which samples were again centrifuged at 13,000 X G for 30 seconds. Samples were then washed twice; each wash was followed by 30 seconds centrifugation at 13,000 X G. DNA was then eluted by centrifugation at 13,000 X G in a microfuge tube by adding the elution buffer.

2.3.3. : RNA Extraction

300 µl of TRI Reagent (Sigma) was added to each sample, mixed and were left standing for 5 minutes at room temperature. Each sample was then added to 100 µl chloroform and again mixed and left to stand for 5 minutes at room temperature. The samples were then centrifuged at 13,000 X G at 4°C for 15 minutes. The supernatants were then mixed with 250µl isopropanol and were left at room temperature for 5 minutes. The mixtures were then centrifuged at 13,000 X G at 4°C for 10 minutes. The pellet was then washed with 200µl 75% ethanol and was centrifuged at 13,000 X G at 4°C for 5 minute. The samples were then left for air-dry for 5 minutes. 20 µl of water were added to resuspend the pellet. 1X buffer and 2 µl of DNAse (Invitrogen) were added to each sample. Samples were then incubated at room temperature for 30 minutes. After this incubation, 25mM EDTA were added to each samples and incubated at 65°C for 10 minutes. 1µl of each samples were then taken for RNA quantification. RNA samples were then used straight away for cDNA synthesis or frozen down at -80°C.

2.3.4. : cDNA synthesis

0.25 µM of random primer mix (Promega) was added to at least 2 µg of RNA. To each sample 0.1 µM DNTP (Bioline) and water was added to make a total volume of 13 µl. Samples were then heated at 65°C for 5 minutes before putting them on ice for 1 minute. Then, 5X buffer, 0.1M DTT and 100U/µl of reverse transcriptase (all Invitrogen) were added except for samples used as a negative reverse transcriptase control. All samples were then subjected to 1 cycle on a thermal cycler (25°C for 5 minutes, 50°C for 45 minutes and 75°C for 15 minutes).

2.3.5. : Polymerase Chain Reaction (PCR)

20 µl PCR reaction was carried out by mixing 100 ng genomic DNA with 1X Buffer (Invitrogen), 2.5 mM $MgCl_2$ (Invitrogen), 0.8 mM dNTP (Bioline), 0.1 µM forward and reverse primer and 0.05 U/µl Platinum Taq (Invitrogen). Samples were then subjected to PCR with a cycling condition as follows: 94°C for 5 minutes then 40 cycles of 94°C for 1 minute, 58°C for 1 minute and 72°C for 1 minute with a final extension for 5 minutes at 72°C.

2.3.6. : Reverse-Transcriptase PCR

25 µl PCR reaction was carried out by mixing approximately 100 ng cDNA samples with 1X Buffer (NEB), 0.5 mM dNTP (Bioline), 0.5 µM reverse and forward primer (refer table 2.1) and 0.05 U/µl Taq Polymerase (NEB). Samples were then subjected to 40 cycles of PCR (95°C for 1 minute, 58°C-60°C for 1 minute and 72°C for 1 minute). The PCR products were then run on 1.0 – 2% agarose gel depending on the expected product size.

2.3.7. : Real-Time PCR

Mastermix for each primer pair (refer table 2.1) were prepared by mixing 2X SYBR Green Mastermix (Applied Biosystem), 100 nM of forward and reverse primer, 30 nM of 1:500 reference dye (Applied Biosystem) and water up to 25 ml/well. 25 ml of those matermix were then transferred into each designated well of 96-well plate before adding 50 ng cDNA into each well.

Samples were then subjected to PCR using Stratagene MxPro 3005P machine. The PCR cycling condition are as follows: 95°C for 10 minutes followed by 40 cycles of 95°C for 30 seconds, 60°C for 40 seconds and 72°C for 40 seconds. All data were then analyzed using MxPro software.

2.3.8. : DNMT3B Knockdown using microRNA29b

3-4 X 10^7 YSTs cell lines were trypsinized into a single cells using 0.05% (1X) Trypsin-EDTA (Gibco) for 5 minute. Trypsin-EDTA was then neutralized by adding DMEM medium at three times the volume of Trypsin-EDTA. Cells were then centrifuged at 106 X G for 5 minutes. Cell pellets were then resuspended using 500 µl DMEM medium before being transferred into a 4mm/22mm electroporation cuvette (Yorkshire Bio Science). MicroRNA-29b plasmid (Open Biosystem) was then added to the medium at a concentration of 7 µg and was left at room temperature for 5 minute. The cuvette was then transferred into an electroporator and one pulse of electrical current was given at 250V for 10 miliseconds. Cells were then transferred into a new medium and were incubated for 48 hours. After that, cells were either pelleted down by centrifugation at 106 X G for 5 minutes and stored in the minus 80 freezer or trypsinized using Trypsin-EDTA to be used for other experiments.

Experiment	Sequences		Expected product size (bp)	Annealing temperature (°C)
A) Real-time PCR	Forward (5'→3')	Reverse (5' → 3')		
Beta-actin	CTGGCACCCAGCACAATG	GGACAGCGAGGCCAGGAT	92	58
DNMT3B	CGACAAGAGGGACATCTCACG	CAGAAACTTTGATGGCATCAATCA	68	58
EZH2	TAGGGAAGCAGGGACTGAAA	CACAACCGGTGTTTCCTCTT	354	58
SUZ12	TGCAGTTCACTCTTCGTTGG	TGCTTCAGTTTGTTGCCTTG	269	58
B) RT-PCR				
Beta-actin	TCTACAATGAGCTGCGTGTG	ATCTCCTTCTGCATCCTGTC	682	58
DNMT3B	CCAGGACTCGTTCAGAAAGC	CGTCTGTGAGGTCGATGGTA	237	58
CASP8	CTCTATCAGATTTCAGAAGAA	CCAACTTTCCTTCTCCCAGG	150	58
PYCARD	GAGCTCAAGAAGTTCAAGCT	CGTAGGTCTCCAGGTAGAA	138	58
FUT4	CAAGAGCATACGGAACTTG	GGTGTTTGAAGAACTTACTC	150	58
CDX1	CAGCCGTTACATCACAATCC	TTCTTGTTCACTTTGCGCTC	120	58
PRDM1	TTGCGCCAAGACTTTCGG	TGTGTACCAGGTAGTGTTTC	140	58
PARP12	TGCAAGTTCCTGAGAGCCG	TTGGCAAATTTCTGGCAAAAG	165	58
RPRM	TAATGCGCGTGGTGCAGAT	TCCGGTCCTTCACGAGGAA	129	58
C)LINE-1 Assay				
LINE-1	fam-TTGAGTTGTGGTGGGTTTTATTTTAG	TCATCTCACTAAAAAATACCAAACA	421	55
D) PYROSEQUENCING				
PYCARD	TTAGTTGTTATGATTTTAAGATTT	biotin-CCAAACCTCTAAATTAAAACC	294	55
PYCARD (sequencing primer)	TTGTTATGATTTTAAGATTTTA		100	55

Table 2.1: List of primers used in the analysis

2.3.9. : DNA methylation inhibition using 5-deoxyazacytidine (5-AzadC)

All four cell lines were grown in T75 flask until they were 70% confluent. Each cell line was then treated with a range of 5-AzadC (Sigma) concentrations (0.01 μM until 50 μM) for 24 hours. After 24 hours, medium were changed with a fresh medium without 5-AzadC added into them. Once the cell reached confluence, they were trypsinized and stored for further analysis.

2.3.10. : DNA Sequencing

2.3.10.1. *: Sequencing Set-up*

200 ng DNA was added to a 0.5 μl PCR tube and dried down at 80°C (if necessary). To this tube, 10 pmol of primer, 2 μl of 1:4 Big Dye (Applied Biosystem) and water were added to make up to 5μl.

In relation to CG-rich product, this protocol was modified to include 1M betaine (Sigma) and 2.5% DMSO in 10 μl reaction.

Sequencing was performed using G-Storm thermocycler with cycling condition as follows: 95°C for 5 minutes followed by 99 cycles of 95°C for 30 seconds, 50°C for 20 seconds and 60°C for 4 minutes.

2.3.10.2. *: Sequencing Clean-up*

Sequencing reactions were transferred into a 0.5 ml eppendorf tube. 95 μl of water was added to make up to 100 μl. 10 μl 3M Na acetate pH 5.2, 1 μl glycogen and 200 μl 100% ethanol were added to the mixture and incubated at -20°C for 15 minutes. Samples were then centrifuged at 13,000 X G for 15 minutes. The DNA pellets were then washed with 100 μl of 75% ethanol and were centrifuged at 13,000 X G for 5 minutes. Samples were air-dried for a few minutes before being sent to Source Biosciences, Nottingham for sequencing analysis. Data obtained from that sequencing were analysed using FinchTV version 1.4.

2.4. : LINE-1 Assay

A 60 µl PCR reaction was carried out according to (Yang et al., 2004), with a slight modification. Briefly, 30ng of bisulfitetreated DNA were mixed with a final concentration of 1X Buffer (Invitrogen), 0.8 mM dNTP (Bioline), 1.5mM $MgCl_2$ (Invitrogen), 0.06M Tris-HCl, 0.1 µM of forward and reverse primer (refer Table 2.1) and 0.05U/µl of Platinum Taq (Invitrogen). PCR cycling conditions were 94°C for 1 minute, 55°C for 1 minute and 72°C for 40s for 45 cycles. The final PCR products were digested with Hinf1 (New England Biolabs) restriction enzymes for 1 hour and were separated by electrophoresis on 1.4% agarose gel. Some of the digested products (20 µl) were sent for analysis using Genescan quantification. Data obtained from Genescan were then analyzed using PeakScanner software version 1.0.

2.5. : Amelogenin XY identification assay

This assay was performed according to the protocol supplied by Promega. 10 ng DNA was mixed with 10X STR Buffer, 10X primer pair (buffer and primer were supplied in GenePrint Fluorescence Sex Identification system kit, Promega) and 5 U/µl Kapa Taq (GRI) in 25 µl PCR reaction. Samples were then subjected to 2-step PCR. The first step involves 10 cycles of PCR (94°C for 1 minute, 64°C for 1 minute and 70°C for 1 minute and 30 seconds) and the second step involves 19 cycles of PCR (90°C for 1 minute, 64°C for 1 minute and 70°C for 1 minute and 30 seconds). 15 µl of PCR products were run on 1.5% agarose gel. The remaining 10 µl PCR products were sent for Genescan quantification. Data obtained from Genescan were then analysed using PeakScanner software version 1.0.

2.6. : Methylation Arrays

2.6.1. : Illumina GoldenGate methylation array

2.6.1.1. : *Methylation microarray analysis and quality control*

Methylation array analysis was conducted at the Wellcome Trust Centre for Human Genetics, University of Oxford, using the Illumina GoldenGate Cancer Panel I assay. This was done according to the maufacturer's instruction.

All quality controls were carried out using Beadstudio version 3.2 methylation module (Illumina) and *beadarray*, a program in Bioconductor R package (Dunning et al., 2007) which identifies outliers in the array. In addition to this, confounding spatial artefacts were also identified using the Beadarray Subversion of Highlight (BASH) (Cairns et al., 2008). All these outliers and confounding artefacts were excluded from subsequent analysis.

After background signal normalisation, β-values for each probe were reported and these values are in a range of 0 to 1, in which 0 means unmethylated while 1 represent a fully methylated probe.

2.6.1.2. : *Cluster analysis and identification of differentially methylated loci*

Using *pvclust*, another program from the R package, bootstrapped hierarchical clustering were performed (Suzuki and Shimodaira, 2006). Subgroups with a p-value of ≤ 0.05 were considered significant. The clustering pattern was then assessed using principal component analysis and k-mean analysis. For the later, a predetermined optimal number of cluster for analysis were determined using Scree plot.

To determine differentially methylated loci, a Mann-Whitney U-test was performed. Loci with p-value ≤ 0.05 after Benjamini-Hochberg false discovery rate correction were considered to be differentially methylated. An additional filter was also used for this purpose by using the average change in β-value between subgroups of more than 0.2. This value was then increased to more than 0.34 when comparing between tumour subtypes.

2.6.2. : Infinium Methylation450K arrays

Infinium methylation450 arrays were carried out at Queen's Mary University of London according to the manufacturer's protocol. Basically, genomic DNA was first subjected to bisulfite modification before being denatured into single-strand DNA and neutralized. These denatured samples were then isothermally amplified overnight at 37°C before being subjected to fragmentation using a controlled enzymatic process. The fragmented DNA was collected via isopropanol precipitation and subsequently resuspended in the hybridization buffer. These DNA samples were then subjected to

hybridization by applying them to the Illumina BeadChips. All unhybridized and non-specifically hybridized DNA was then washed away. Hybridized DNA were then underwent the extension process. Beadchips were then scanned where the fluorescence emitted from the single base extension products were recorded. This data were then extracted using Genome Studio Methylation Module software where the fluorescence intensity values were measured and represented as β-values ranging from 0 (not methylated) to 1 (fully methylated). Data normalization was then performed by this software using the internal control present in the beadchip.

2.7. Affymetrix gene expression array

2.7.1. RNA extraction

All four GCT cell lines were trypsinized and the number of cells was counted using a haemocytometer. 2 X 10^6 cells from each cell lines were pelleted by centrifugation at 300 X G for 5 minutes. RNA extraction was performed using the RNeasy extraction kit (Qiagen) according to the manufacturer protocol. Briefly, cells were lysed with lysis buffer (Qiagen) with added β-mercaptoethanol (Sigma). Cells were homogenized by passing the cell lysate into a shredder column (Qiagen) and centrifuged at 13,000 X G for 2 minutes. Ethanol was added to the homogenized cells lysate to promote RNA binding to the column supplied. Using the wash buffer supplied, columns were washed before RNA was eluted with RNAse-free water.

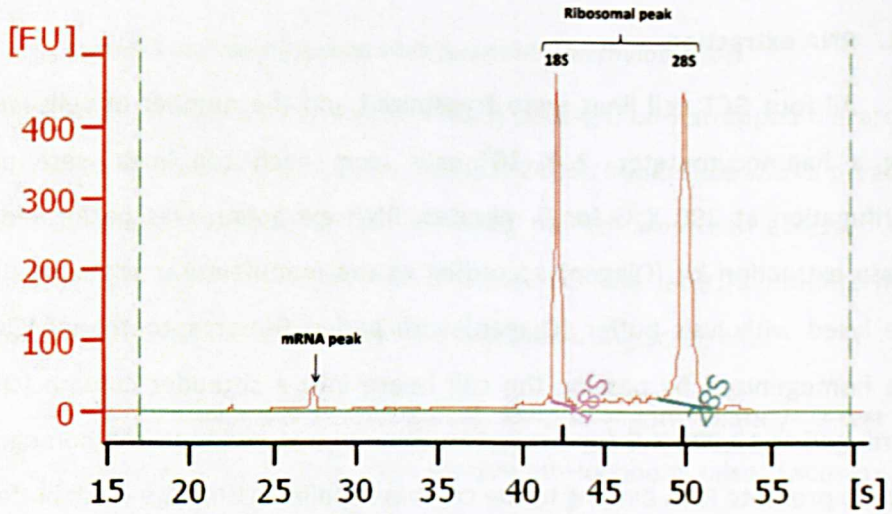
2.7.2. RNA Quality Measurement

It is a requirement for RNA to be of high quality in order to perform Affymetrix Gene expression arrays successfully. Therefore, to determine the quality of GCT RNA samples, we analyzed the RNA using a Bioanalyzer 2100 (Agilent) as suggested by the manufacturer.

Briefly, machine's electrodes were decontaminated with RNAseZap (Ambion Technology) to avoid sample decomposition. A gel-dye mix was made by mixing RNA 6000 Nano dye supplied by manufacturer with a filtered gel prepared earlier by the technician and was centrifuged at 13,000 X G for 10 minutes. Once chips were set up in

the priming station, gel-dye mix was put into the designated chip wells. RNA 600 Nano markers were then placed into wells designated for ladder and the samples. Ladders were supplied by the manufacturer and RNA samples were then placed into their designated wells. Chips were then placed into the Bionalayzer 2100 for analysis.

A successful sample run should feature one mRNA peak and two ribosomal peaks (Figure 2.1). Measurement was calculated using 2100 expert software version B.02.07 and displayed as RNA concentration, the ribosomal ratio and the RNA integrity number (RIN). For the purpose of selecting samples for Affymetrix Gene Expression array, samples with RIN > 9.0 were selected as this implies a high quality RNA samples.



Overall Results for sample 5 : yst1

RNA Area:	1,605.8
RNA Concentration:	1,203 ng/µl
rRNA Ratio [28s / 18s]:	1.7
RNA Integrity Number (RIN):	9.5 (B.02.07)

Figure 2.1: Representative result for RNA quality measurement in one of GCT samples which showed a high concentration of high quality RNA determined by the high RIN number.

2.7.3. Affymetrix Array

Arrays were performed at the Nottingham Arabidopsis Stock Centre, University of Nottingham Sutton Bonington Campus using GeneChip Human Genome U133 Plus 2.0 arrays.

All data were then analysed using GeneSpring GX (Agilent Technologies). Comparison between cell lines was made and significant differences of expression between cell lines were determined using a default setting of fold change > 2.0.

2.8. : Pyrosequencing

50µl PCR reactions were set-up for each sample individually (as each tumor concentration varies) with a final concentration of 1X buffer (2mM MgCl), forward and reverse primer (500nm each), 0.8mM dNTP, Dream Taq (Fermentas) 0.05 U/µl and around 100-120ng DNA. Samples were then subjected to 45 cycles of PCR. The PCR cycling conditions were 94°C for 10 minutes, followed by 45 cycles of 94°C for 60 seconds, 55°C for 60 seconds, and 72°C for 40 seconds with a final 5 minutes extension at 72°C.

10 µl of PCR products were then run on 2% agarose gel to check for product amplification and its quality. The remaining 40 µl PCR products were purified using PCR purification kits (Qiagen) and were sent to Queen Mary's University of London for pyrosequencing analysis.

2.9. : Clonogenic Survival Assay

2.9.1. : Cell's preparation

Media were prepared in advance by adding 60 ml DMEM or RPMI-1640 into a T75 flask. This amount of medium was needed to plate 3 X 6-well plates in which 3 ml medium is needed for each well. These T75 flasks containing the medium were then pre-heated to 37°C in the incubator.

A confluent T75 flask containing around 3-4 $X10^6$ cells was trypsinized using Trypsin-EDTA (TED) and incubated for 5 minutes in the incubator. Once cells had been

trypsinized into a single cell suspension, medium (DMEM or RPMI-1640) containing fetal bovine serum (FBS) was added in an amount of three times of that to the volume of TED to neutralize the TED. Cell suspension was then centrifuged at 106 X G for 5 minutes. After removing the supernatant, cell pellets were then resuspended thoroughly with 3 ml medium to make sure all the cells remained as single cells. Cells were then passed through a 40 µM cell strainer (MWG) into 50 ml Falcon tube.

2.9.2. : Cell counting

10 µl of single cells were taken out from the 50ml Falcon tube and mix with 10µl of Tryphan blue and the average number of cells was then determined using a haemocytometer (A X 10^4 cells/ml). To determine the amount of volume needed to plate 100 cells/ well, calculation was done using the formula below:

$$C_1V_1 = C_2V_2$$

$$(A \times 10^4 \text{ cells/ml}) \times V_1 = (100 \text{ cells}/ 3\text{ml}) \times 60\text{ml}$$

$$V_1 = \frac{33.3 \text{ cells/ml} \times 60\text{ml}}{A \times 10^4 \text{ cells/ml}}$$

$$= (0.1998/ A) \text{ ml}$$

$$= (199.8/A) \text{ µl}$$

The calculated amount was taken out from the single cell suspension in the 50 ml Falcon tube and transferred into a T75 flask containing 60 ml medium prepared earlier. This cell suspension media was mixed thoroughly by pipetting a few times before transferring 3 ml into each well of a 6-well plate. Plates were then incubates at 37°C in the incubator with 5% CO_2 for 1-2 hours to allow cells to attach on the plate.

2.9.3. : Cisplatin treatment

Once cells had adhered following plating, Cisplatin (Accord) was added at a range of 2.5 μM to 12.5 μM into each designated well. Cells were exposed to this treatment for 2 hours after which time medium was changed. Cells were then incubated for 7-10 days (depending on the cells' doubling time).

2.9.4. : Cell fixation and staining

Medium was removed from cells and these were then fixed with 1ml/well 4% paraformalaldehyde (PFA, Sigma) for 20 minutes at room temperature. PFA was then removed and cells were stained with 0.5 ml/well of 0.1% crystal violet (Sigma) for 5 minutes. Wells were then washed with distilled water and left to dry at room temperature.

2.9.5. : Migration Assay

1.5 – 2 X 10^3 cells were plated on each well of a 6 well-plate and were left in the incubator at 37°C with 5% CO_2. Once the cells reach 70-80% confluence, a scratch was made in straight line using a 10μl tip. Medium was then removed and cells washed with PBS for 5 minutes. After removing the PBS, fresh medium was added to each well. Cell migration was then observed under the microscope and pictures taken everyday for 5 days or until the scratch had closed. The distance between the sides of the gap created from the scratch was measured using ImageJ software.

2.10. : Statistical Analysis

All statistical tests were conducted using GraphPad Prism version 4.0 software.

Since tumour samples are from different patients, we assumed all data were not normally distributed. Therefore, non-parametric Mann-Whitney test were used to analyse the significance of the tumour data. The same applies when comparing between GCT cell lines.

For experiments investigating the difference before and after treatment of each cell line, we used a parametric paired t-test to analyse the significance of the findings.

3. METHYLATION PROFILE OF PAEDIATRIC GERM CELL TUMOUR SAMPLES

3.1. Introduction

There have been many methylation studies carried out in adult germ cell tumours (GCTs), but these studies were only conducted in testicular GCTs and based on a small number of candidate genes. The methylation profile of GCTs in the paediatric population and also GCTs that are formed outside of the gonads has not been fully explored. Thus, our study set out to investigate the methylation profile in these populations. To do this, two methods were employed, a LINE-1 assay and the Illumina GoldenGate methylation array.

Both of these methods use bisulfite treated DNA as the basis to differentiate between methylated and unmethylated DNA. This technique pioneered by (Frommer et al., 1992), is based on the principle that uracil is a thymine analog. During bisulfite treatment (Figure 3.1), addition of bisulfate will caused sulphonation of cytosine residue into cytosine-6-sulphonate. This process is reversible and the reaction equilibrium shifts towards formation of cytosine-6-sulphonate in acidic solution. Cytosine-6-sulphonate will then be converted into uracil-6-sulphonate by irreversible process of hydrolytic deamination. In an alkali condition, rapid desulphonation process will then result in the conversion of uracil-6-sulphonate into uracil.

LINE-1 elements are repeat elements abundantly found in the human genome. The measurement of LINE-1 repeat elements has been found to be applicable as a surrogate in determining genome-wide methylation (Estecio et al., 2007). Thus, this method has been widely used for such purposes. Here, we used an established LINE-1 assay (Yang et al., 2004)that employed a combination of bisulfite treatment and restriction analysis. By converting unmethylated cytosine to thymine, the recognition sites for the restriction enzymes, in our case Hinf1, are lost (Figure 3.2). Therefore, only methylated cytosines are digested, providing a simple and quick way to determine the methylation state of a sample.

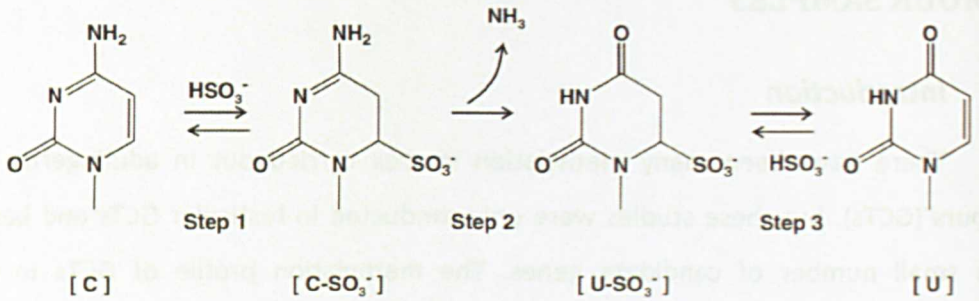


Figure 3.1: Steps involved in cytosine conversion to uracil. In step 1, sulphonation of the cytosine (C) residue by addition of bisulfite causes it to be converted to cytosine-6-sulphonate ($C\text{-}SO_3^-$). This process is reversible with the equilibrium shifted towards ($C\text{-}SO_3^-$) in acidic solution. Step 2 is the irreversible hydrolytic deamination of ($C\text{-}SO_3^-$) to uracil-6-sulphonate ($U\text{-}SO_3^-$). This molecule is stable in neutral solution but desulphonation process (Step 3) occurs rapidly in alkali condition resulting in the formation of uracil (U). Diagram was taken from Hayatsu (2008).

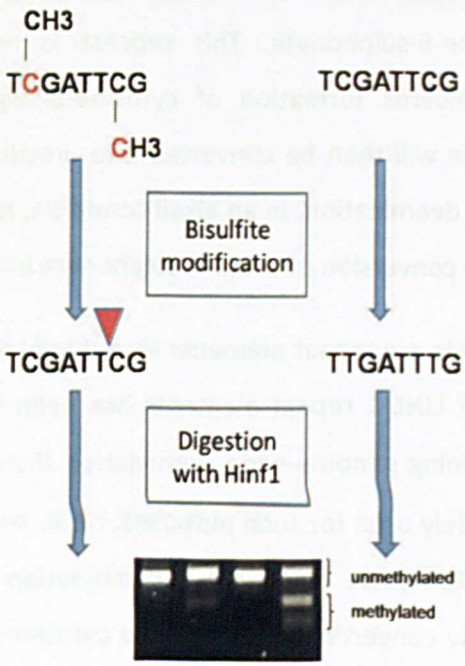


Figure 3.2: Steps in LINE-1 assay. In LINE-1 Assay, only unmethylated cytosine will be converted to thymine by bisulfite modification. This caused loss of Hinf1 recognition site (GANT/C, represented by the red triangle) and this site remains undigested.

The Illumina GoldenGate methylation array offers a genome-wide methylation perspective covering 1505 CpG sites that corresponds to 807 genes. Unlike previous methods that used restriction enzymes, Golden Gate arrays use two allele specific primers that can distinguish between the methylated and unmethylated sites and one primer containing the Illumina address code. All three primers will then hybridize to the DNA sample. The allele-specific primer that is complementary to the interrogating site will then undergo extension and ligate to the primer containing the Illumina address code. This full length product will then served as a template for subsequent PCR process. In this PCR, universal primer (P1, P2 and P3) is used. P1 and P2 are labeled with either red or green fluorescence that specifically recognized methylated or unmethylated allele, respectively. After downstream processing, single stranded, dye-labelled DNA will be subjected to hybridization to their complement bead type according to the Illumina address code. Successful hybridization to the BeadChip will produce the signal readout which will then be further analysed using computer software.

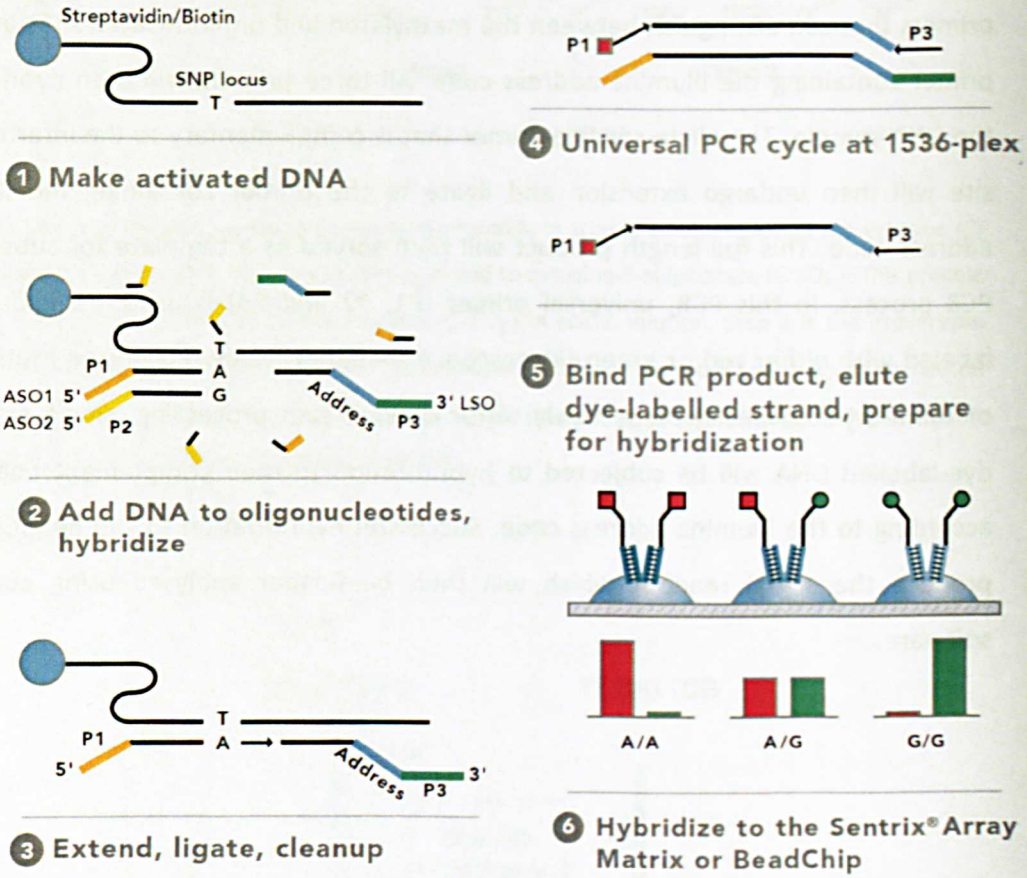


Figure 3.3: Illumina GoldenGate assay process. 1) DNA was activated to enable it to bind to the paramagnetic particle. 2) Three oligonucleotide primers (ASO1, ASO2 and LSO) were added to the DNA. ASO1 and ASO2 are specific primer that binds to either methylated or unmethylated allele while LSO is a universal primer that contains Illumina address code sequence attached to it.3) Primers that complement the interrogating site will then extend and ligate to the LSO primer. This product will then serve as a template for subsequent PCR. 4) In the subsequent PCR, three universal primers, P1, P2 and P3 will be used. P1 and P2 are fluorescently labeled and each of them is specific to either methylated or unmethylated allele (only P1 is shown in the figure) and they will then extend and ligate to P3. 5) After further downstream processing, single-stranded, dye-labelled DNA will then be subjected to hybridization to their complement bead type according to the Illumina address code. 6) Successful hybridization to the BeadChip will result in signal readout. Images taken from Illumina Inc. website (www.illumina.com)

3.2. Results

3.2.1. Investigating global methylation levels of paediatric germ cell tumours

It is generally believed that all cancers haveglobal genomic hypomethylation. Currently, few studies have been conducted in paediatric GCTs addressing this matter. The most recent study of a 'so-called' paediatric population was done in testicular GCTs samples with mean age of 35 years old (Ushida et al., 2011). Therefore, we would like to know whether global hypomethylation is also a feature of all types of GCTs in a younger population. In order to do so, we performed a LINE-1 assay on the 32 GCTs samples in our tumour bank.These samples were kindly given to us by our collaborator in Cambridge and originate from the UK Children Cancer and Leukemic Group (CCLG) bank. Out of these 32 samples, 12 were seminomas, 20 YSTs and 18 controls (Table 3.1). These samples were taken from patients of different ages with an average age of eight years old (average of five, thirteen and seven years old for YSTs, seminomas and controls respectively). To address the global methylation features of extragonadal GCTs, samples taken from the brain, sacrococcygeal (SCT), liver and abdomen are also included in our samples.

Sample ID	Histology	Site	Age (years old)
X59	Seminoma	Ovary	12
X33	Seminoma	Ovary	12
X17	Seminoma	Ovary	14
X110	Seminoma	Ovary	3
X45	Seminoma	Ovary	8
X112	Seminoma	Testis	15
X111	Seminoma	Testis	9
X22	Seminoma	Testis	25
X42	Seminoma	Brain	12
X67	Seminoma	Brain	16
X35	Seminoma	Brain	11
X30	Seminoma	Brain	16
X8	YST	Ovary	12
X19	YST	Ovary	12
X2	YST	Ovary	12
X15	YST	Ovary	12
X14	YST	Ovary	5
X76	YST	Testis	2
X6	YST	Testis	1
X36	YST	Testis	1
X38	YST	Testis	1
X12	YST	Testis	1
X43	YST	Testis	1
X34	YST	Brain	12
X73	YST	Brain	3
X48	YST	Abdomen	12
X79	YST	Abdomen	<1
X113	YST	Liver	3
X54	YST	SCT	2
X57	YST	SCT	2
X11	YST	SCT	16
X46	YST	SCT	2
X16	Control	Ovary	<1
X89	Control	Ovary	<1
X91	Control	Ovary	43
X92	Control	Ovary	7
X87	Control	Ovary	<1
X88	Control	Ovary	<1
X96	Control	Testis	<1
X100	Control	Testis	<1
X93	Control	Testis	9
X97	Control	Fetal Yolk Sac	<1
X101	Control	Fetal Yolk Sac	<1
X90	Control	Fetal Yolk Sac	<1
LBI3	Control	Blood	1
EWI2	Control	Blood	1
MDI1	Control	Blood	2
A3	Control	Buccal	30
A2	Control	Buccal	22
A1	Control	Buccal	20

Table 3.1: List of paediatric germ cell tumour samples and controls used in LINE-1 Assay.

Samples were subjected to bisulfite modification and 40 cycles of PCR using LINE-1 specific primers. Each of the PCR products was divided into two, and one of them was digested with Hinf1 for 1 hour. Both digested and undigested samples were run on an agarose gel. We observed that most of the tumour samples showed a low signal for the digested product compared to the controls (Figure 3.4). This indicates that most of the tumour samples have low methylation of LINE-1 repeat elements.

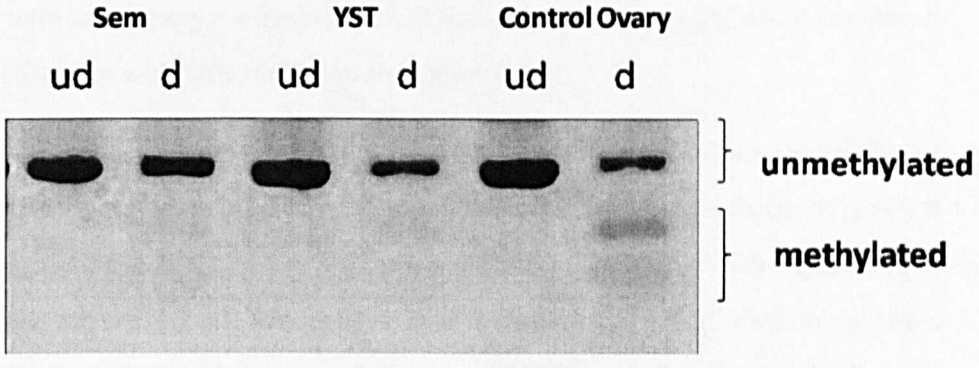


Figure 3.4: Representative gel picture of LINE-1 assay on paediatric GCT samples. It clearly showed here that there are less amplification of the digested product in both YST and seminoma. ud is for undigested and d is for digested

However, we found that this method did not give reliable quantitative data. To improve this method and make it more quantitative, we designed a FAM-labelled forward primer for the PCR. The digested PCR products were then analysed using Genescan, where the fluorescence intensity from both digested and undigested products were measured. Using Peak Scanner software, the digested and undigested PCR products were detected according to their sizes (Figure 3.5). The methylation level of each samples were then calculated by using the equation below:

$$\text{LINE-1 methylation level} = \frac{\text{Area in point for the digested peak (methylated)}}{\text{Area in point for the digested peak (methylated)} + \text{area in point for the undigested peak (unmethylated)}}$$

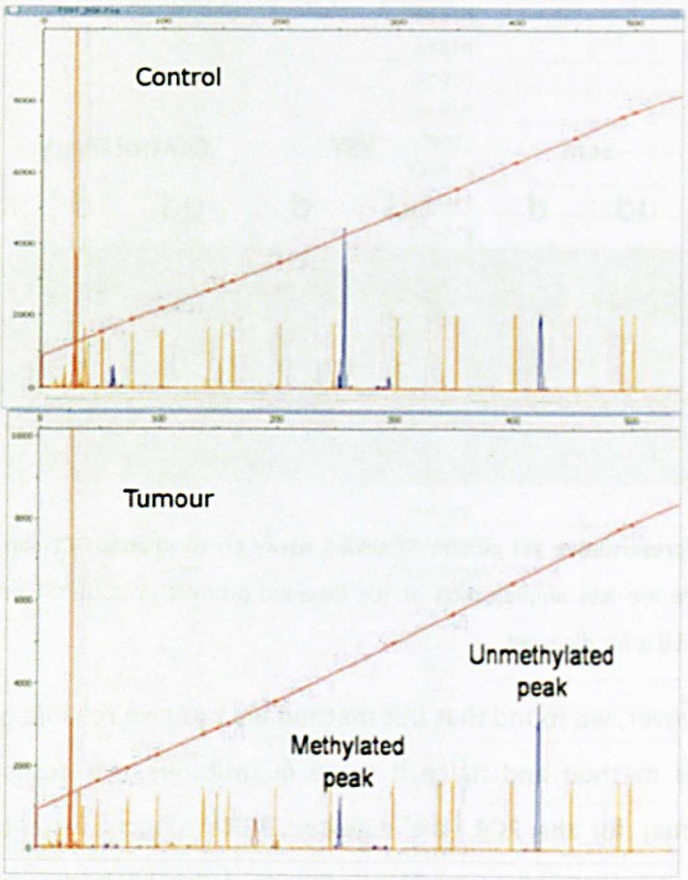


Figure 3.5: LINE-1 assay analysis using Genescan. Representative diagram of Genescan using Peak Scanner software showing the methylated and unmethylated peak of digested LINE-1 assay PCR products.

Using the above formula to calculate the methylation level of each sample, it was evident that the majority of both seminoma and YST sampleshave lower methylation levels than the controls (Figure 3.6). Due to the limited amount of DNA available we were unable to complete replicates on all of the samples.

In order to determine the significance of hypomethylation seen, we grouped these samples according to their tumour type. As expected, we found that both seminomas and YSTs showed significant hypomethylation of LINE-1 repeat elements ($p<0.0001$) with an average methylation of 32% and 42% respectively, when compared to the control group with 68% methylation (Figure 3.7 A).

We then compared these tumour samples to their matched tissue. We found that both seminoma and YSTs were hypomethylated when compared to control samples (Figure 3.7 B & C). However, this hypomethylation was only significant in ovarian tissue (Figure 3.7 B). We believe this is due to the small number of testis controls and in addition that one of these controls exhibited an unusually low methylation value (labelled as X in Figure 3.6). However, given that no prior history was available for this volunteer's sample, we could not rule out whether other health factorscould have influenced this observation.

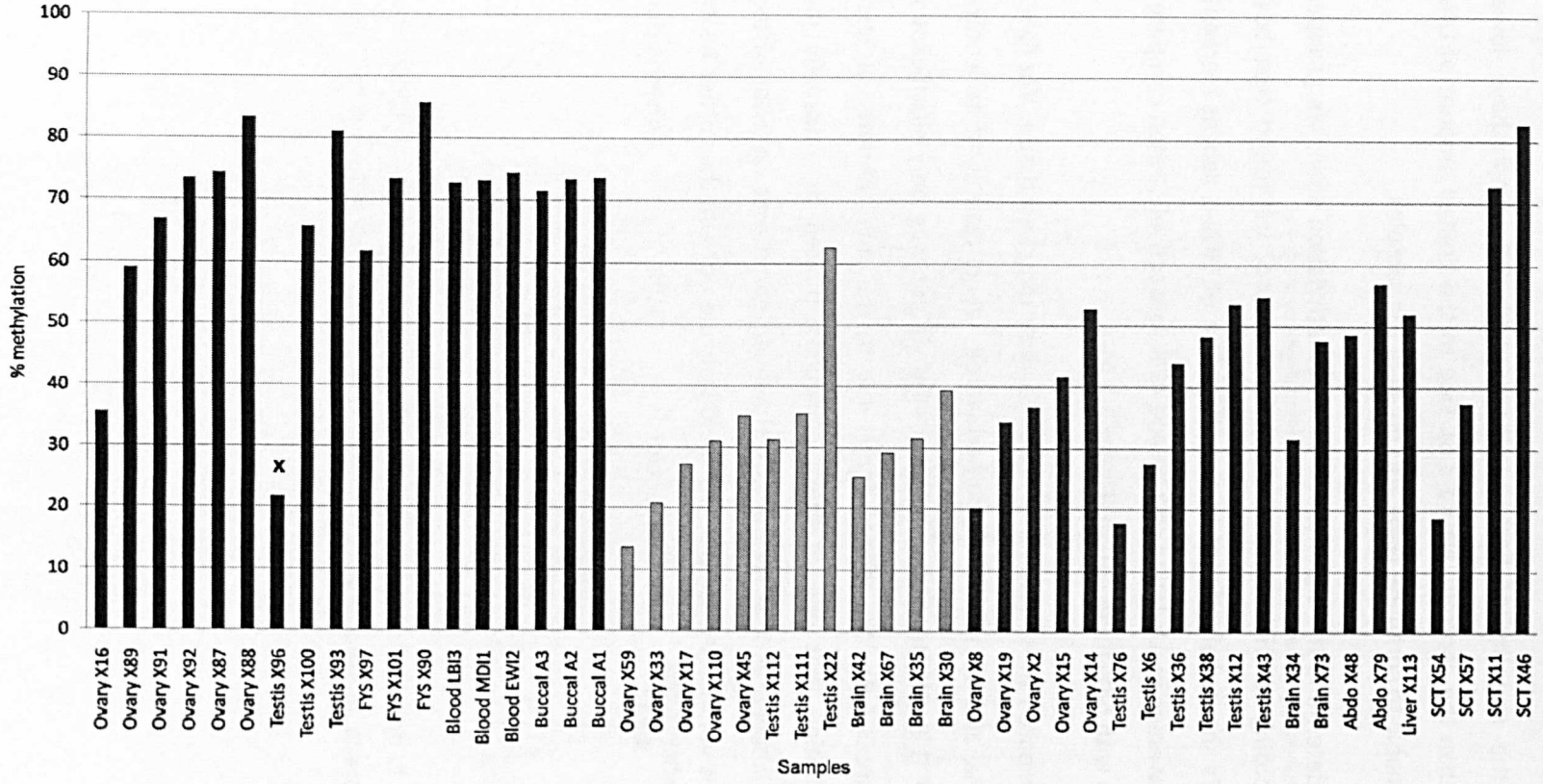


Figure 3.6:Bar graph showing methylation level of LINE-1 repeat elements in the GCTs and the controls.Majority of seminoma and YST samples displayed low methylation of LINE-1 repeat elements compared to the controls.FYS represents fetal yolk sac. The black bars represent the control, lighter grey represents seminoma and darker grey represents the YST. X denotes the presence of a control testis sample with low methylation.

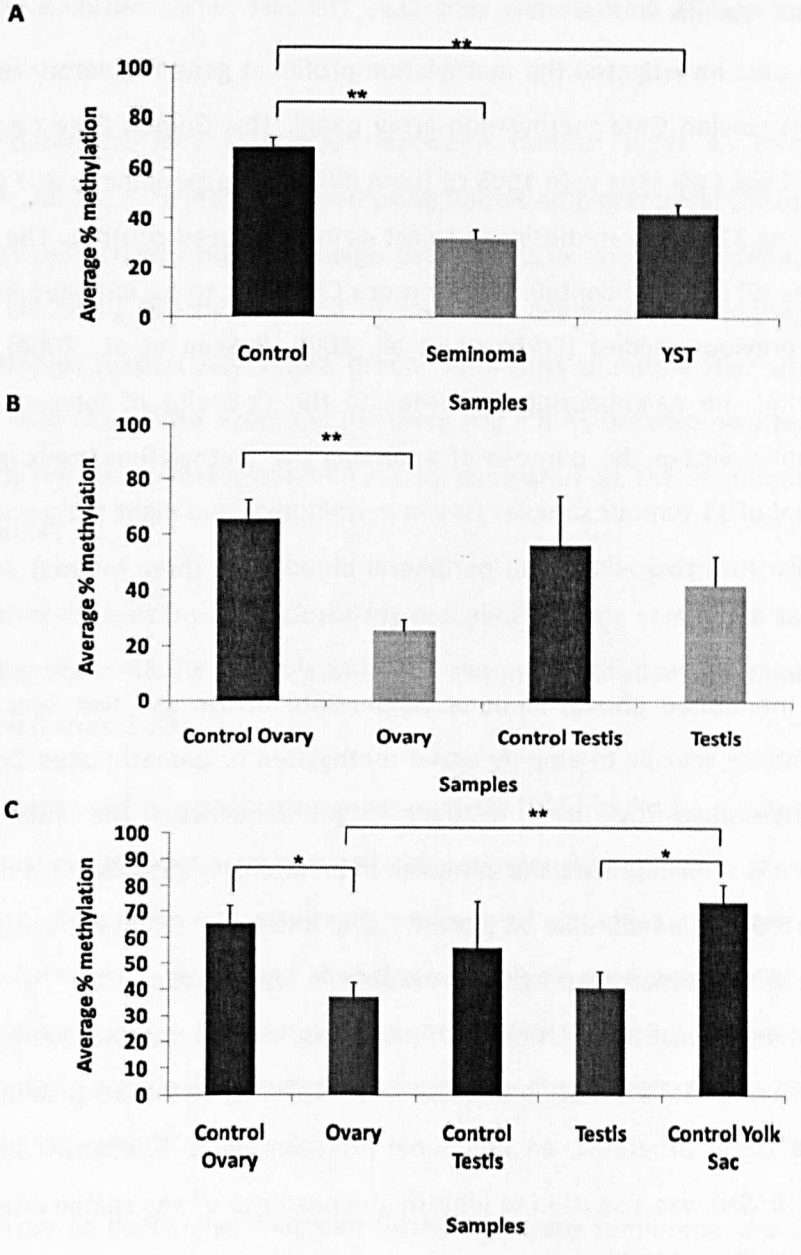


Figure 3.7: LINE-1 methylation level in A) each tumour group, B) matched tissues in seminomas, C) matched tissues in YST. Both seminoma and YST samples displayed significant hypomethylation of LINE-1 repeat elements compared to the controls. However in both tumour types, only ovarian samples showed significant hypomethylation of LINE-1 repeat elements compared to its matched tissue controls. * denotes p-value <0.01 while ** denotes p-value <0.001using Mann-Whitney t-test.

3.2.2. : Methylation level at the gene regulatory region

3.2.2.1. : *Data Analysis*

We next investigated the methylation profile at gene regulatory regions using the Illumina Golden Gate methylation array panel. This Golden Gate Cancer Panel I consists of 1536 CpG sites with 1505 of them (98%) corresponding to 807 genes while the remaining 31 sites were designed to act as internal bead controls. The majority of these genes (576 genes) contain two or more CpG sites to be analysed in the panel. Based on previous studies (Eckhardt et al., 2006, Rakyan et al., 2004), this panel assumed that the neighbouring CpG sites to the CpG site of interest has similar methylation levels.For the purpose of analysing the methylation levels in paediatric GCTs, a total of 15 tumour samples (seven germinomas and eight YSTs) and 5 controls (buccal cells from two adults and peripheral blood from three infants) were initially analysed.

As mentioned above, Illumina GoldenGate arrays use two sets of labelled primers that are specific to amplify either methylated or unmethylated CpG sites and will fluoresce once they bind to their target sequences. The intensity of the fluorescence is then captured and compiled in an intensity data file. This data file was then exported into BeadStudio v3.2 methylation module™ (Illumina, San Diego, USA) and along with beadarray program available in the Bioconductor®, probe quality assessment was performed. Using both programs, tumour samples were excluded if more than 1% of 1505 target probes showed a detection probe p-value > 0.05. In addition to these programs, an additional program called Beadarray Subversion of Hashlights (BASH) was also used to identify the presence of any spatial effect that may undermine further analysis.

After quality control assessment, bead summary signal taken from the green and red fluorescence channels were analysed with the beadarray program. After background normalisation, the corrected methylation scores or β-values were calculated which produced a value between zero and one; zero represent unmethylated CpG site while one represent fully methylated CpG site.

Once the β-value was corrected, 84 probes that represent 39 X-chromosome genes were excluded due to discrepancies between male and female methylation levels at the X-chromosome. This left 1421 sites representing 708 genes for further analysis.

To determine any association between tumour types in terms of their methylation, all the data were analysed using bootstrap hierarchical clustering (Fig 3.8 B). This was performed using R package pvclust (Suzuki and Shimodaira, 2006). The generated clustering heatmap consist of rows and columns, representing the genes and the samples respectively where green represents unmethylated site while red represents methylated site. From the heatmap (Fig 3.8 A), we observed that there are areas which are more methylatedin YSTs as compared to the seminomas and the control samples.

Further analysis from the clustering revealed that the seminoma samples were clustered together with the controls while YST samples formed an independent cluster on their own (Figure 3.8B).

We next used principal component analysis (PCA) to find out whether we can separate our samples into smaller variable components. First, a scree plot was measured to determine the minimum amount of variables that are considered meaningful for PCA. It was found that three variables were enough for PCA in our paediatric samples. From the PCA, we found that the clustering between seminoma and the controls were still retained. Interestingly, there seem to be two separate groups in the YST samples (Figure 3.9A).

In order to determine if we can further separate seminoma and the controls into separate entity, another type of cluster analysis, k-mean analysis was used. From this analysis we failed to separate seminomas and controls, which strongly suggest the similarities shared between these two sets of samples in terms of their methylation. Interestingly, we managed to further separate YSTs into two groups (Figure 3.9B). This suggests the presence of two YST subtypes that can be differentiated by their methylation levels.

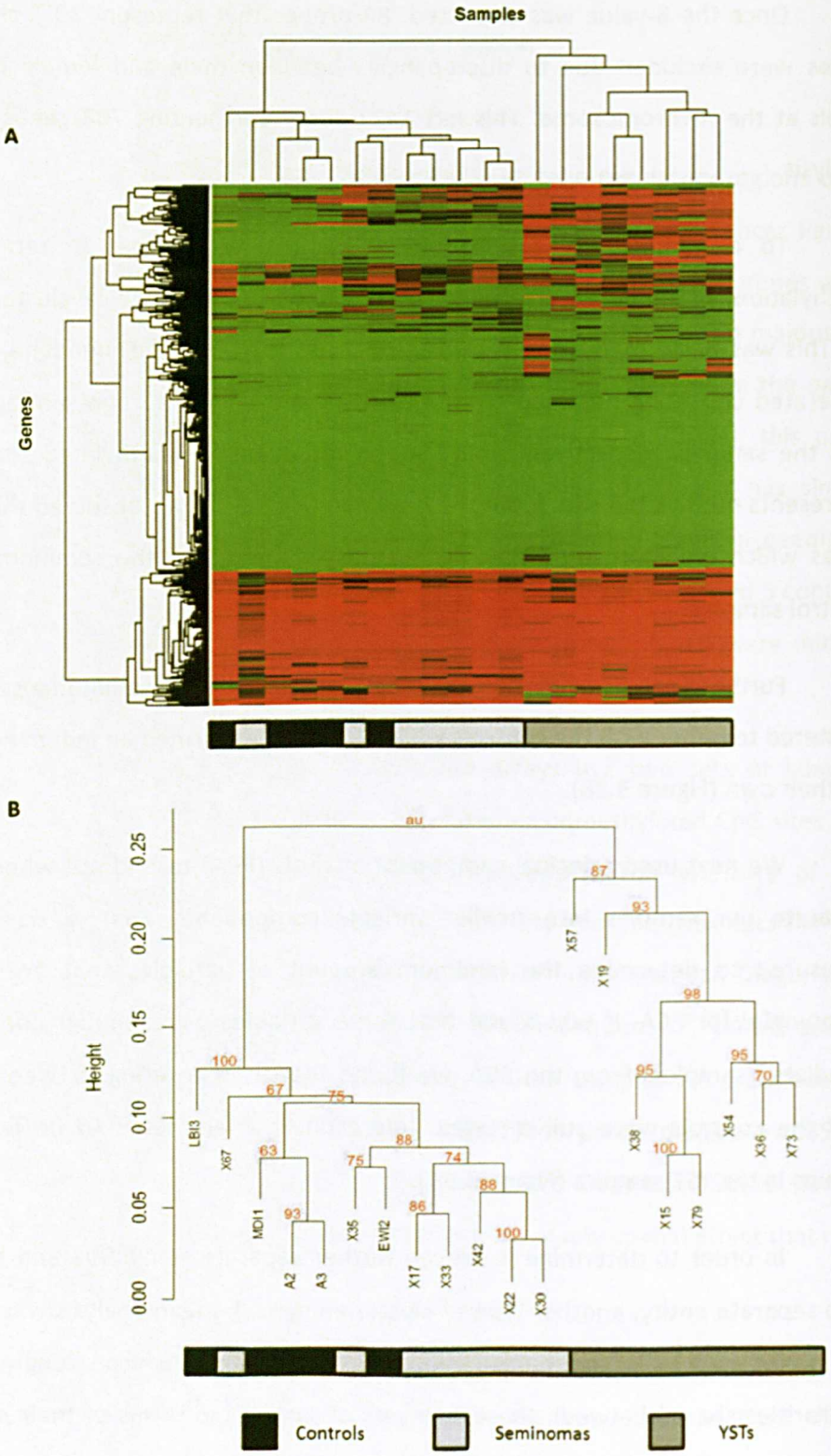


Figure 3.8: Heatmap and bootstrapped hierarchical clustering analysis. A) Heatmap produced from Infinium analysis showing differentially methylated area in YST when compared with seminomas, B) bootstrap hierarchical clustering showing seminomas clustering together with the controls.

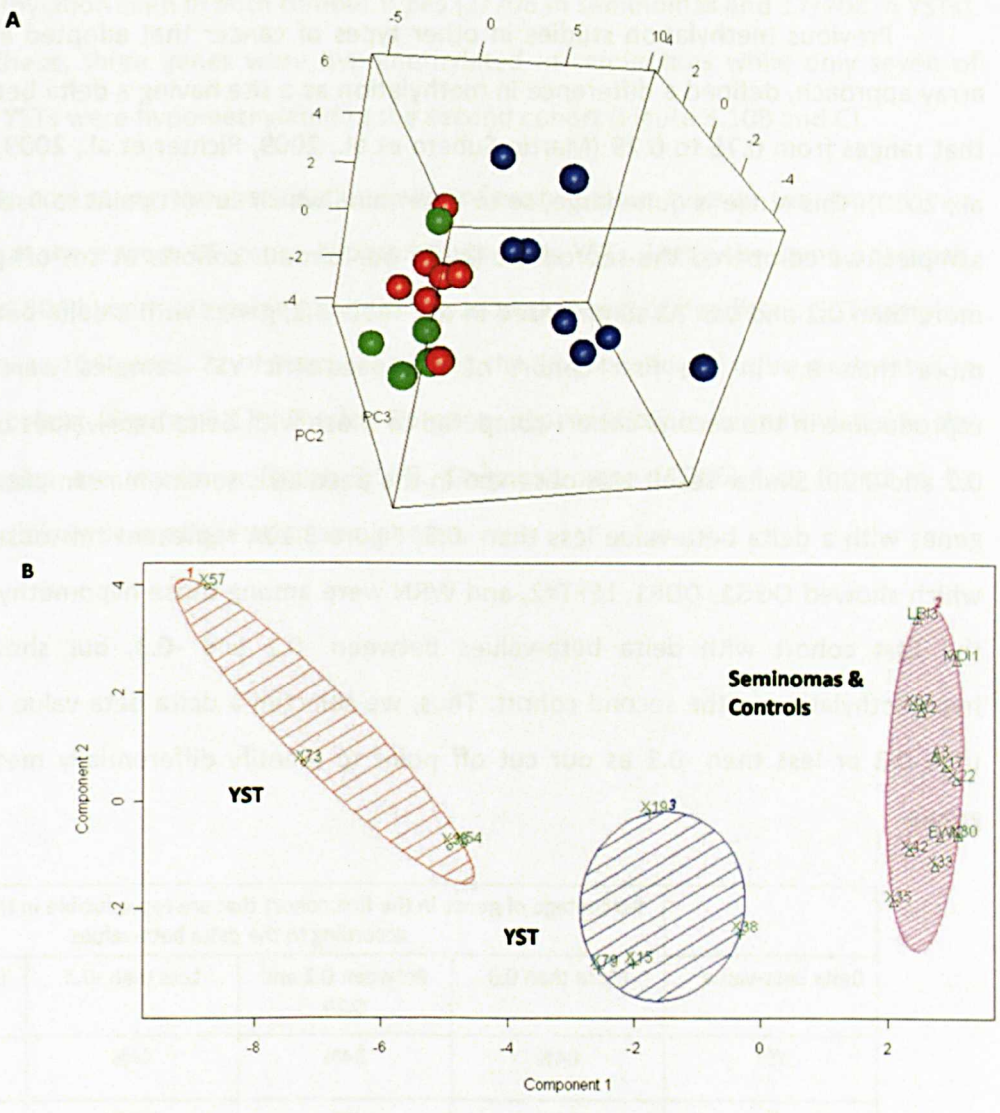


Figure 3.9: Further clustering analysis using principal component analysis and k-mean analysis still showed seminomas clustered with the controls. A) Principal component analysis showing YST (blue circles) forming an independent cluster while seminomas (red circles) are clustering together with the controls (green circles), B) k-mean analysis showing YST can be further clustered into two methylation group.

3.2.2.2. ***: Identification of differentially methylated genes***

In order to help identify genes that are differentially methylated between seminoma and YSTs, an additional independent cohort of samples were analysed. This independent cohort consisted of nine seminomas and eleven YST samples.

Previous methylation studies in other types of cancer that adopted a similar array approach, defined a difference in methylation as a site having a delta beta-value that ranges from 0.75 to 0.29 (Martin-Subero et al., 2009, Richter et al., 2009, Ang et al., 2010). This range is quite large, so to determine which cut-off point to use for our samples, we compared the reproducibility of our tumour cohorts at cut-off point of more than 0.2 and 0.3. As summarized in the Table 3.2, genes with a delta beta-value more than 0.3 in the first cohort of the paediatric YST samples were highly reproducible in the second cohort compared to those with delta beta-values between 0.2 and 0.29. Similar result was observed in the paediatric seminoma samples, and in genes with a delta beta-value less than -0.3. Figure 3.10A represent this observation which showed OGG1, DDR1, LEFTY2, and WRN were among those hypomethylated in the first cohort with delta beta-values between -0.2 and -0.3, but showed no hypomethylation in the second cohort. Thus, we selected a delta beta-value of more than 0.3 or less than -0.3 as our cut off point to identify differentially methylated genes.

	Percentage of genes in the first cohort that are reproducible in the second cohort according to the delta beta-values			
Delta beta-value	More than 0.3	Between 0.2 and 0.29	Less than -0.3	Between -0.2 and -0.29
YST	84%	34%	44%	36%
Seminoma	25%	7%	75%	33%

Table 3.2: Majority of genes with delta beta-value more than 0.3 or less than -0.3 are reproducible. Table shows the percentage of genes in the first cohort that pass the same delta beta-value thresholdin the second cohort.This shows that genes with delta beta-values of more than 0.3 or less than -0.3 are highly reproducible which leads us to use this value to define differentially methylated genes.

In order to identify genes that were hyper or hypomethylated, we sorted the raw data in an Excel file and ranked them from the highest to the lowest delta beta-value. Only delta beta-values of more than 0.3 or less than -0.3, were considered to show significant difference in methylation. In the first cohort there was little hypomethylation seen in both tumour types (3/708 in seminomas and 17/708 in YSTs). Out of these, three genes were hypomethylated in seminomas while only seven of those in YSTs were hypomethylated in the second cohort (Figure 3.10B and C).

By comparing the methylation value of each tumour type to the controls, we found that there were 85 genes hypermethylated in YSTs. Using the same approach, we found 104 hypermethylated genes in the second cohort of paediatric GCT samples. Out of these 104 genes, 71 of them are among the 85 genes found to be methylated in the first cohort (Figures 3.11). The top 25 genes reproducibly hypermethylated in the YST samples are shown in Figure 3.11B. Only one gene (*CD86*) was found to be reproducibily hypermethylated in seminomas.

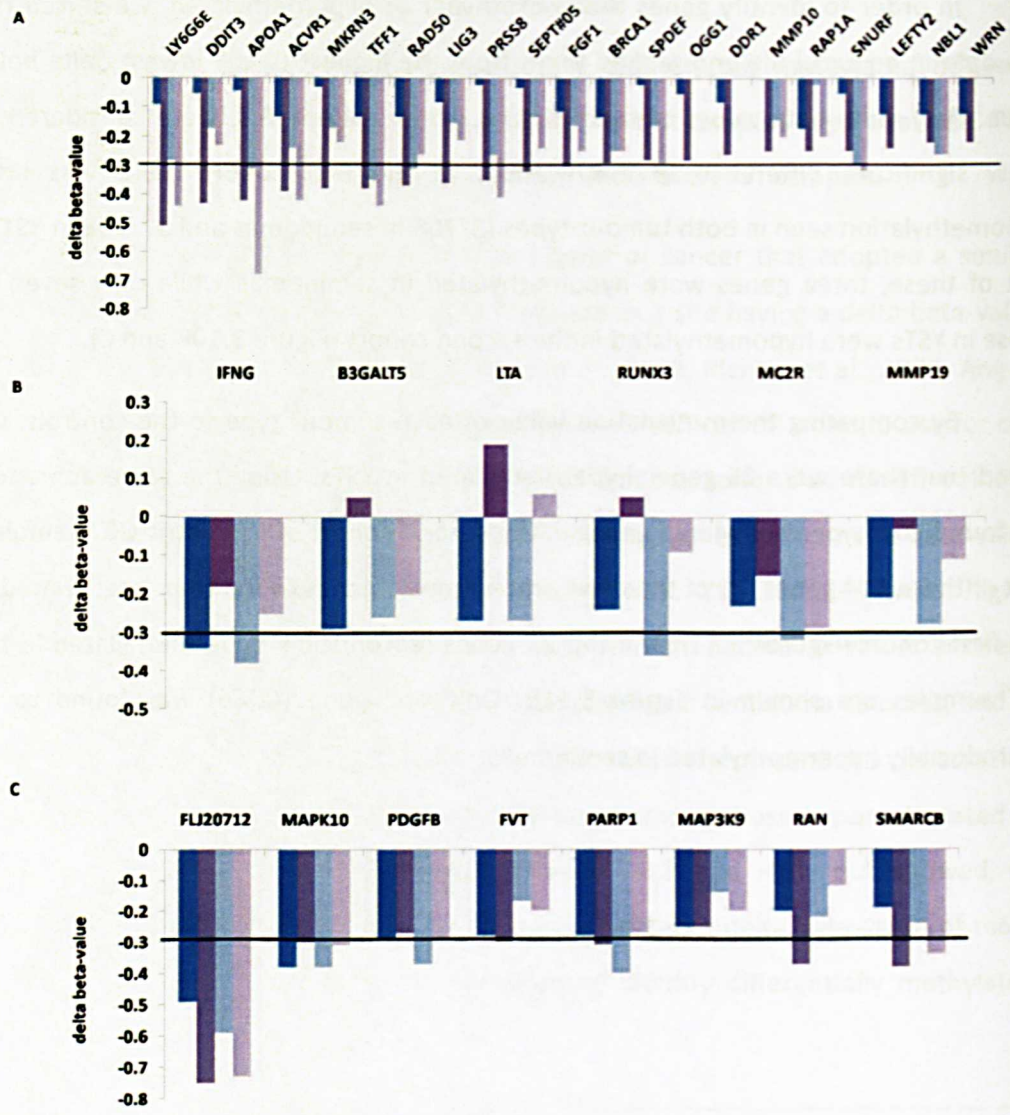


Figure 3.10:Relatively small amount of genes are reproducibly hypomethylated (with delta beta-value less than -0.3) in both tumour types. Graph showing comparison of differentialy methylated genes between the two cohorts of tumours A) hypomethylated in YSTs and B) hypomethylated in seminomas C) hypomethylated in both YSTs and seminomas.Figure 3.10A represent observation that genes with delta beta-value of more than 0.3 or less than -0.3 are reproducible. For example OGG1, DDR1, LEFTY2, and WRN that displayed delta beta-values between -0.2 and -0.29 in the first cohort (dark purple) showed no hypomethylation in the second cohort (pale purple).This figuresalso showed that there was little hypomethylation observed in both tumour type.Blue bars represent seminomas while purple bars represent YSTs. Black line represents the cut-off point of 0.3. Darker colour bars represent the first cohort while pale colour bars represent the second cohort of tumours

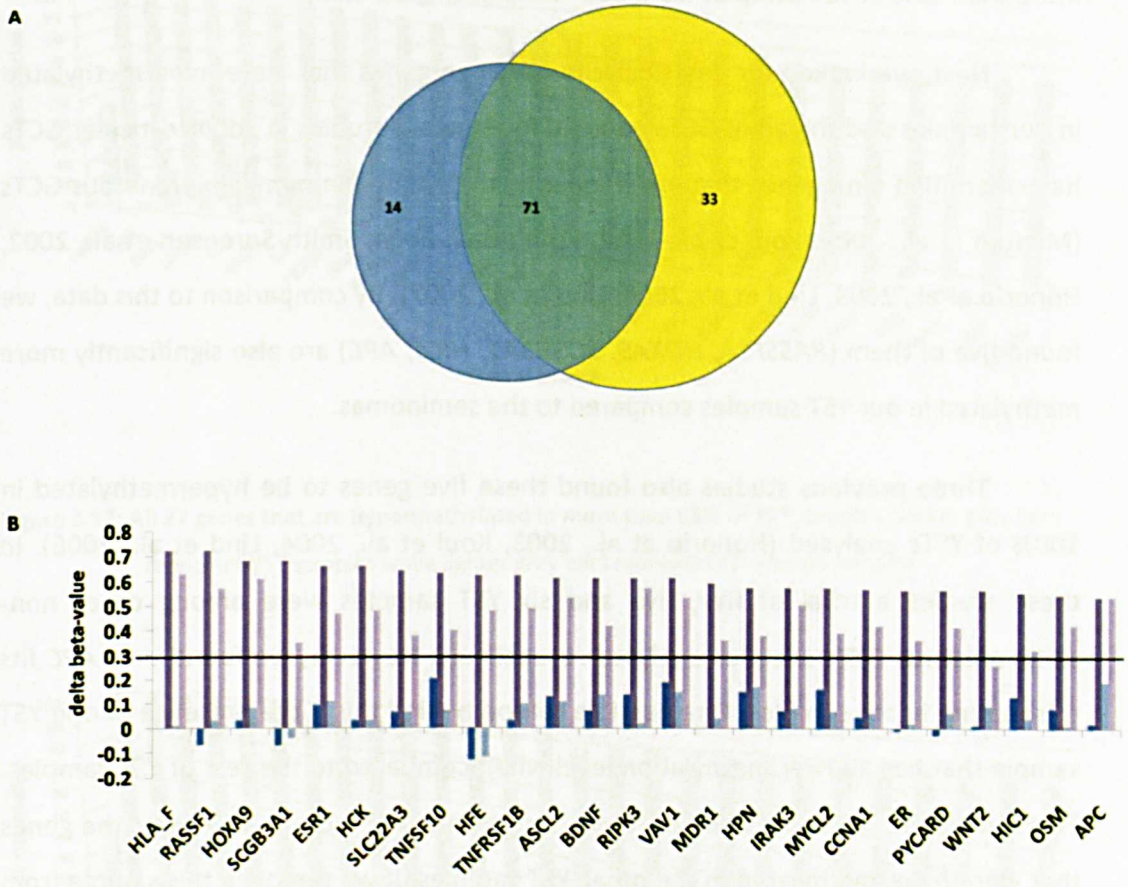


Figure 3.11Majority of genes hypermethylated in YSTs in the first cohort were also found to behypermethylated in the second cohort. A) Venn diagram summarizing the number of genes hypermethylated in YSTs in the first (blue) and second (yellow) cohort showing an overlapped of 71 similar genes. B) Top 25 hypermethylated genes in YSTs in the first cohort (dark purple) and reproducibly hypermethylated in the second cohort (pale purple). Out of those 25 genes, only one gene, *WNT2*, did not maintain delta belta-value more than 3.0 in the second cohort. Blue bars represent seminomas.

We then questioned whether the observed hypermethylation is due to inclusion of a few samples in which those genes are highly methylated that skewed the data. To answer this, we looked at methylation level for the 85 genes initially identified in each tumour samples. We found that 27 of these genes were hypermethylated in more than 85% of YST samples from both cohorts (Figure 3.12).

Next, we looked for any similarity between genes that were hypermethylated in our samples and the adult GCTs population. Previous studies in adult testicular GCTs have identified nine genes that are frequently methylated in non-seminomatous GCTs (Manton et al., 2005, Koul et al., 2002, Koul et al., 2004, Smith-Sorensen et al., 2002, Honorio et al., 2003, Lind et al., 2006, Lind et al., 2007). By comparison to this data, we found five of them (*RASSF1A, HOXA9, SCGB3A1, HIC1, APC*) are also significantly more methylated in our YST samples compared to the seminomas.

Three previous studies also found these five genes to be hypermethylated in 100% of YSTs analysed (Honorio et al., 2003, Koul et al., 2004, Lind et al., 2006). In these studies a total of five, five and six YST samples were among other non-seminomatous GCTs samples analysed respectively. However, we found only *APC* fits this feature in our samples. One possible reason behind this is the presence of one YST sample that has a lower methylation level when compared to the rest of YST samples. This sample did not show significant hypermethylation in more than 70% of the genes that were hypermethylated in the other YST samples. If we removed this sample from our analysis, we found an additional two genes, *HOXA9* and *PYCARD*, to be hypermethylated in 100% of YST samples (Figure 3.13).

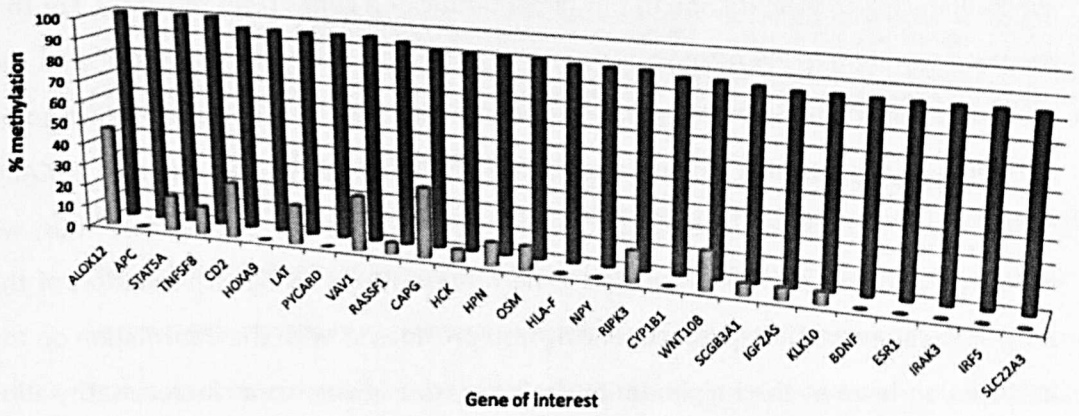


Figure 3.12: All 27 genes that are hypermethylated in more than 85% of YST samples. Darker grey bars represent YST samples while lighter grey bars represent seminoma samples

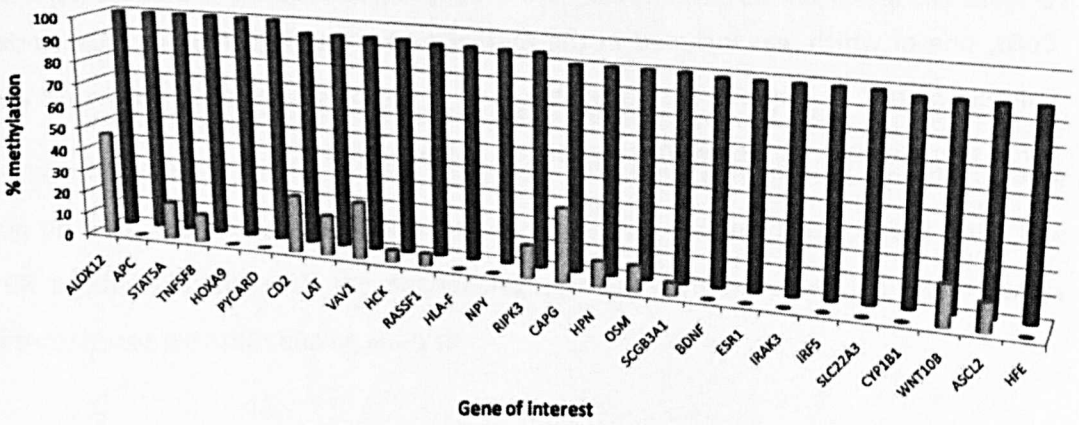


Figure 3.13: Additional 2 genes (*HOXA9* and *PYCARD*) found to be hypermethylated in 100% of YST samples after removing one YST sample that lacked a methylator phenotype. It also showed 21 out of 30 genes that are methylated in more than 80% of YST samples. Darker grey bars represent YST samples while lighter grey bars represent seminoma samples.

3.2.3. : Validation using pyrosequencing

In order to verify that the methylation level seen in the array reflected the real situation, and not due to bias from the method used in the array, we used pyrosequencing to validate one of our hypermethylated genes from the array. For this purpose, we choose *PYCARD* as our gene of choice. This is because it is one of the genes that were significantly hypermethylated in all YSTs and it has been shown to be differentially expressed in seminomas (will be discussed below). Despite this, *PYCARD* only showed a moderate methylation level between YSTs and seminomas. Thus, we believed validation performed on this gene represented a stringent validation of the array. In addition to that, pyrosequencing also provides us with the information on the methylation level at the neighbouring CpG site, thus giving us a clearer methylation profile across the CpG islands. Furthermore since the GoldenGate probe was designed with an assumption that CpGs neighbouring the methylated CpGs are also methylated, pyrosequencing also serves as a test of this assumption.

This pyrosequencing of the *PYCARD* promoter region started at -234bp upstream of the transcription start site. It amplified a 100bp region that covered six CpGs, one of which was included in the methylation array (Figure 3.14). Due to the limited amount of genomic DNA available, only 5 samples were used (two YSTs and three seminomas) and they were only carried out in duplicate.

Figure 3.14: Diagram showing the location of *PYCARD* pyrosequencing primers. The upper sequence is *PYCARD* genomic DNA sequence before bisulfite treatment. The sequence underlined is the sequence of interest which is represented by the lower sequence after bisulfite treatment. The forward, reverse and sequencing primers are represented by the arrow. The underline in the lower sequence represents the sequence analysed by the pyrosequencing. CG in bold red represents the methylated CpG which are maintained as CpG even after bisulfite treatment.

Selected GCT samples that had been bisulfite treated were subjected to 40 cycles of PCR (95°C for 1min, 56°C for 1 min, 72°C for 1 min). The PCR products were run on 1.5% agarose gel to make sure there were no contaminants (Figure 3.15). The PCR products along with the sequencing primer were then sent to Queen Mary University for pyrosequencing analysis.

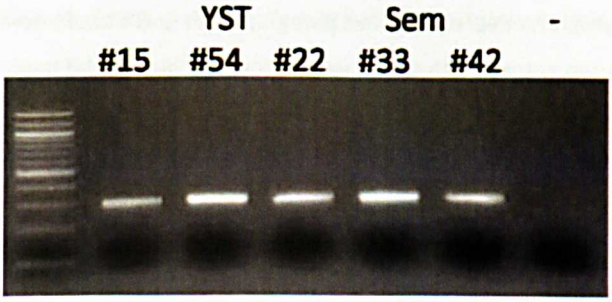


Figure 3.15: Representatitive gel showing amplification of *PYCARD* pyrosequencing PCR products.

Completed pyrosequencing results were sent back in a form of a graph called a pyrogram. Each pyrogram represents an analysis of all 6 CpGs for each tumour samples which showed the percentage of methylation at each CpG of interest (Figure 3.16). Data obtained from the pyrogram were then tabulated and represented on a graph. From the graph, we saw that the YSTs showed a higher methylation level compared to the seminomas at all six CpG sites that were covered (Figure 3.17 A). When we compared the one CpG site that was also included in the methylation array, the level of methylation pattern was found to be similar between pyrosequencing and the array (Figure 3.17 B). In fact from this same graph, it can be seen that pyrosequencing is more sensitive than the array in picking up the methylation signal in places where the array could not.

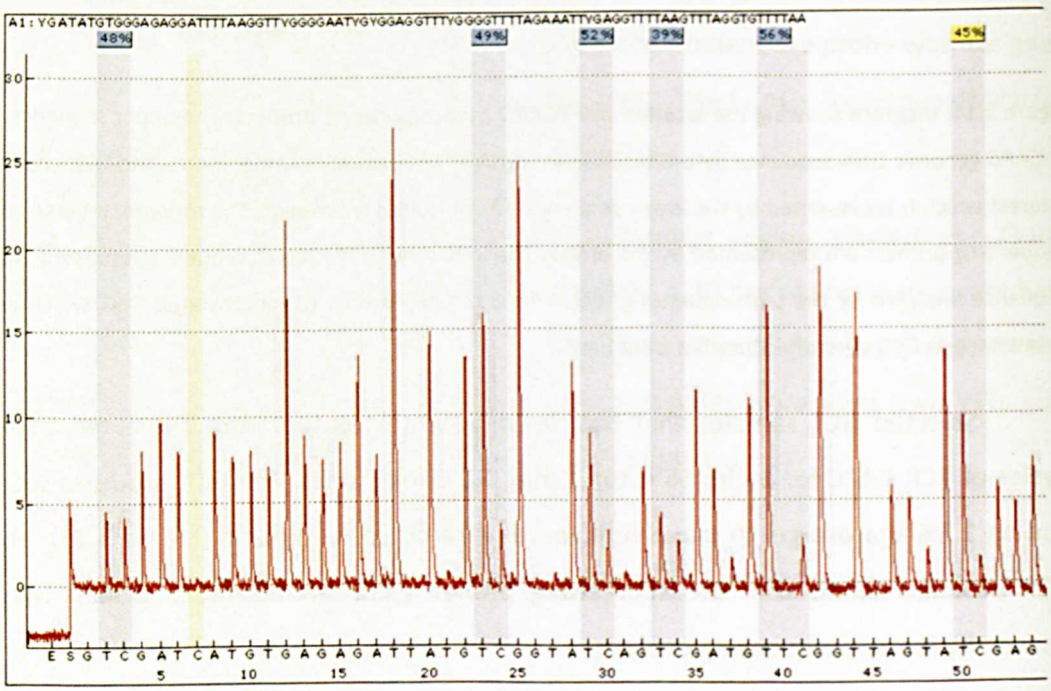


Figure 3.16: *PYCARD* pyrogram. Representatitive pyrogram with CpG site of interest being highlighted in grey and were given its percentage methylation value above the highlighted region.

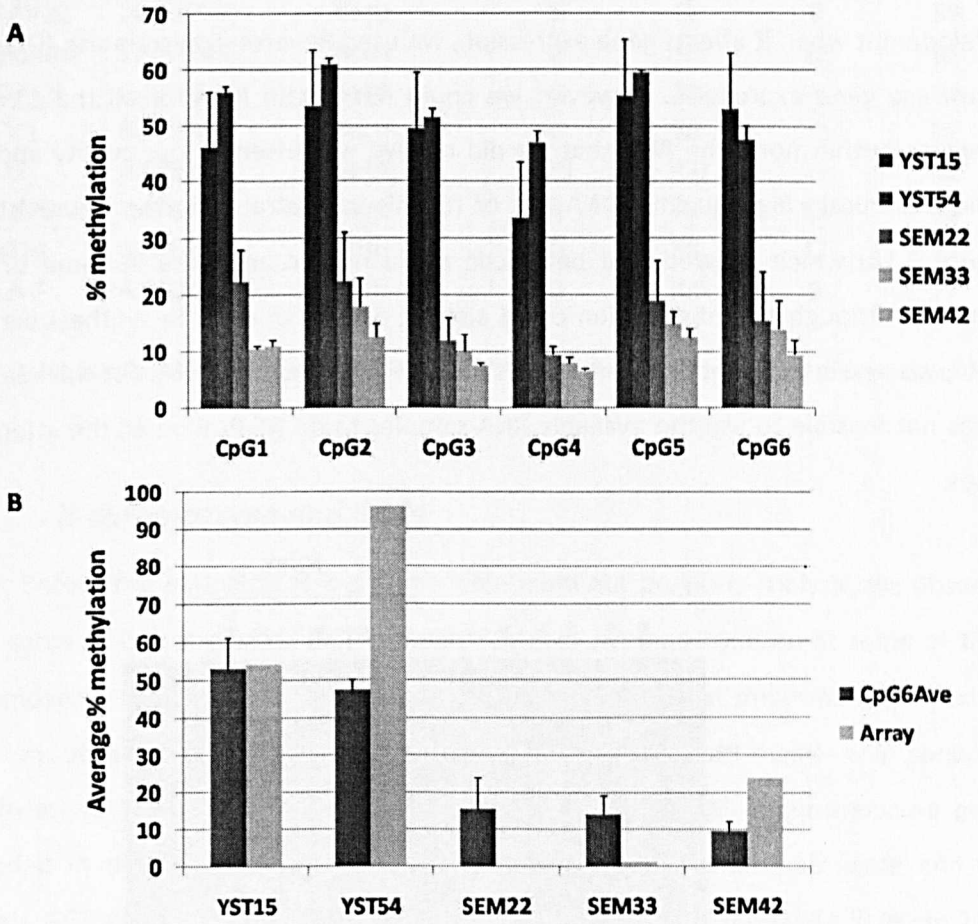


Figure 3.17: **Pyrosequencing showed that YST samples exhibit higher methylation at all PYCARD CpG sites analysed**. A) Methylation level at CpG sites included in the pyrosequencing in the tumour samples which showed that YST samples have higher percentage of methylation level at all CpG sites compared to seminoma samples B) Comparison between methylation level of CpG#6 that was included in both pyrosequencing and the methylation array which suggests that pyrosequencing is more sensitive than the array in picking up the methylation signal.

3.2.4. : Methylation and gene expression

Since DNA methylation is generally considered to be influential in tumour development when it affects gene expression, we used Reverse-Transcriptase (RT) PCR to analyse gene expression. However, we could not obtain RNA for all the tumour samples. Furthermore, the RNA that we did receive was often in low quality and we could not obtain high quality cDNA out of it. This is illustrated in the figure below (Figure 3.18) which showed that beta-actin could not be amplified in some of the samples. Although this observation could also be due to an error in synthesizing the cDNA, we had insufficient RNA to optimise and get good quality cDNA. For this reason, it was not feasible to use the available RNA samples to do RT-PCR on all the intended genes.

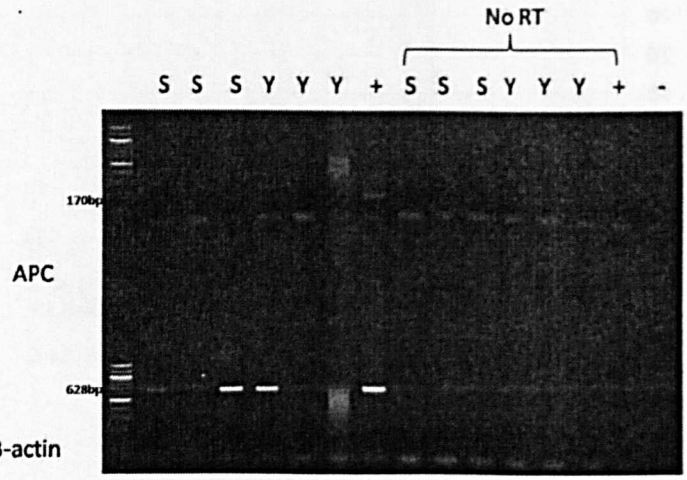


Figure 3.18: RT-PCR of *APC* expression in the paediatric GCT samples. It clearly shows that beta-actin could not be amplified in some of the samples suggesting the possibility of RNA degradation. S indicate seminoma samples, Y indicate YST samples.

Therefore, we instead referred to the Affymetrix expression array data from our collaborators that used the same bank of tumour samples. By comparing this to our Illumina GoldenGate methylation array data, we found that the majority of the genes that were hypermethylated in our analysis were not differentially expressed between YSTs and seminomas. Only 9 of the genes were found to be differentially expressed (average LODS value of more than 4) according to their methylation status. Of these, 8 showed a significantly higher level of expression in seminomas (Table 3.3).

Genes	ave LODS	Tumor expression	% tumor methylated			
			1st Array		2nd Array	
			Germ	YST	Germ	YST
TFAP2C	28.71057	Sem	0	88	0	38
PYCARD	**11.98783**	**Sem**	**0**	**100**	**22**	**85**
HDAC9	9.563456	Sem	0	38	0	54
ETV1	8.929844	Sem	0	38	11	31
CD2	8.079104	Sem	29	100	22	92
CASP8	**7.686273**	**Sem**	**0**	**75**	**11**	**85**
EVI2A	4.376357	Sem	0	88	11	77
HLA-F	4.280225	Sem	0	100	0	77

Table 3.3: List of genes hypermethylated in YST and differentially expressed in seminomas

3.3. : X-chromosome and GCTs

Before the exclusion of X-chromosome from our previous analysis, we observed that some of our paediatric GCT samples features hypomethylation at some of the X chromosome genes (Figure 3.19). Normally it is expected that the females will exhibit 50% methylation of all X-chromosomes genes while the males will show no methylation. When analyse further, we found 19 out of 39 X-chromosome genes included in the GoldenGate array were hypomethylated among six male and one female GCT samples. These hypomethylated X-chromosome genes are listed in Table 3.4.

Patients with an extra X chromosome, such as those with Klinefelter's syndrome, have been found to have an increased risk of developing extragonadal germ cell tumours (Aguirre et al., 2006, Gilbert et al., 2011). To investigate whether any samples within our bank of tumours do have an extra X chromosome, we used amelogenin assay to analyse our samples.

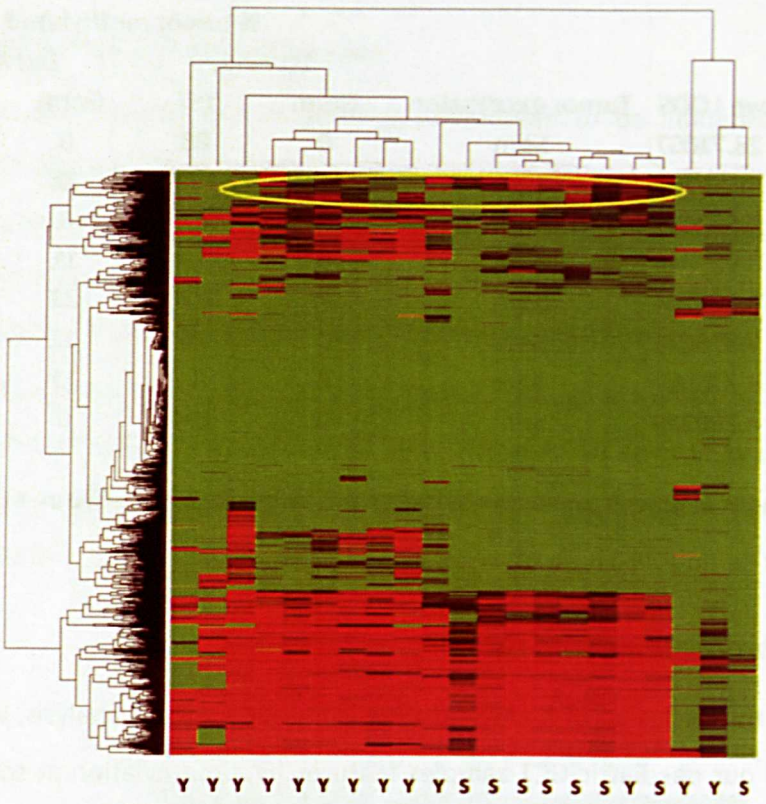


Figure 3.19: Heatmap showing the presence of hypomethylated X-chromosome genes in our paediatric GCT samples which are seen in the area marked in yellow circle.

List of X-chromosome genes found to be hypomethylated in the paediatric GCT samples			
ELK1	*VBP1*	*DNASE1L1*	*IRAK1*
ARAF	*SLC6A8*	*EFNB1*	*MYCL2*
GLA	*HDAC6*	*MECP2*	*TIMP1*
G6PD	*DLG3*	*BGN*	*FHL1*
BIRC4	*FMR1*	*GPC3*	

Table 3.4: List of 19 X-chromosome genes hypomethylated in paediatric GCT samples

The amelogenin assay is a PCR-based sex-identification assay based on the amplification of amelogenin gene alleles. Amelogenin gene is known for its role in enamel development and is homologous at both X and Y sex chromosomes. Despite being homologous, they are different in sizes which make this gene useful for sex-identification. For this purpose we used a kit supplied by Promega that comes with a labelled primer that will amplify a 212bp X-specific and 218bp Y-specific product. Due to the closeness of these two bands, we were unable to reliably distinguish them on a gel (Figure 3.20). Therefore we subjected the samples to Genescan analysis using PeakScanner software version 1.0. As illustrated in Figure 3.21, we determined the number of X and Y chromosome by calculating the ratio of height of peak at 212bp and height of peak at 218bp.

Using NT2D1 cell line that are known to have an extra X-chromosome, we found that some of our samples showed a presence of an extra X-chromosome (Table 3.5) and this includes tumours from gonadal sites. We also observed that the only female sample included in the analysis showed a presence of Y chromosome. The reason behind this is still unclear although female cases with Y chromosome have been reported before (Saavedra-Castillo et al., 2005, Schmid et al., 1992).

It is also unknown whether extra X chromosome observed in some of the paediatric samples are due to the features of Klinefelter's Syndrome itself or the features of the tumours on their own. To investigate this, we attempted to obtain constitutional DNA from our collaborators in Cambridge. Unfortunately, none were available. Therefore, this issue was not pursued further.

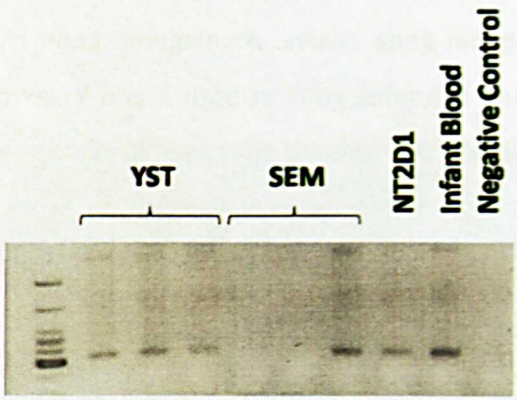


Figure 3.20: Representative gel picture of amelogenin assay using paediatric germ cell tumour samples

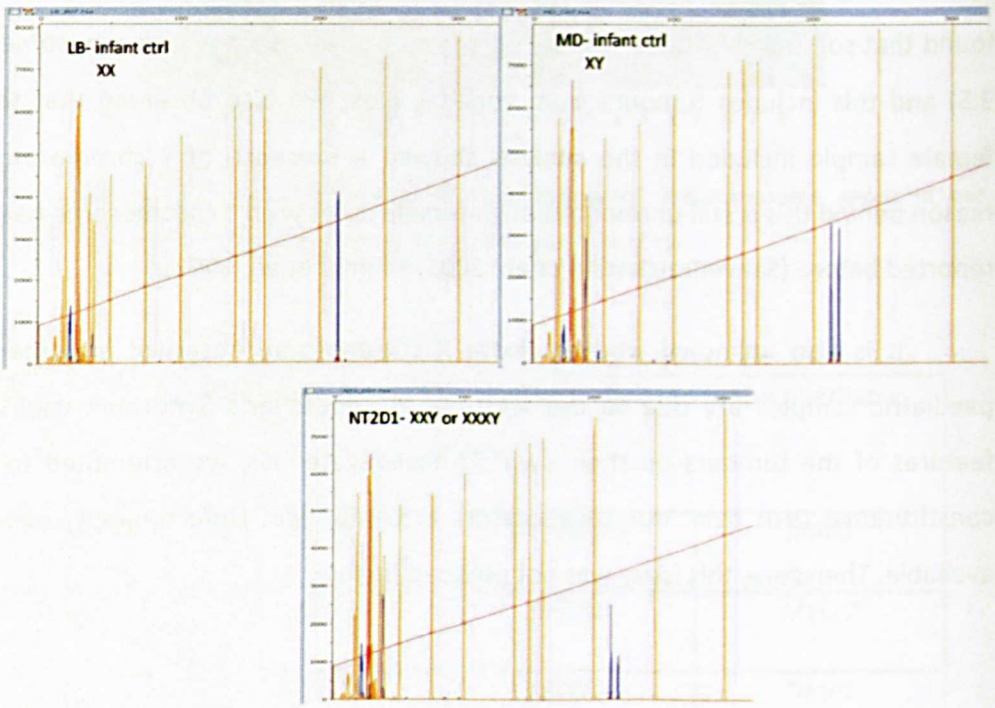


Figure 3.21: Genescan diagram showing the peak for X and Y chromosomes (blue peaks). Only one peak (212bp) was observed in female LB infant control which corresponds to X chromosome. In male MD infant control, two peaks (212bp and 218bp) represent X and Y chromosome respectively were observed having a similar height of peak suggesting similar dosage of sex chromosome. In NT2D1 cell line, a male testicular GCT cell line, were observed to have higher peak at X chromosome, almost double in size than Y chromosome peak suggesting the presence of an extra X chromosome.

Sample	Gender	Location	Heights at 212bp (X-chr)	Heights at 218bp (Y-chr)	Ratio between heights (X chr:Y chr)
Seminoma	M	Testis	605	168	04:01
Seminoma		Brain	3256	2792	01:01
Seminoma		Brain	1457	935	02:01
Seminoma		Brain	531	406	01:01
Seminoma	M	Testis	5747	413	14:01
Seminoma	M	Testis	496	421	01:01
YST	M	Testis	124	88	01:01
YST		SCT	284	163	02:01
YST	F	Ovary	125	130	01:01
YST	M	Testis	3779	1189	03:01
NT2D1	M	Testis	175	84	02:01

Table 3.5: Ratio of X chromosome to Y chromosome in paediatric GCT samples as determined using amelogenin assay and measured using PeakScanner software. From this measurement, we observed some of the paediatric samples showed a presence of an extra X chromosome.

3.4. Discussion

3.4.1. : Paediatric GCT's global methylation

Our measurement of global methylation of LINE-1 repeat elements as a means of assessing global methylation level reveals that both of germinomas and yolk sac tumours (YSTs) are significantly less methylated than the controls. This finding showed similarity with the result found by Ushida *et al.* (2011) who used the same technique in their study of patient with testicular germ cell tumours. In this study, the mean age of patients involved was 35 years old for both seminoma and non-seminoma cohort. However, our results showed that hypomethylation of LINE-1 repeat element can occur even in a younger age patient, suggesting the phenomenon is not age dependent. Furthermore, we also showed that this hypomethylation can be observed regardless of sex and the location of the GCTs. Looking at other global methylation analysis approach done on other type of tumours (Baba et al., 2010, Daskalos et al.,

2009) it is clear that in all types of tumour hypomethylation of intergenic DNA is a common feature.

From the Golden Gate array analysis, we did not find many genes showing promoter hypomethylation despite the global intergenic hypomethylation. It seems to suggest that there is no correlation between global hypomethylation and the state of promoter methylation, which is in agreement with the current consensus (De Smet and Loriot, 2010). Therefore, the most plausible contribution for this global hypomethylation towards tumourigenesis is due to its effect on repeat elements. At the moment, two mechanisms have been proposed to explain how repeat element hypomethylation might be involved in tumouregenesis. The first is that hypomethylation of repeat elements causes their mobilisation along the genome and creates genomic instability (Schulz et al., 2006).This was seen in an experiment using *Dnmt1* hypomorphic mutation mice which only expressed 10% of wild-type *Dnmt1*. These mice were found to develop lymphoma with a high frequency of chromosome 15 trisomy (Gaudet et al., 2003) which suggests an involvement of chromosomal instability. Further experiment from this group also showed that among these mice, there was evidence of an insertion of intracisternal A particles (IAP), a transposable element, into the genomic locus of *Notch1*(Howard et al., 2008). This further suggests that chromosomal instability in these mice was also accompanied with an activation of the transposable elements.

Meanwhile, the abundant number of repeat elements in the human genome and the fact that repetitive elements can be found at almost 30% of the transcription start sites (Faulkner et al., 2009) suggests an involvement of repeat elements in some gene regulation. For instance, human *cMET*, a proto-oncogene, contains an intronic LINE-1 element between exon two and three (Weber et al., 2010a).This L1-cMet gene was found to be heavily methylated in normal cells but showed significant hypomethylation in bladder cancer (Wolff et al., 2010). This observation correlates well with the high expression of the LINE-1 anti-sense promoter called L1-MET. Furthermore, they also demonstrated that demethylation of this anti-sense promoter in bladder fibroblast cell lines led to an increase in the expression of L1-MET that is

usually repressed in this cell line. This suggests an involvement of DNA methylation in silencing this promoter. There is no doubt about the involvement of DNA methylation in silencing the repeat element but how repeat element hypomethylation might contribute to GCTs development still needs to be investigated.

3.4.2. YST samples showed higher methylation level at the gene regulatory region

From our genome-wide methylation analysis in two cohorts of paediatric GCTs using Illumina GoldenGate array, we found that seminomas showed an almost normal methylation level when compared to controls while YSTs showed a high methylation level at many of the CpG sites. This is in agreement with previous findings using restriction landmark genome sequencing (RLGS) in adult testicular GCT which showed almost no methylation at all CpG site in seminomas (Smiraglia and Plass, 2002). This is interesting since similarity observed between seminoma and the normal controls suggests that seminoma is a totally different type of tumour as compared to other solid tumours. We also found that the CpGs of approximately 10% of genes included in the array panel are hypermethylated in YSTs as compared to the seminomas. Unlike previous studies that chose a candidate gene approach (Furukawa et al., 2009), our data showed that the gene regulatory regions in the YSTs are more hypermethylated than the seminomas across the genome. Interestingly, five of the genes (*SCGB3A1, HOXA9, APC, RASSF1A, HIC1*) found to be methylated in our YST samples are among the genes commonly found to be methylated in adult non-seminomatous testicular GCTs (Koul et al., 2002, Koul et al., 2004, Smith-Sorensen et al., 2002, Honorio et al., 2003, Lind et al., 2006, Lind et al., 2007, Manton et al., 2005).

Previously, *SORBS1* and *GSTP1* have been reported to be hypermethylated in seminoma samples (Koul et al., 2002, Lind et al., 2006). However we found that none of the seminoma samples (0/16) included in our Golden Gate analysis showed hypermethylation of *GSTP1*. This is in agreement with the findings by (Kawakami et al., 2003a) and (Honorio et al., 2003) who also showed no GSTP1 methylation in their seminoma samples. Given that previously only one out of 29 seminoma samples showed hypermethylation of *GSTP1*(Koul et al., 2002), our current observation might suggest that this hypermethylation could just be an isolated case. Unfortunately, we

could not assess the methylation level of *SORBS1* in the paediatric seminoma samples since this gene is not included in the array panel.

3.4.3. Hypermethylated genes in YST samples are not entirely silenced

It seems highly unlikely that all genes that are methylated could have a role in tumour development, as seems to be suggested by previous study (Lind et al., 2007). It is known that not all genes that are methylated will be silenced. At the moment, only *RASSF1A* has been found to have any correlation between methylation and gene silencing in adult testicular GCTs (Koul et al., 2002). Therefore, we referred our methylation result to the gene expression data performed on the same cohort of paediatric GCT samples by our collaborators (Palmer et al., 2008). Interestingly, of 85 genes that are hypermethylated in YSTs, only eight genes were differentially expressed in seminomas and strikingly *RASSF1A* was not one of them. It is also interesting to see that only 13 out of 29 genes involved in TGF-β/BMP signalling that were found to be differentially expressed in paediatric YSTs (Fustino et al., 2011) are differentially expressed in our samples. However, only two out of these genes are included in our methylation array and both of them (*COL1A1 and IGFBP3*) are hypermethylated in YST. Although we found only eight genes hypermethylated in our YSTs samples that show differential expression, this small number has really narrowed down the search for possible candidate gene involved in paediatric GCTs development.

3.4.4. : Genes hypermethylated in YST and differentially expressed in seminoma

3.4.4.1. *TFAP2C*

Transcription factor AP-2 gamma, *TFAP2C* (other aliases *AP-2 GAMMA, ERF1, TFAP2G, hAP-2G*) is a member of the AP2 transcription factor family known to be involved in activation of many developmental genes. It has been found to be methylated in chronic lymphocytic leukemia (Tong et al., 2010). In the same study, treatment with a DNA methylation inhibitor could reactivate *TFAP2C* expression in two leukemic cell lines which suggested involvement of DNA methylation in *TFAP2C* regulation.

Interestingly, *TFAP2C* was found to have a significant role in primordial germ cells (PGCs), the cells thought to be the origin of germ cell tumours. Conditional deletion of *Tfap2c* resulted in loss of primordial germ cells (PGCs) soon after specification (Weber et al., 2010b). Besides losing a significant number of PGCs from this knockout, the remaining PGCs were found to have impairment in their migration ability. This might support the migration theory that suggests extragonadal GCTs arising from PGCs that fail to complete their migration into the gonad. In addition to which, this deletion also leads to upregulation of markers related to mesoderm differentiation. This fits well with the development of more differentiated germ cell tumour such as YSTs.

While impairment of migration ability was found in PGCs after deletion of *Tfap2c*, a recent study in a mouse model of melanoma showed a contrast observation. In this study, silencing of *Tfap2c* with short-hairpin RNA caused an increase in cell migration and extravasation (Penna et al., 2011) which fits well with the aggressiveness of YSTs. This contrasting observation might be due to a different role played by *Tfap2c* at different developmental stages and/or in different cell types.

3.4.4.2. *PYCARD*

PYD (Pyrin) and CARD containing domain, *PYCARD* (also known as ASC or TMS-1) is an adaptor protein that has been shown to interact with CASP8 and is involved in apoptosis (Masumoto et al., 2003, Masumoto et al., 1999). A study done in prostate cancer cell lines, LNCap, showed that *PYCARD* was fully silenced (Das et al., 2006). This is due to DNA methylation since treatment with 5-aza and not TSA, partially demethylated and reactivated the gene.

Using methylation-specific PCR, Hervout *et al.* (2010) found that only 30% and 22% of their 27 glioblastoma multiforme patients showed methylation of *PYCARD* and *CASP8* respectively. Interestingly, by measuring caspase 3 protease activity to determine intra-tumor apoptosis levels and correlating them to the gene methylation status, they found that there was no correlation between methylation level of these two genes and the caspase activity. This might be due to low methylation of both

genes and since no expression analysis was done, this low methylation might not have had any effect on their expression.

3.4.4.3. *HDAC9*

Histone deacetylase 9, *HDAC9* is a zinc-dependent HDAC class IIa located in a chromosome region implicated in many cancers. Higher expression of *HDAC9* has been linked to poor prognosis for some tumour types (Moreno et al., 2010, Milde et al., 2010); however the exact role played by *HDAC9* in tumourigenesis is still not well-studied. One study (Milde et al., 2010) showed that silencing of *HDAC9* using siRNA caused reduction in medulablastoma cell growth and viability.

3.4.4.4. *ETV1*

ETS variant 1, *ETV1* is a submember of the PEA3 group of transcription factors and it has been found to be overly expressed in many type of cancers either in their full length or as a fusion with another gene (Tomlins et al., 2007, Gasi et al., 2011, Jane-Valbuena et al., 2010). In melanoma, primary melanocytes were found to be transformed and develop tumours in mice after overexpression of *Etv1*(Jane-Valbuena et al., 2010). In addition to this, *ETV1* expression is essential in interstitial cells of Cajal (ICC) development and this expression is regulated by *KIT*(Chi et al., 2010). In the same study it was found that constitutive activation of *KIT* by mutation will cause stabilization of ETV1 protein which leads to ICC hyperplasia and gastrointestinal stromal tumour development. It is interesting to see this strong relationship between *KIT* and *ETV1* might also exist in GCTs development, particularly in seminomatous GCTs since numerous studies have shown the presence of activating KIT mutations in seminomas (Kamakura et al., 2006, Sakuma et al., 2003, Sakuma et al., 2004, Kemmer et al., 2004). Indeed, preliminary sequencing experiments performed in our lab revealed that some of the seminoma samples also exhibited *KIT* mutations. What is more striking is that *ETV1* is among the genes that are differentially expressed in our seminoma samples. This suggests the possibility that these two genes are involved in seminoma development.

3.4.4.5. *CD2*

CD2 is an immune response gene but how its silencing affects tumour cells is not well documented. However,*CD2* ligation has been shown to be one mechanism to prime and subsequently activate resting NK cells (Bryceson et al., 2006). In addition to that, a recent study in a leukemic cell line showed that blockade of *CD2* caused inhibition of cell lysis (Sabry et al., 2011).This might suggest that *CD2* methylation and its low expression in YST might provides the cell's with an ability to evade lysis.

3.4.4.6. *CASP8*

CASP8 is a member of the caspase family and involved in the apoptosis signalling pathways (Fulda, 2009, Kim et al., 2001), which could either be through the death-receptor or the mitochondrial pathway. Beside its apoptotic function, *CASP8* has also been shown to play other non-apoptotic roles such as in embryonic development, blood cell formation and immune response (Maelfait and Beyaert, 2008). In addition to that, *CASP8* can play an important role in resistance to chemotherapy (de Vries et al., 2007).

One study looking at the methylation of p53 target genes in testicular GCTs showed minimal methylation of *CASP8* in both seminoma and non-seminomatous GCT (Christoph et al., 2007). Using quantitative methylation-specific PCR, they only found one seminoma and two non-seminomatous samples to be methylated out of 26 and 20 samples respectively. This is much lower than the methylation level observed in our YST samples (10% vs 70%). However, it should be noted that only one YST sample was included in their non-seminomatous group which might not reflect the true methylation level of most YSTs. Furthermore, their method of using *MYOD1* as a reference gene might also have an influence in their measurement of the methylation level. Nonetheless, they did not perform any expression analysis of *CASP8* due to this low methylation level.

3.4.4.7. *EVI2A*

The function of *EVI2A* (ecotropic viral integration site 2a) remains unknown. It is believed to be an oncogene and has been suggested to be involved in retrovirus induced murine myeloid leukemia in BXH-2 mice (Buchberg et al., 1990). *EVI2A* also has been found in an intron of Nf1, a tumour suppressor, and is believed to have a role in development of neurofibromatosis (Pasmant et al., 2011); however, the exact mechanism is still unclear. Furthermore, how methylation affects *EVI2A* function and how this may contribute to GCTs development has yet to be investigated.

3.4.4.8. *HLA-F*

Major histocompatibility complex class 1 F (*HLA-F*) is important in the immune response and has been found to be methylated in glioblastoma multiforme (Martinez et al., 2009). However, how silencing of this gene might affect tumour development is still unknown.

3.4.5. Paediatric GCTs associated with an extra X-chromosome

We have found evidence of an extra X-chromosome present in some of our paediatric GCT samples. This extra X-chromosome suggests that these samples were taken from Klinefelter's syndrome patients.However no clinical data or constitutional DNA samples were available for these patients, so we could not be certain whether such observation is due to Klinefelter's syndrome or it is the feature of the tumour samples on its own.

Klinefelter's syndrome patients are at risk of getting extragonadal germ cell tumours from mediastinal (Hasle et al., 1992) and to a lesser extent intracranial GCTs (Kaido et al., 2003). From our analysis, we found that these extra X-chromosome can also be seen in gonadal samples which have also been seen in a previous study (Sandberg et al., 1996).

However, how an extra X-chromosome could initiate tumour development especially in patients with Klinefelter's syndrome still needs to be investigated. Among the X-chromosome genes that are hypomethylated in our paediatric GCT samples, ELK1 and ARAF, are known oncogenes (Nishigaki et al., 2005, Kawakami et al., 2003b).

Therefore, activation of oncogenes on the X-chromosome might contribute to GCT development. In addition to these two genes, the hypomethylation of FMR1 and GPC3 genes in our samples have also been reported in a previous study in testicular GCTs (Kawakami et al., 2003b) which seem to suggest common X-chromosome genes hypomethylated in germ cell tumours. However, further research needs to be done to ascertain the involvement of these X-chromosome genes in GCT development.

4. METHYLATION CHARACTERISATION OF GERM CELL TUMOUR CELL LINES

4.1. Introduction

From the Illumina GoldenGate methylation array, we saw that there are distinct methylation properties between seminomas and yolk sac tumours with the latter showing higher methylation at many genes' promoter regions. Nonetheless, we should keep in mind that there are a few caveats around the Golden Gate methylation array.

First, the Golden Gate array only covers 807 genes, and these genes have been pre-selected to be tumour-related genes such as tumour suppressors and oncogenes. Therefore, we cannot really be sure whether such methylation patterns are also similar in other types of genes. Furthermore, only 1505 CpG sites are analysed meaning that only 1 - 2 CpGs are covered for each gene. The fact that such a small number of CpG sites are covered, makes it quite difficult to generalise the methylation level to other neighbouring CpG sites. In addition, CpG sites that were included in the Golden Gate array were selected to be in the CpG islands. The reason behind this is that hypermethylation of CpG islands at the promoter region has long been considered and shown to contribute to gene silencing (Fernandez et al., 2011, Noushmehr et al., 2010, Toyota et al., 1999). Contrary to this, recently Irizarry *et al.* (2009),in their study on colon cancer showed that instead of CpG islands, it was the CpG shores that were usually differentially methylated and this methylation was seen to correlate with gene silencing.

In order to fully understand this matter, we used the Infinium Methylation450 array. This array works on the same principles as the Golden Gate methylation but with the addition of a newly designed assay called InfiniumII, which only uses one probe rather than two for each CpG. The 3' terminus region of this probe will bind to the query site, while the addition of a labelled G or A base that complements either the methylated or the unmethylated site will result in the base extension (Figure 4.1).

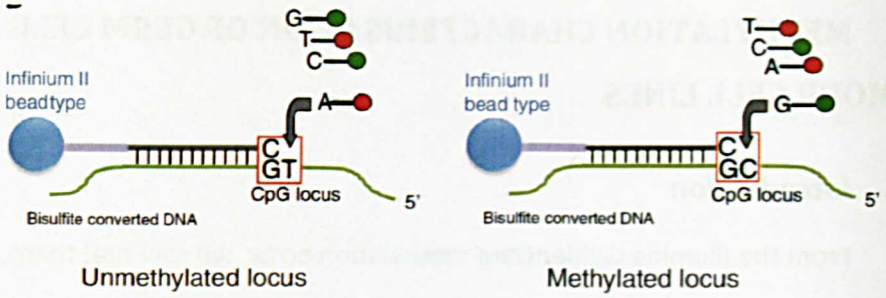


Figure 4.1: InfiniumII assay design which uses only one probe. The 3' terminus of the probes complements the base directly upstream of the query site. Binding of either the methylated or the unmethylated site depends on the addition of labelled G and A bases. Taken from Bibikova *et al.*(2011)

The Infinium Methylation450 array panel covers a total of 485,577 sites where the majorities (482,421 sites) are CpG sites which correspond to 21,231 RefSeq genes in the University of California, Santa Cruz (UCSC) database. This includes an average of 17.2 probes per gene region. These sites are further subclassified according to UCSC classification. Most of the sites (145,272 sites) are concentrated on the CpG islands which is defined as 500bp DNA sequences that have a GC composition of greater than 50% and a CpG observed/expected (O/E) ratio of more than 0.6 (Gardiner-Garden and Frommer, 1987, Takai and Jones, 2002). In addition to this, sites that are adjacent to the CpG islands were also covered. These were named CpG shores (two-kilobase regions flanking up- and downstream of CpG islands) and CpG shelves (two-kilobase regions flanking CpG shores). To ensure in-depth methylation assessment, sites were **also selected to include different gene elements such as 5'UTR, the gene body**, the first **exon and 3'UTR. Sites near the transcription start site (TSS) were also selected and** were labelled as TSS200 (sites 200bp upstream TSS) and TSS 1500 (sites 1500bp upstream TSS).

It is not possible with tumour samples, to perform functional analyses such as studying the consequences of demethylation on cell migration and survival, or studying the role of genes identified to be silenced by methylation. We therefore decided to move to the analysis of GCT cell lines for this array, where we could carry out such follow-up functional studies. In addition, GCT cell lines are widely used; thus data on these cell lines from other laboratories are easily accessible for comparison with our

data. Furthermore, no previous GCT studies have investigated methylation at regions other than the CpG islands. Given that the Infinium array offers coverage beyond the CpG islands, we believed this could provide a better understanding of GCT methylation. However, the only available GCT cell lines at the moment are adult GCT cell lines. Therefore in this chapter, methylation analysis were performed on fouradult testicular GCT cell lines; TCAM2 (seminoma), NT2D1 (teratocarcinoma), GCT27 (embryonal carcinoma, EC) and GCT44 (yolk sac tumour, YST).TCAM2 is a seminoma cell line derived from aprimary lesion of testicular seminoma in a 35 years old male patient (Mizuno et al., 1993). GCT 27 and GCT44 represent the EC and YST cell lines respectively (Pera et al., 1987). GCT27 was derived from a primary teratoma testicular tumour samples which formed embryonal carcinoma when inoculated into nude mice. GCT44, on the other hand, was established from testicular tumour that has metastasized into the para-aortic lymph node. This cell line was found to give rise to a tumour that displayed endodermal sinus yolk-sac morphology when inoculated into nude mice. Finally, the NT2D1 cell line was obtained as ateratocarcinoma cell line (Boucher and Bennett, 2003, Pierce et al., 1999, Dahm et al., 2010, Yao et al., 2007) derived from a 22-year old male patient. However, as I will discuss at the end of this chapter, this cell line has also been considered as pluripotent embryonal carcinoma cell line (Vestergaard et al., 2008, Milia-Argeiti et al., 2012, Caricasole et al., 2000).

4.2. Results

4.2.1. : Methylation analysis on GCT cell lines

The Infinium methylation450 array analysis on these four cell lines was carried out at Queen Mary, University of London according to the manufacturer's protocol. Basically, genomic DNA was first subjected to bisulfite modification before being denatured into single-stranded DNA and neutralized. These denatured samples were then isothermally amplified overnight at 37°C. They were then subjected to fragmentation using a controlled enzymatic process. The fragmented DNA samples were collected via isopropanol precipitation and subsequently resuspended in a hybridization buffer. These DNA samples were then subjected to hybridization by applying them to the BeadChip. Unhybridized and non-specifically hybridized DNA was

then washed away. Hybridized DNA then underwent the extension process. BeadChips were then scanned, and the fluorescence emitted from the single base extension products was recorded. This data was extracted using Genome Studio Methylation Module software where the fluorescence intensity values were measured and represented as a β-value ranging from 0 (not methylated) to 1 (fully methylated). Data normalization was then performed by this software using the internal control present in the BeadChip.

4.2.1.1. *: Data exclusion*

In addition to the β-value, the software also measured the detection p-value for the total signal intensity. According to the manufacturer, a high detection p-value (>0.05), would indicate poor probe hybridization, which could affect the analysis. We only had four samples and at any one time, only one sample could have a detection p-value >0.05. Therefore, we measured the mean detection p-value for all four cell lines. Since a mean detection p-value >0.01 would exclude any site where one cell line had a detection p-value >0.05, we used this as a cut-off point. By taking this measure, we excluded 4,813 sites from future analysis.

We also adopted a procedure proposed by Irizarry *et al.* (unpublished data). They calculated the intensities for both methylated and unmethylated probes. When this was $\log_2$ transformed, they found that probes withsignals with a $\log_2$ intensity of <10.5, indicating low signal intensity which could be due to poor probe binding, were unable to discriminate the differences between samples. Therefore, we removed probes with such low signals. Using this approach, we removed a total of 61,192 sites from further analysis.

As with the GoldenGate methylation analysis, we also excluded all sites that corresponded to the sex chromosomes. By doing so, we lost an additional 8,171 sites (846 genes). We also noticed that quite a number of sites (100,446 sites) were not represented by gene symbols which we believed would make it quite difficult to identify differentially methylated genes. Therefore, we decided to exclude these sites

for such purposes. After making all these exclusions, only 313,824 out of 422,440 sites which corresponded to 20,444 out of 41,059 genes, were selected for future analysis.

4.2.2. Data Analysis

Unlike previous analysis in the GoldenGate array, currently there is no software or R script available to be used to analyse Infinium data in depth. Therefore, analysis was carried out using an Excel spreadsheet.

The UCSC database describes a standard in evaluating the methylation level from the Infinium Methylation450 array. According to this database, a probe with a β-value of 0.6 and above is considered fully methylated while a β-value between 0.2 and 0.59 is considered partially methylated and a β-value below 0.2 is unmethylated. Therefore, we used this as the criteria for the methylation state in our analysis.

4.2.2.1. *Methylation features of GCT cell lines*

Before we set out to characterise the methylation pattern in the GCT cell lines, we wanted to determine the beta-value distribution across the entire GCT cell line genomes. By counting the frequency of occurrence for each beta-value, we found that all four cell lines shared a bimodal distribution of beta-values (Figure 4.2). Despite this, we can clearly see that the distribution differs between all four cell lines. The seminoma and the YST cell lines showed a much higher distribution at beta-values ranging from 0.2 to 0.7 (up to 0.8 in seminoma) but a low distribution at a beta-value of 0.9 to 1.0 (except YST which show high frequency at beta-value of 1.0). In contrast, both the EC and the teratoma cell lines displayed higher distribution at beta-values above 0.8, showing that higher methylation is a feature of EC and teratoma cell lines.

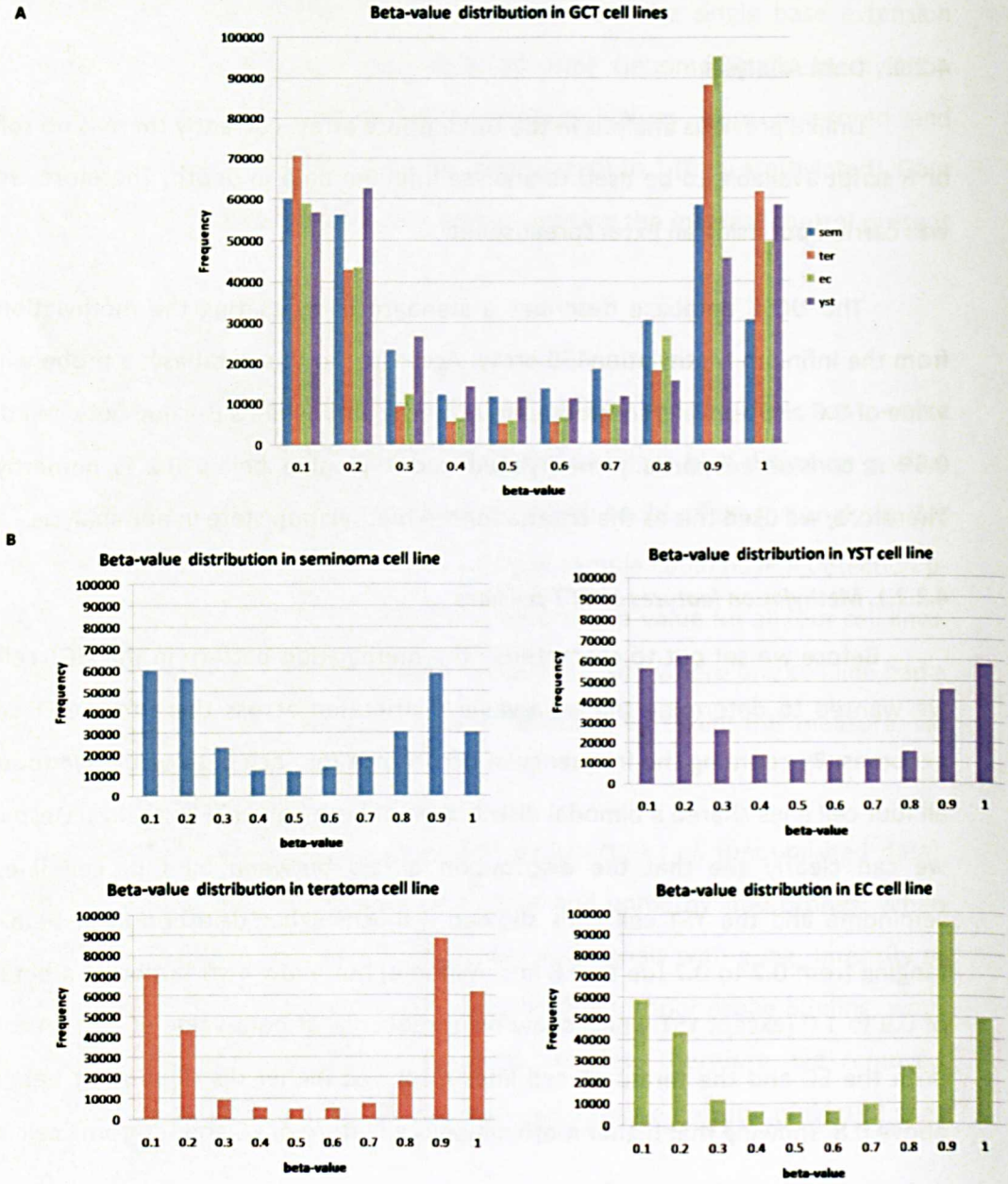


Figure 4.2: Bimodal distribution of beta-values in A) all four GCT cell lines, and B) each of the cell lines. This shows that probes' in EC and teratoma tend to concentrate at higher methylation levels suggesting these two cell lines feature higher methylation as compared to the other two cell lines.

This was far more evident when we tried to determine methylation according to chromosome region using a distribution plot. Using the SVS v7.0 software (Golden Helix), we plotted probes' distribution according to their beta-value across chromosome regions. In general we did not see any difference in methylation at any chromosome region in all four cell lines. Strikingly however, we observed that there were fewer probes distributed between beta-values of 0.2 and 0.6 (and to a lesser extent between 0.6 and up to 0.8) in the EC and the teratoma cell lines as compared to the seminoma and the YST cell lines (Figure 4.3). Since beta-values ranging from 0.2 to 0.6 are considered partially methylated, it seems thatseminoma and YST cell lines hadmore partially methylated sites as compared to the EC and teratoma cell lines. Since the partial methylation observed resembles the expected properties of imprinted genes, we investigated the methylation profile of all imprinted genes included in the array for all four cell lines. Imprinted genes are expected to have one allele silenced by methylation leaving only one allele functioning (50% methylated). However, there could also be instances when both alleles are silenced (100% methylated) leading to completegene inactivation. Therefore, we expected to observe these imprinted genes to have beta-values of 0.2 to 0.59 or more than 0.6 which corresponds to partial methylation or fully methylated respectively, as defined by theUCSC database. Using the GeneImprint database (www.geneimprint.com) which lists 84 known imprinted genes in humans, we identified 34 that were included in our Infinium methylation analysis. This low number is likely to be due to the exclusion of imprinted genes at the X-chromosomes made earlier in the analysis. Looking at the beta-value distribution of all CpG probes for these 34 imprinted genes, we found that most of the probes in the YST and seminoma cell lines accumulated at beta-values of 0.2 to 0.8, with more probes in the YST cell line than the seminoma cell line exhibitingat beta-value of 0.2 to 0.5, which fits with the partial methylation features of animprinted gene (Figure 4.4) while many of the probes in the seminoma cell line displayed a beta-value of 0.6 to 0.8 as compared to the YST cell line. Strikingly, for both EC and teratoma cell lines, the majority of probes were concentrated at beta-values of more than 0.9, while only a few exhibiteda beta-value of less than 0.6. This might explain the lack of probe

distribution between beta-values of 0.2 to 0.6 that was observed on the Manhattan plot in both EC and teratoma cell lines (Figure 4.3). However, when we performed the same analysis across the CpG islands of the imprinted genes rather than for individual CpG probes, we found that there was not much difference between partial and fully methylated imprinted genes (Figure 4.5). But, it is obvious that many more imprinted genes in the seminoma and EC cell lines were fully methylated (i.e. both alleles methylated) while most of the imprinted genes in the YST cell line were partially methylated, consistent with only one allele being methylated. Nonetheless, we realise that the partially methylated imprinted genes only represent a subset of the partially methylated loci. Therefore, there could be other reasons that could contribute to this phenomenon and this may be worth investigating in the future.

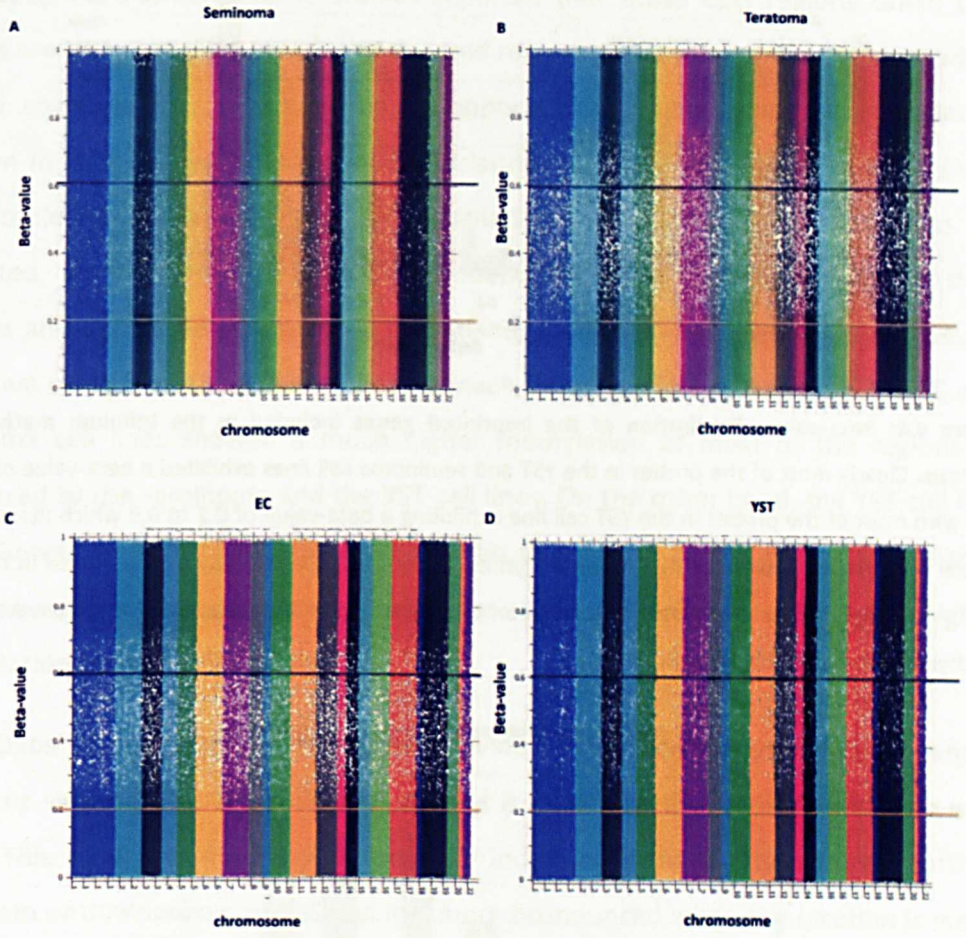


Figure 4.3: Distribution plots showing probe distribution at different chromosome regions (represented by multicoloured channels) in A) seminoma B) teratoma C) EC and D)YST cell lines. In general, no differences in methylation were observed at any chromosome region in all four cell lines. However, fewer probes were found to be distributed between beta-value of 0.2 to 0.6 in teratoma and EC cell lines as compared to the seminoma and YST cell lines at all chromosome regions. A beta-value of >0.6 (black line) is considered fully methylated; a beta-value <0.2 (orange line) is considered unmethylated; while a beta-value between 0.2 and 0.6 is considered partially methylated.

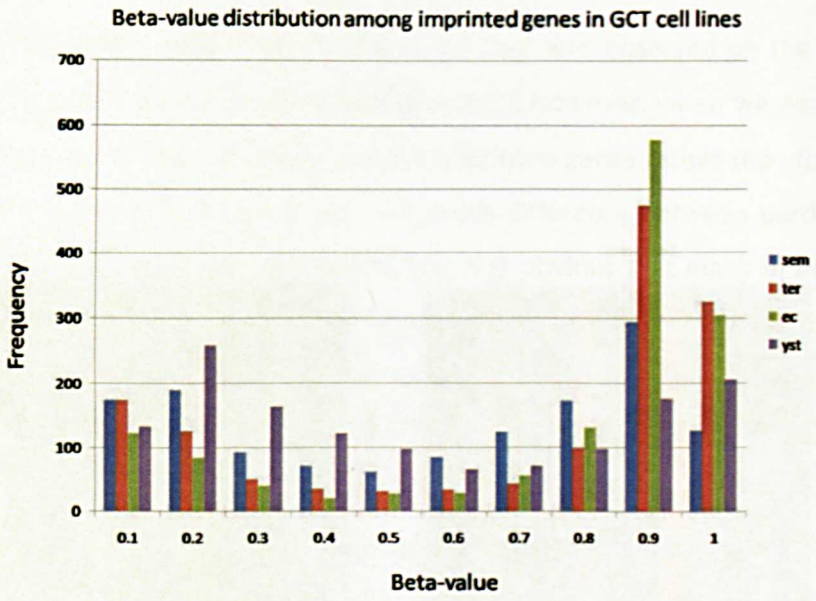


Figure 4.4: Beta-value distribution of the imprinted genes included in the Infinium methylation analysis. Clearly most of the probes in the YST and seminoma cell lines exhibited a beta-value of 0.2 to 0.8, with most of the probes in the YST cell line exhibiting a beta-value of 0.2 to 0.5 which fits with the partial methylation feature of the imprinted genes. In contrast, the majority of the probes in both EC and teratoma cell lines displayed a beta-value of more than 0.9, which suggests that they were highly methylated and probably silenced.

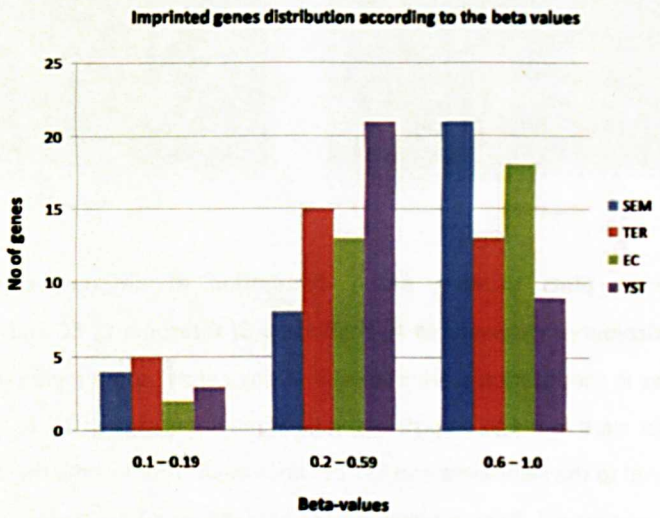


Figure 4.5: Bar graph showing the distribution of the imprinted genes according to the average beta-values across their CpG islands. It is obvious from the graph that many more imprinted genes in the seminoma and the EC cell lines were concentrated at a beta-value of 0.6 to 1.0, which implies that both alleles were fully methylated and silenced. In contrast the majority of the imprinted genes in the YST cell line were partially methylated which fits with only one allele being methylated.

CpG islands, especially those in the promoter region have long been considered to have been affected by methylation (Noushmehr et al., 2010, Fernandez et al., 2011, Toyota et al., 1999). However, recently CpG sites outside of CpG islands were also found to be aberrantly methylated in colorectal cancer (Irizarry et al., 2009, Ushida et al., 2011). Furthermore, these studies reported that these CpG regions called CpG shores are the most differentially methylated regions. Since Infinium arrays offer wider region coverage, this also gave us an opportunity to determine the methylation pattern in GCT cell lines outside of CpG islands. For this purpose, we used an Excel pivot table to calculate the average methylation level at different gene regions. As expected, we found that in general, the methylation level was highest in the shelf regions and decreased as it reached CpG islands in all cell lines (Figure 4.6). However, when we compared all of the cell lines to each other, we found that both the EC and teratoma cell lines showed a much higher methylation at most of the regions as compared to the seminoma and the YST cell lines. On the other hand, the YST cell line only showed a much higher methylation than the seminoma cell line at CpG islands while at regions distal from CpG islands, the seminoma cell lines exhibited a higher methylation than the YST cell line.

Ogoshi *et al.* (2011) in their genome-wide methylation analysis of colon cancer cell lines showed that the methylation level is lowest at the transcription start site (TSS). This methylation level was found to increase as the location moves further upstream or downstream of TSS and it is more pronounced when the location is more than two kilobases from the TSS. Using the same approach as before, we calculated the average methylation level in GCT cell lines at different gene elements. Indeed, we found that in all cell lines, methylation was at its lowest near the TSS and started to increase as it reached the 3'UTR (Figure 4.7). In addition to this, we found that there was little variation in methylation level at the gene element (ie TSS, 5'UTR, gene body, 3'UTR) as compared to the gene region (ie CpG shelf, CpG shores CpG islands). This is especially true between seminoma and YST cell lines. Nonetheless as in methylation at the gene region, EC and teratoma cell lines displayed a much higher methylation at all gene elements as compared to the seminoma and the YST cell lines.

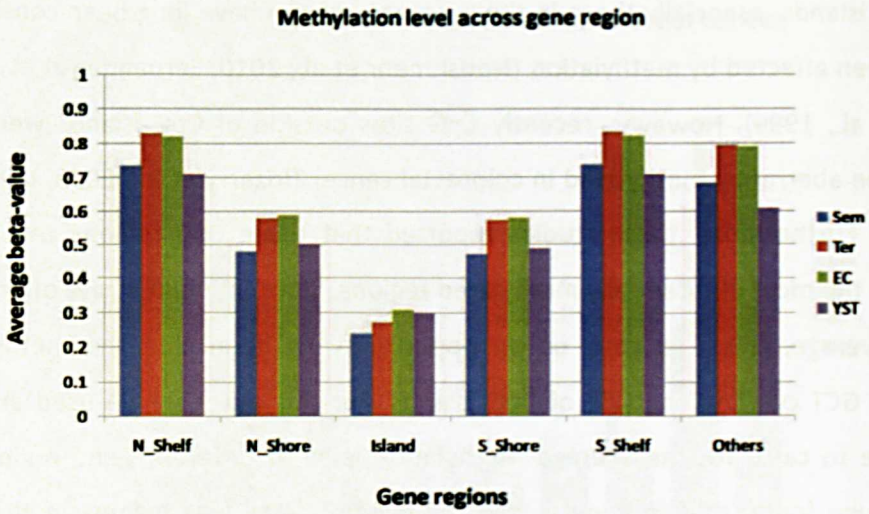


Figure 4.6: Methylation level across gene region. Average methylation across gene regions in GCT cell lines which shows that EC and teratoma had a higher methylation at most regions as compared to seminoma and YST. Meanwhile, YST only showed a higher methylation than seminoma in the CpG islands region.

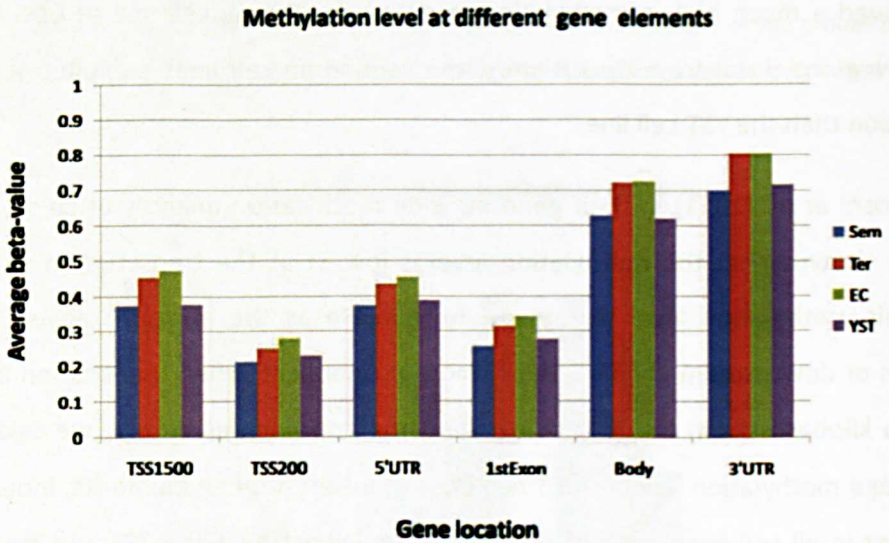


Figure 4.7:Methylation levels at different gene elements showed much less variation between cell lines. It is evident from here that methylation in GCT cell lines was at its lowest near TSS and increased when it moved towards 3'UTR. Note that methylation level in teratoma and EC cell lines were higher than the seminoma and the YST cell lines at all gene elements, similar to the finding in the gene regions.

4.2.2.2. *Differentially methylated genes at CpG islands and other gene regions*

Previously we found that while EC and teratoma cell lines were highly methylated in all gene regions, YST only showed a substantial increase in methylation as compared to seminoma at CpG islands. Thus, we believe genes that are methylated at CpG islands in YST might be important for YST development. Our first attempt to identify these genes was by selecting probes in the CpG islands with a beta-value above 0.6 (considered fully methylated by the UCSC database) and ranking them. However, since each gene could have more than two probes for CpG islands, we found that this method is not reliable, as sometimes a high methylation level in one probe does not translate in the other probes.

Therefore, we used an Excel pivot table and calculated the average beta-value at CpG islands, again using the cut-off point of above 0.6 to identify differentially methylated genes in GCT cell lines especially in the YST. Comparing all four cell lines we found that on average, EC cell line showed the highest methylation level at CpG islands followed by the YST, the teratoma and lastly the seminoma cell lines (Figure 4.8A).

Interestingly when we looked at the number of genes uniquely methylated in each cell line (defined as genes having an average beta-value >0.6 and a difference in beta-value, or delta beta-value, >0.3), we found that the YST cell line had the highest number of uniquely methylated genes at CpG islands, followed by the EC, the teratoma and lastly the seminoma cell lines (Figure 4.8B). In order to determine any similarities or differences in the methylated genes in all four cell lines, we looked at the number of overlapping genes in all four cell lines. We found that the majority (97-98%) of genes methylated in the seminoma cell line were also methylated in the other three cell lines (Figure 4.9). In contrast, only about half of the genes methylated in YST (50.1%), EC (53.4%) and teratoma (62.6%) cell lines were also methylated in seminoma.

Analysis on the other gene regions (ie CpG shores and shelf) also displayed similar features where, in accordance with the methylation level seen in Figure 4.6, EC and teratoma cell lines displayed the highest percentage of methylated genes at all regions. On the other hand, the YST cell line only showed a higher percentage of methylated genes at the islands and to a lesser extent in the shores as compared to

the seminoma (Figure 4.10A). Nevertheless, the YST cell line featured the highest number of uniquely methylated genes at all regions as compared to the other cell lines with the vast majority concentrated at island regions (Figure 4.10B)

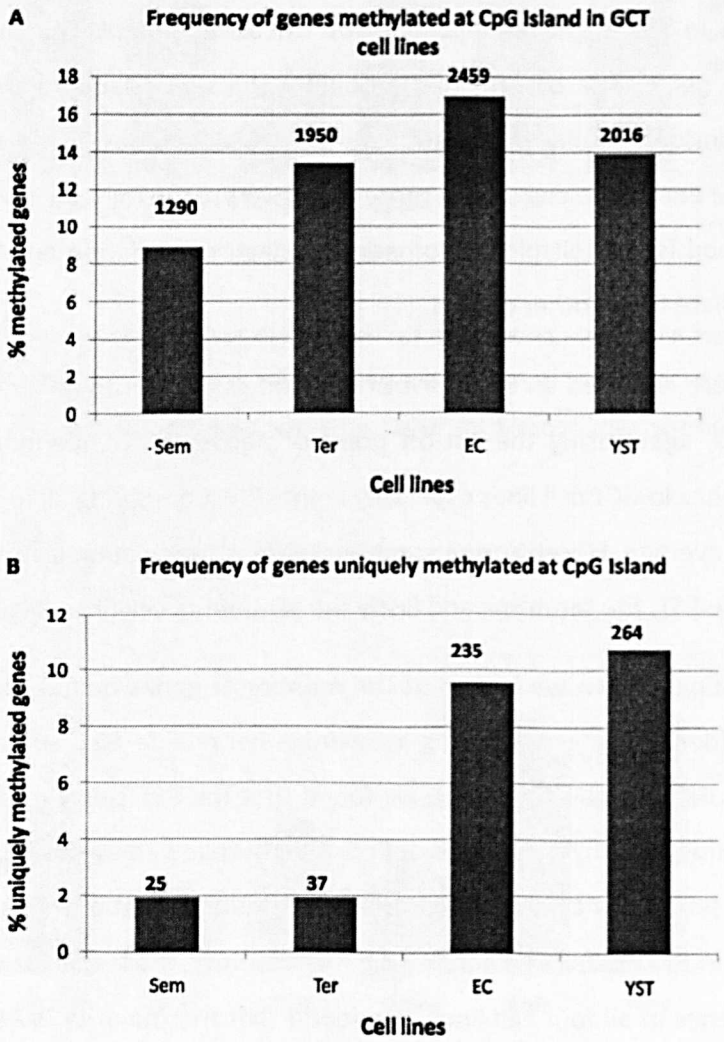


Figure 4.8: Bar graphs showing A) the frequency of genes methylated (average beta-value >0.6), and B) the frequency of genes uniquely methylated (average beta-value >0.6 and have difference in beta-value >0.3 to other cell lines) at the CpG islands in all four cell lines. It shows that EC had the highest number of methylated genes at the CpG islands. Note that despite having lower number of genes methylated as compared to EC, YST displayed much higher number of uniquely methylated genes at the CpG islands.

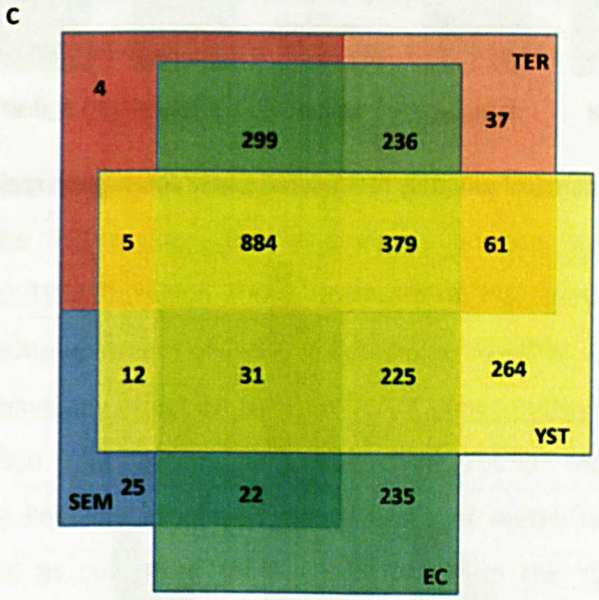


Figure 4.9: Diagram showing the overlapping genes that were methylated in all four cell lines. It shows that the majority of genes methylated in seminomas were also methylated in the other three cell lines.

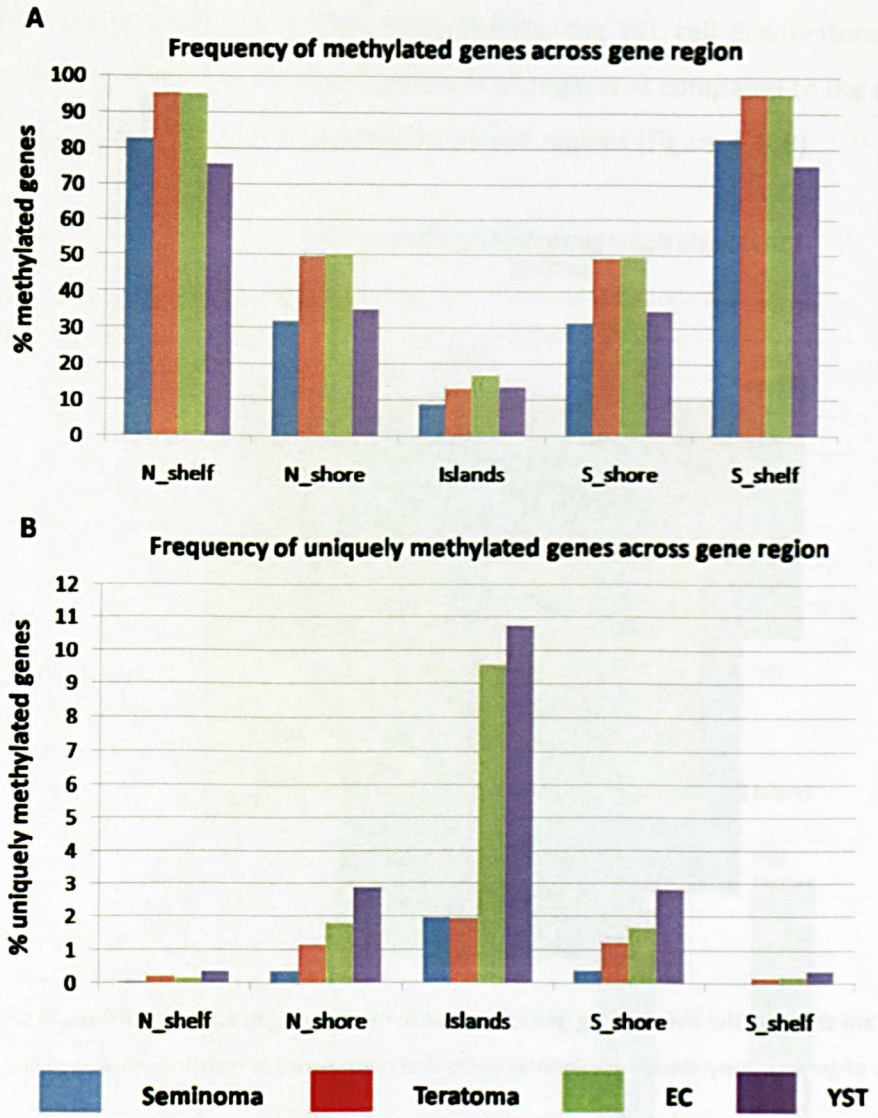


Figure 4.10: Graphs showing the frequency of A) methylated genes and B) uniquely methylated genes across the gene regions in all four cell lines. They clearly show that YST possessed the highest number of uniquely methylated genes at all regions although it did not display the highest number of methylated genes as compared to EC and teratoma cell lines.

4.2.2.3. *Differentially methylated genes at the transcription start sites (TSS) and other gene elements.*

Previous methylation studies have focused on the CpG islands in the promoter region, which can be found in the vicinity of transcription start sites. However, recently non-CpG island regions have also been found to be methylated (Han et al., 2012). Indeed, in our previous analysis, we focused on CpG islands such that we overlooked almost 40% of genes where there was no CpG island in the region of the TSS. Since methylation at TSSs has been shown to be important for gene regulation, we next turned our analysis to this region.

Using the same approach as before, we tried to identify genes uniquely methylated near the TSS regions. In our previous analysis we found that the methylation levels at TSS200 (region 200bp upstream of TSS) were much lower than TSS1500 (region 1500bp upstream of TSS). To determine whether such a difference in methylation would have any effect on the number of genes methylated, we analysed TSS200 and TSS1500 methylation separately. We found that both EC and teratocarcinoma cell lines still displayed higher levels of methylated genes at both TSS200 and TSS1500 as compared to the seminoma and the YST cell lines. It is interesting to see that unlike in CpG islands, YST had the lowest percentage of methylated genes at both TSS regions (Figures 4.11 A& B). Consistent with the higher methylation level at the distal region (shown earlier in Figure 4.7), we found that the number of genes methylated in all four cell lines at TSS1500 was double that at the TSS200 region (Figures 4.11 A&B). Despite this, the number of uniquely methylated genes was much lower in the former region. Surprisingly despite having the lowest percentage of methylated genes, we found that YST displayed the highest number of uniquely methylated genes at both TSS regions as compared to the other three cell lines (Figures 4.12A & B).

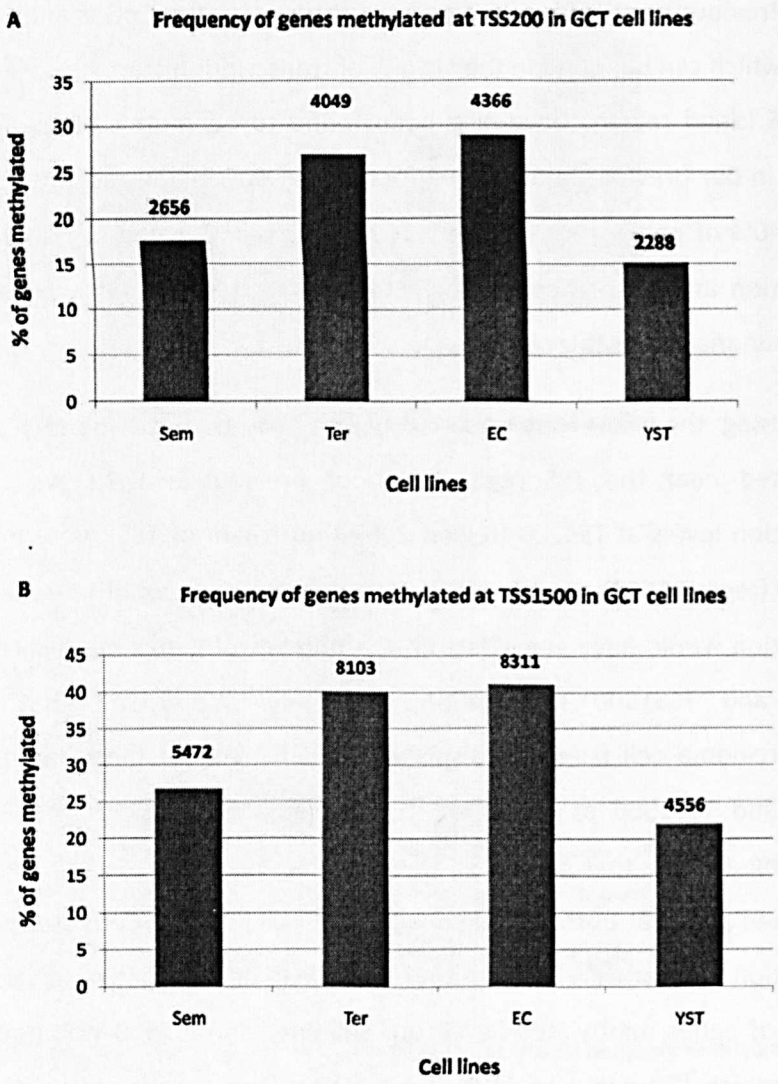


Figure 4.11: Graphs showing the percentage of methylated genes at A) TSS200 and B) TSS1500. It can clearly be seen that at both TSS200 and TSS1500 regions, EC and teratocarcinoma cell lines possessed the highest percentage of methylated genes as compared to the seminoma and YST cell lines. In all four cell lines, a higher percentage of methylated genes can be seen in TSS1500 as compared to the TSS200 region and the number of genes methylated doubled in this region as well. Interestingly, YST showed the lowest percentage and number of methylated genes as compared to the other three cell lines. The percentage of methylated genes was calculated by dividing the number of genes with an average beta-value >0.6 by the total number of genes. The values on top of each bar represent the gene count.

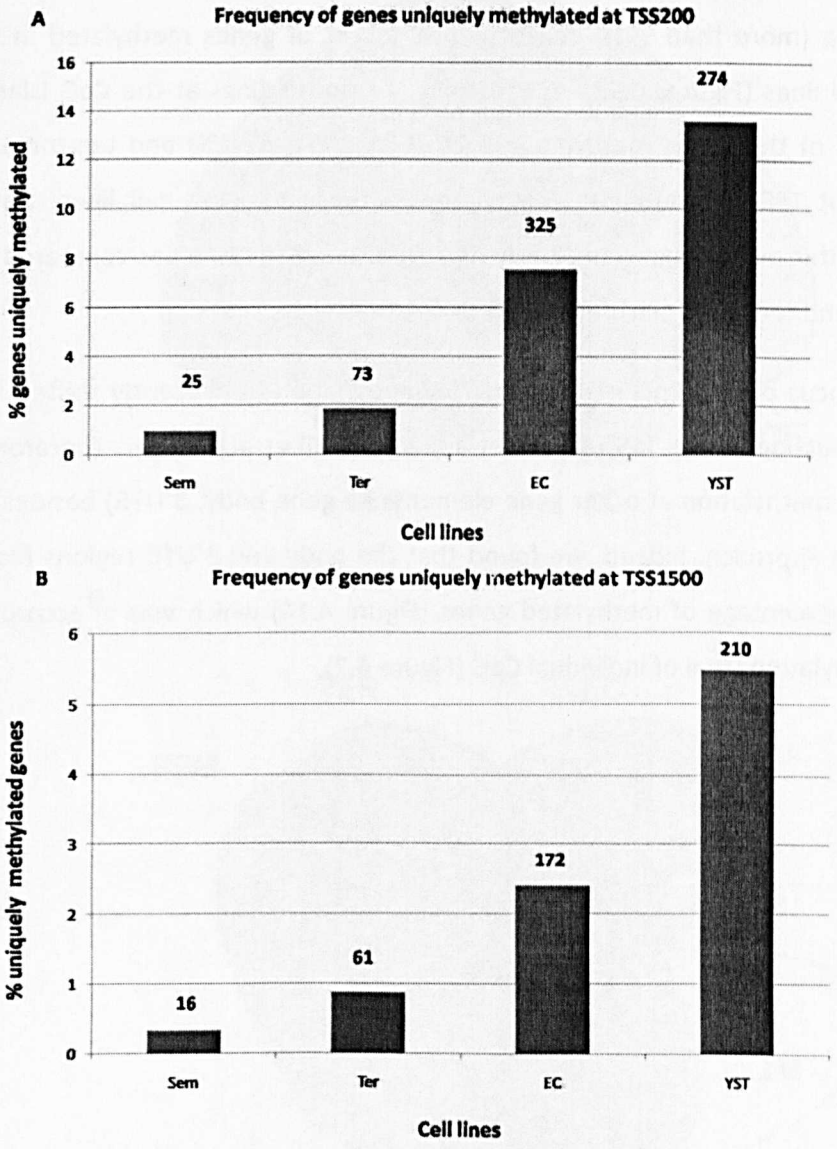


Figure 4.12: Graphs showing the percentage of uniquely methylated genes at A) TSS200 and B) TSS1500. Uniquely methylated genes were determined by calculating genes that were methylated (average beta-value >0.6) either at TSS200 or TSS1500 only and had a difference in beta-value (delta beta-value) >0.3 in the other three cell lines. The value on each bar represents the gene count.

Again when we looked into the similarities between all four cell lines in terms of genes methylated at these two gene elements, virtually all genes methylated in seminoma (more than 99%) constituted a subset of genes methylated in the other three cell lines (Figures 4.13A & B). Similar to the findings at the CpG islands, more than half of the genes methylated in EC (60%), YST (59.2%) and teratoma cell lines (64.2%) at TSS200 were also methylated in the seminoma cell lines. However, at TSS1500 this percentage was slightly higher in the YST (72.1%) as compared to the EC (67.7%) and teratoma cell lines (67.5%).

Focus on the CpG islands and TSS methylation has recently shifted into other regions outside of the TSS (Brenet et al., 2012, Ball et al., 2009). Therefore we also analysed methylation at other gene elements (ie gene body, 3'UTR) besides TSS using the same approach. Indeed, we found that the body and 3'UTR regions featured the highest percentage of methylated genes (Figure 4.14) which was in accordance with the methylation level of individual CpG (Figure 4.7).

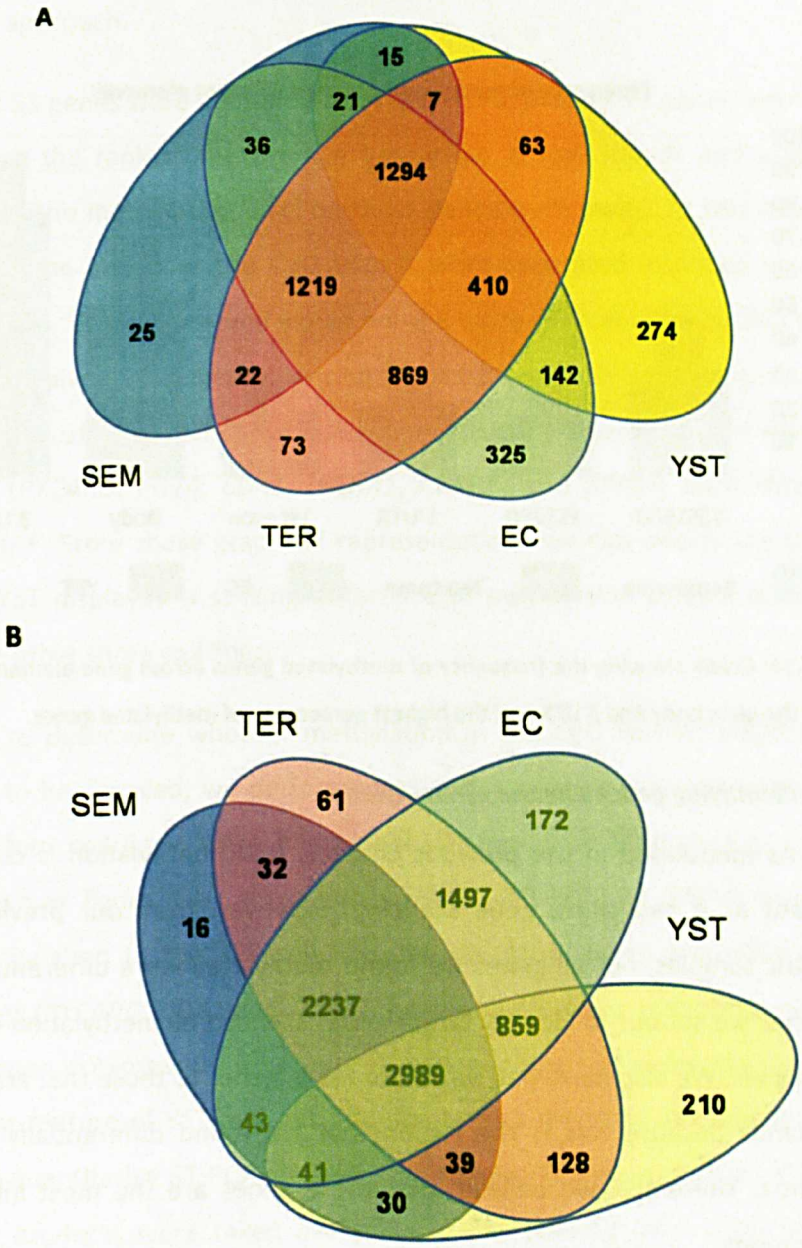


Figure 4.13: Diagrams showing the overlapping genes in all four cell lines that were methylated at A) TSS200 and B) TSS1500 regions. Again, genes methylated in seminoma represented a major subset of genes methylated in the other three cell lines. At TSS1500, no methylated genes were overlapping between EC and YST cell lines only.

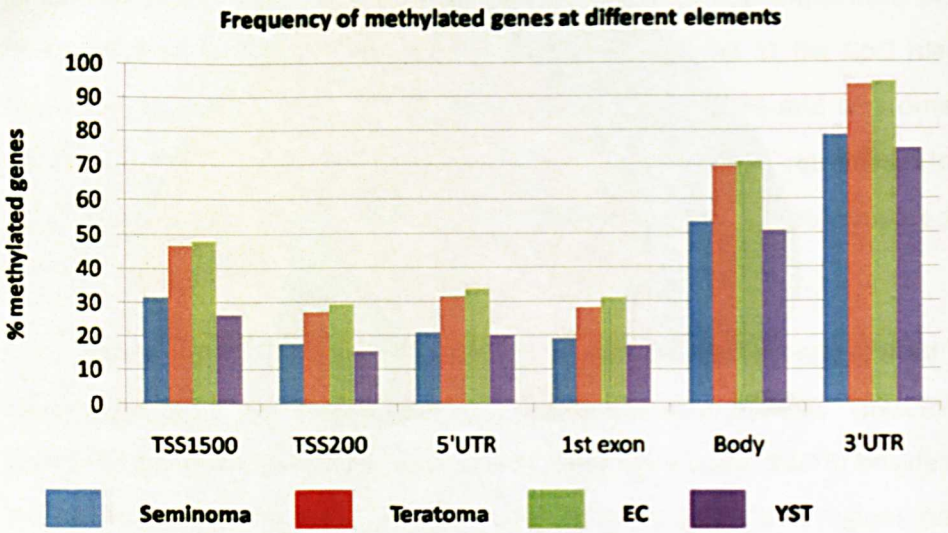


Figure 4.14: Graph showing the frequency of methylated genes across gene elements. We can clearly see that the gene body and 3'UTR had the highest percentage of methylated genes.

4.2.2.4. *Identifying genes silenced by methylation*

As mentioned in the previous chapter, DNA methylation is considered to be important as it can cause gene silencing. However, from our previous analysis of paediatric samples, not all genes we found methylated were differentially expressed. Therefore, we set out to identify target genes silenced by methylation focusing on the YST cell lines. We also narrowed down our list of genes to those that are methylated at CpG islands because this is the region that we found differentially methylated to seminoma. Therefore, we believe, that these genes are the most influential in YST development.

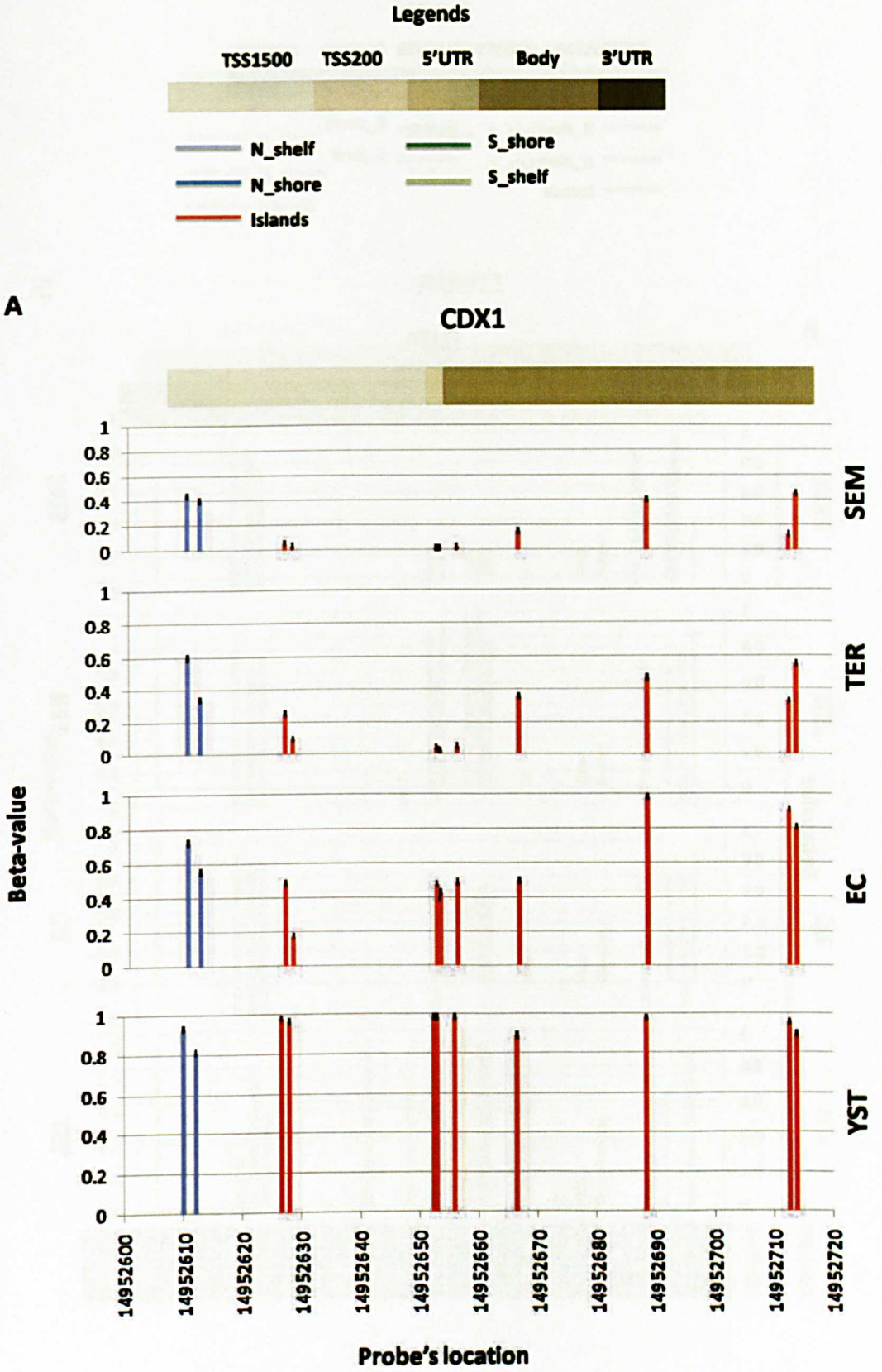
Prior to receiving the Affymetrix data, we decided to select genes of interest via two approaches. The first approach was by correlating the cell lines' methylation data to the expression analysis done in the paediatric samples (Palmer et al., 2008). The second approach was by screening all 264 genes uniquely methylated in YST at CpG islands and selecting those which are known to be methylated in other types of cancer. The subsequent works were divided between my colleague, Martin Cusack, and me, in

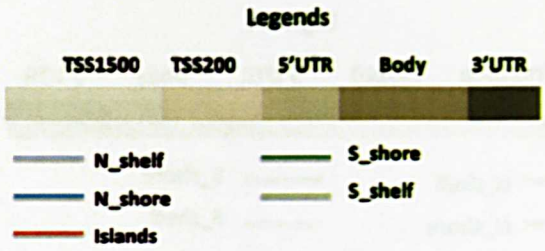
which I am using the first approach in identifying genes of interest while my colleague used the second approach.

A total of 53 genes were identified from the first approach. 21 genes were then selected based on the rank of the average beta-value at CpG islands and graphical representations of the methylation level on these genes were made. At this stage we realised that in some instances the CpG islands were separated into two or more clusters (Appendix I). This we believed would make it quite difficult to be certain which cluster might be involved in the gene silencing. Therefore, we only selected genes that showed one cluster of CpG islands for easier analysis. Graphical representations of these six genes (*PYCARD, FUT4, CDX1, PRDM1, PARP12* and *RPRM*) were shown in Figures 4.15 A to F. From these graphical representations we can clearly see that in most of them, YST displayed a striking difference in methylation at CpG islands as compared to the other three cell lines.

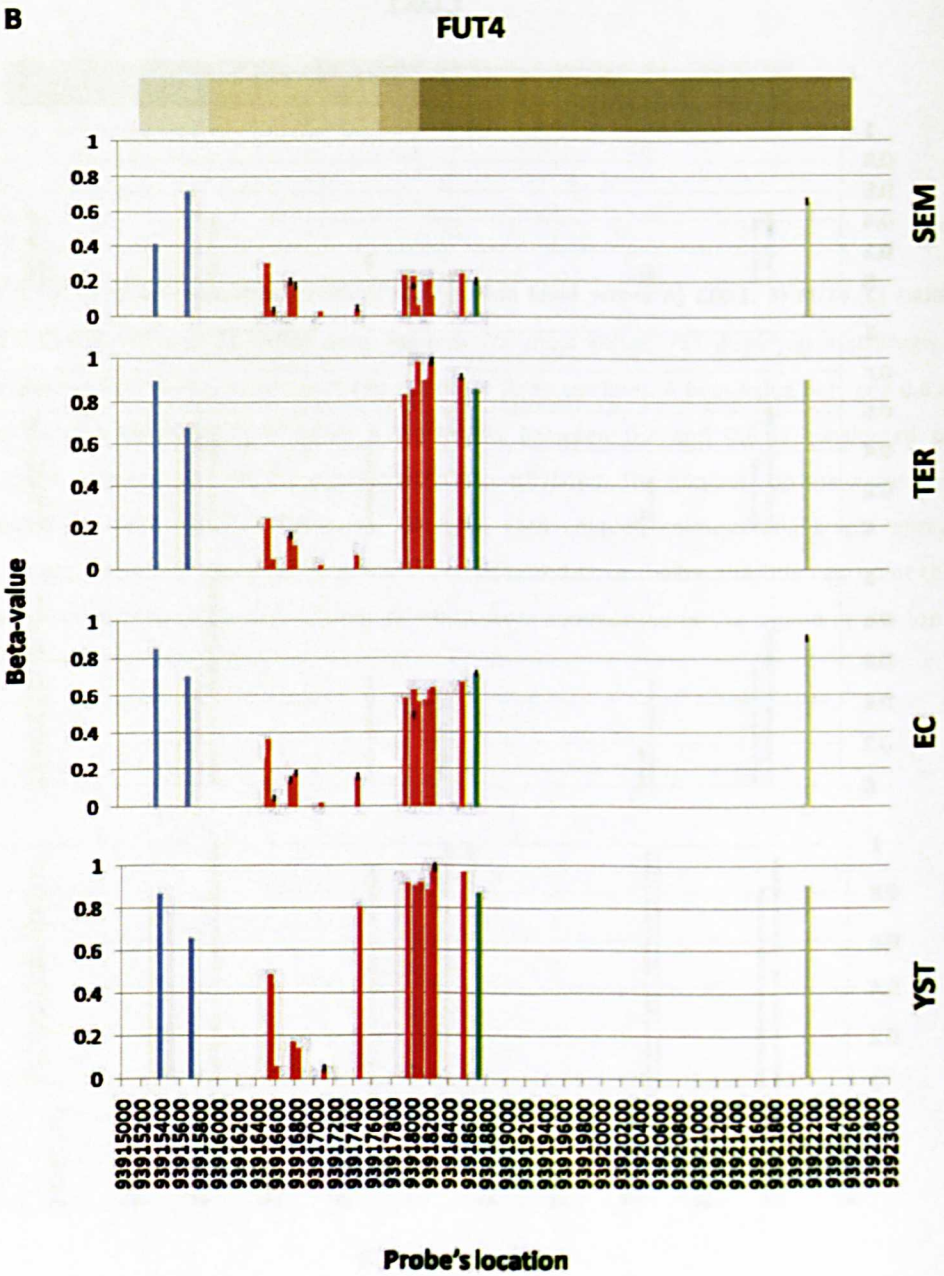
In order to determine whether methylation at the CpG islands would cause these six genes to be silenced, we performed 40 cycles of reverse-transcriptase PCR (RT-PCR) on all four cell lines, using human neural stem cells (hNSCs) as a positive control. As shown in figure 4.16, three genes (*FUT4, PRDM1* and *RPRM*) were not silenced by methylation in the YST cell line. On the other hand, the other three methylated genes (*PYCARD, CDX1* and *PARP12*) were found either to be fully silenced or to have reduction in expression. To ensure that the silenced and reduced expression was a prominent feature of YST and not just due to late template amplification, we performed semi-quantitative RT-PCR on these three genes. PCR were performed for 45 cycles and PCR products were taken every five cycles starting from cycle 30. We observed that *CDX1* still showed a reduced expression in YST as seen previously even at 45 cycles (Figure 4.17.A) while *PARP12* and *PYCARD* were completely silenced in the YST cell lines (Figures 4.17B and 4.17C respectively)

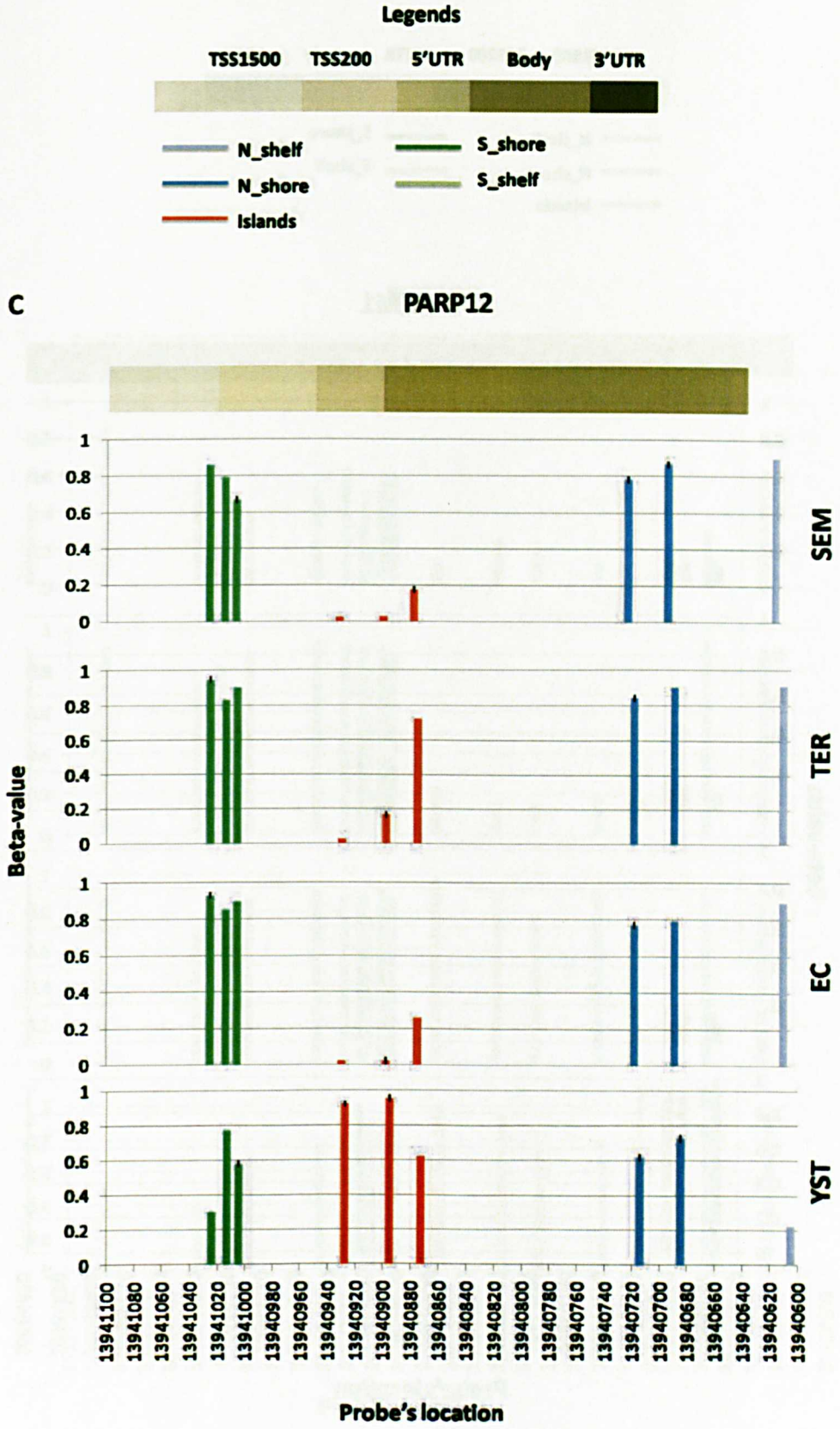
Figure 4.15: Graphical representations of methylation level across A) *CDX1*, B) *FUT4*, C) *PARP12*, D) *PRDM1*, E) *PYCARD* and F) *RPRM* gene regions. For most genes, YST displayed a strikingly higher methylation at CpG islands as compared to the other three cell lines. A beta-value between 0.6 and 1.0 is considered fully methylated, while a beta-value between 0.2 and 0.6 is considered partially methylated. Beta-value below 0.2 is considered unmethylated. The numbers on the x-axis show the location of the probe used in the human genome. Each uniquely coloured line graph represents a different gene region analysed while the uniquely coloured boxes below the title represent the gene elements analysed by the probes, details of which were summarized in the legend at the top of the page.

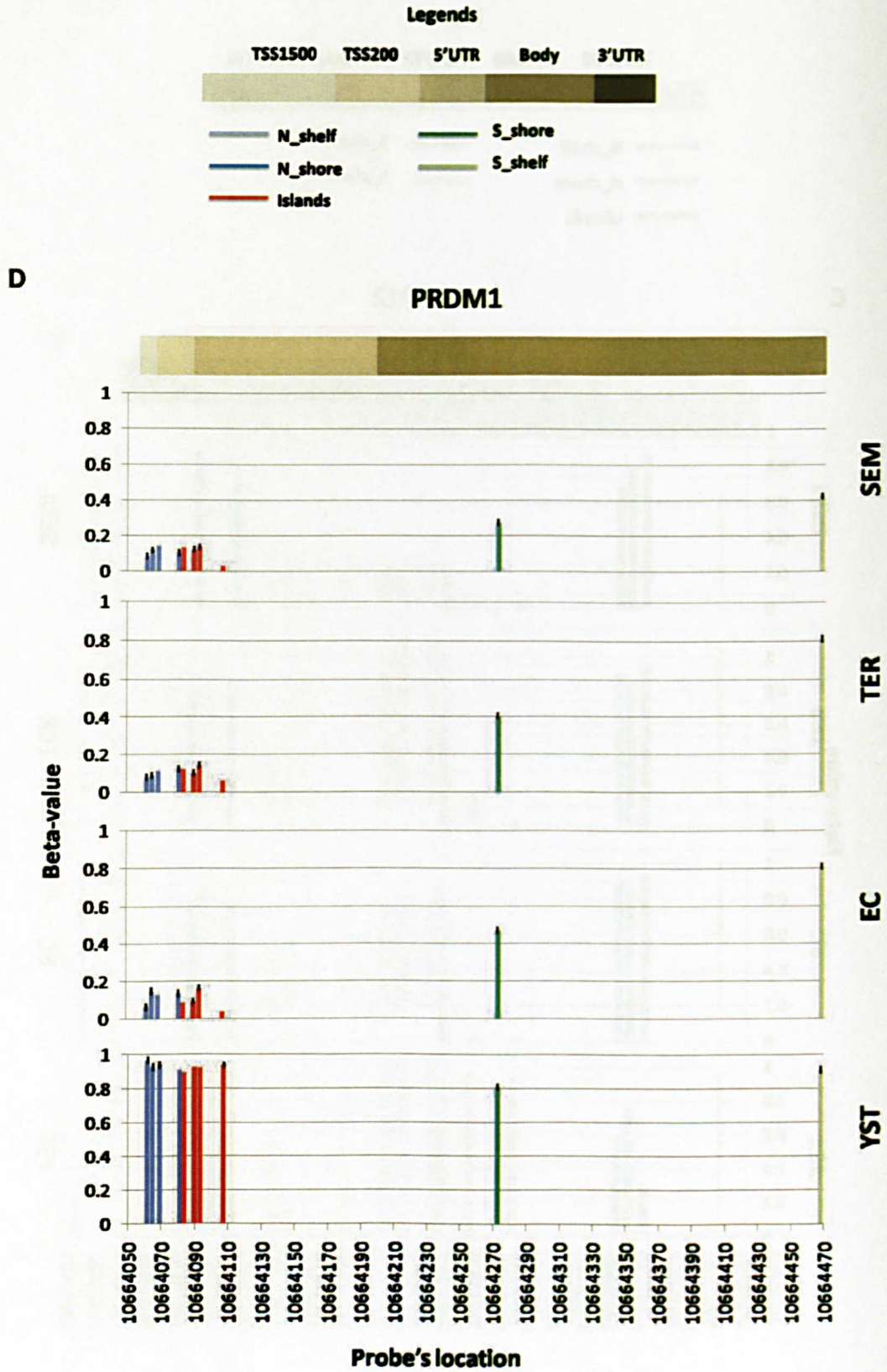

Legends
TSS1500
TSS200
5'UTR
Body
3'UTR
N_shelf
N_shore
Islands
S_shore
S_shelf
A
CDX1
Beta-value
SEM
TER
EC
YST
1
0.8
0.6
0.4
0.2
0
14952600
14952610
14952620
14952630
14952640
14952650
14952660
14952670
14952680
14952690
14952700
14952710
14952720
Probe's location

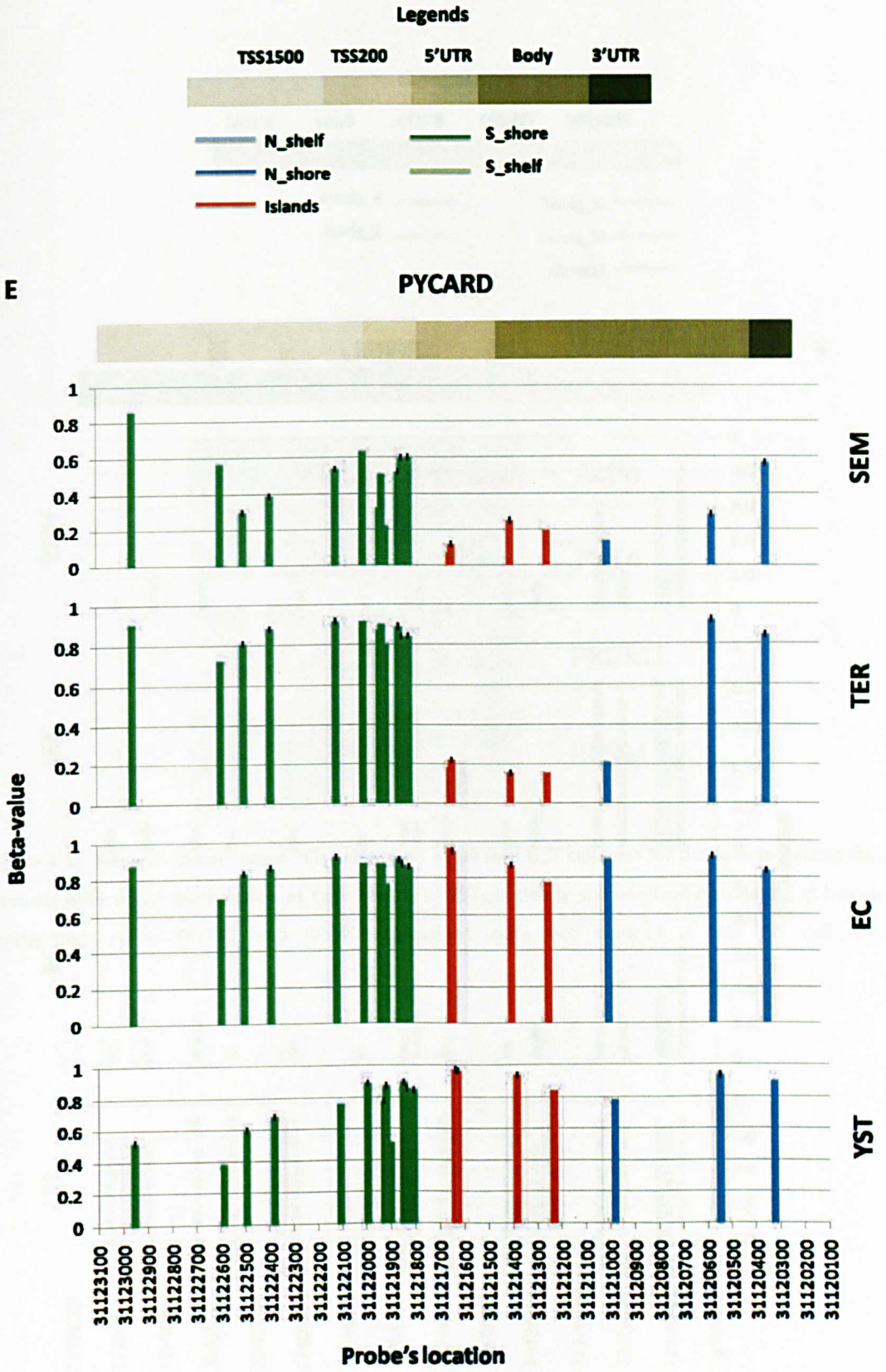

Legends
TSS1500
TSS200
5'UTR
Body
3'UTR
N_shelf
N_shore
Islands
S_shore
S_shelf

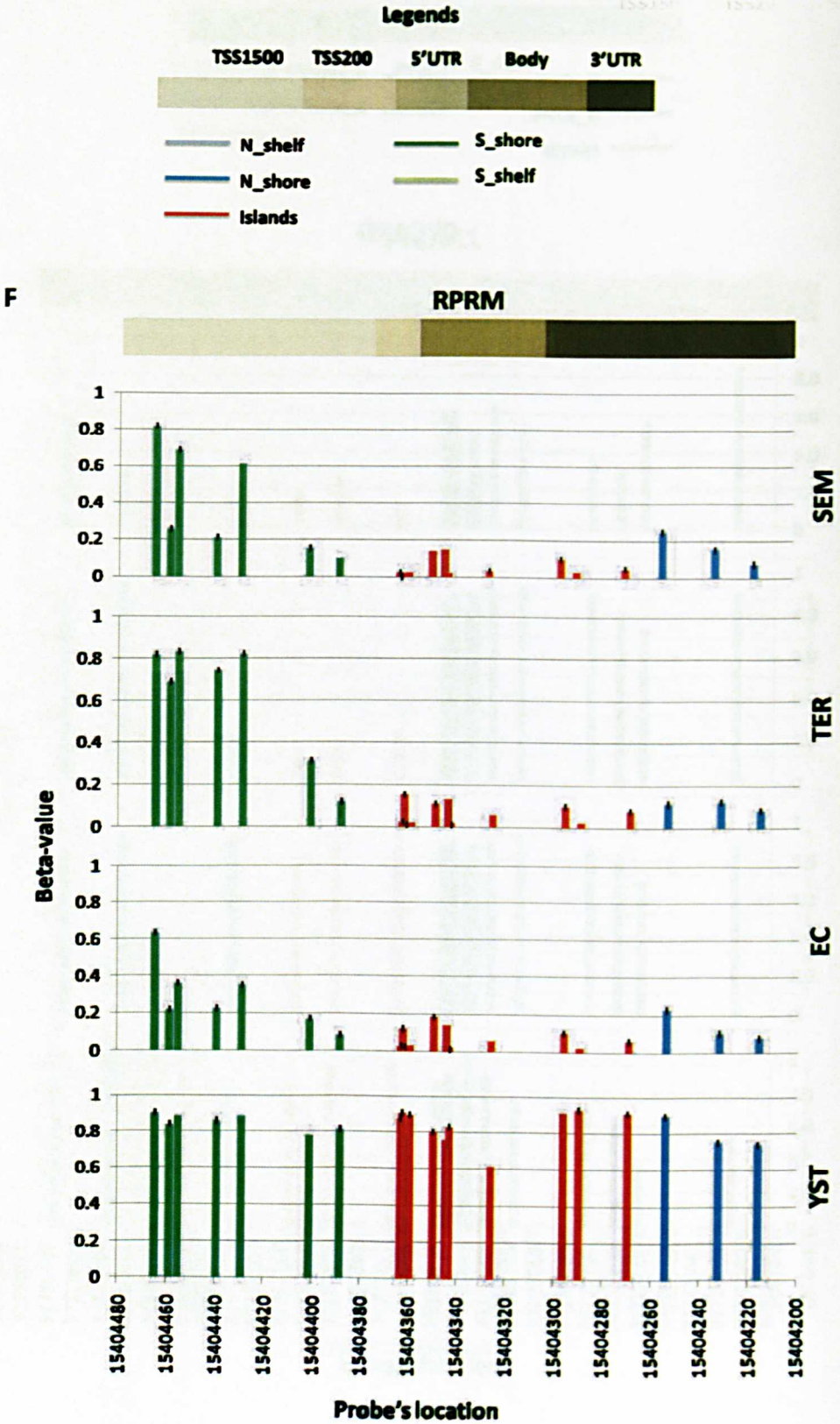
B


FUT4
Beta-value
SEM
TER
EC
YST
Probe's location


Legends
TSS1500
TSS200
5'UTR
Body
3'UTR
N_shelf
N_shore
Islands
S_shore
S_shelf
C
PARP12
Beta-value
SEM
TER
EC
YST
Probe's location


Legends
TSS1500
TSS200
5'UTR
Body
3'UTR
N_shelf
N_shore
Islands
S_shore
S_shelf
D
PRDM1
Beta-value
SEM
TER
EC
YST
Probe's location
10664050
10664070
10664090
10664110
10664130
10664150
10664170
10664190
10664210
10664230
10664250
10664270
10664290
10664310
10664330
10664350
10664370
10664390
10664410
10664430
10664450
10664470


Legends
TSS1500
TSS200
5'UTR
Body
3'UTR
N_shelf
N_shore
Islands
S_shore
S_shelf
E
PYCARD
SEM
TER
EC
YST
Beta-value
Probe's location
31123100
31123000
31122900
31122800
31122700
31122600
31122500
31122400
31122300
31122200
31122100
31122000
31121900
31121800
31121700
31121600
31121500
31121400
31121300
31121200
31121100
31121000
31120900
31120800
31120700
31120600
31120500
31120400
31120300
31120200
31120100


Legends
TSS1500
TSS200
5'UTR
Body
3'UTR
N_shelf
N_shore
Islands
S_shore
S_shelf
F
RPRM
SEM
TER
EC
YST
Beta-value
Probe's location
15404480
15404460
15404440
15404420
15404400
15404380
15404360
15404340
15404320
15404300
15404280
15404260
15404240
15404220
15404200

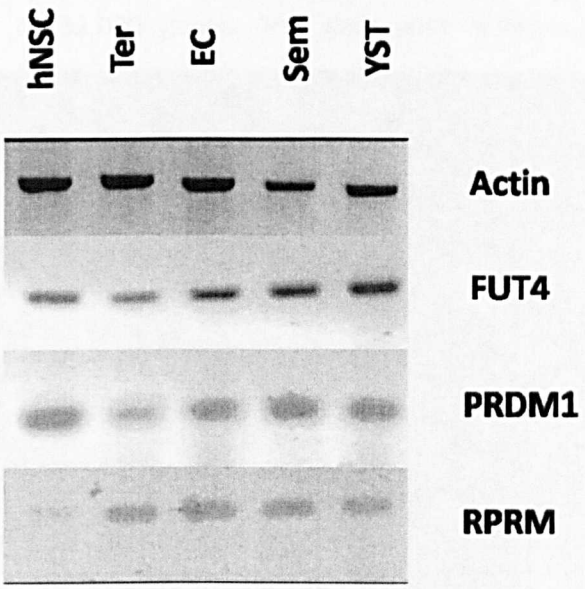


Figure 4.16: Reverse-transcriptase PCR performed on all four GCT cell lines for the selected genes that showed differential methylation at CpG islands in YST. It clearly showed that despite being heavily methylated, *FUT4*, *PRDM1* and *RPRM* expressions were not silenced in the YST cell line.

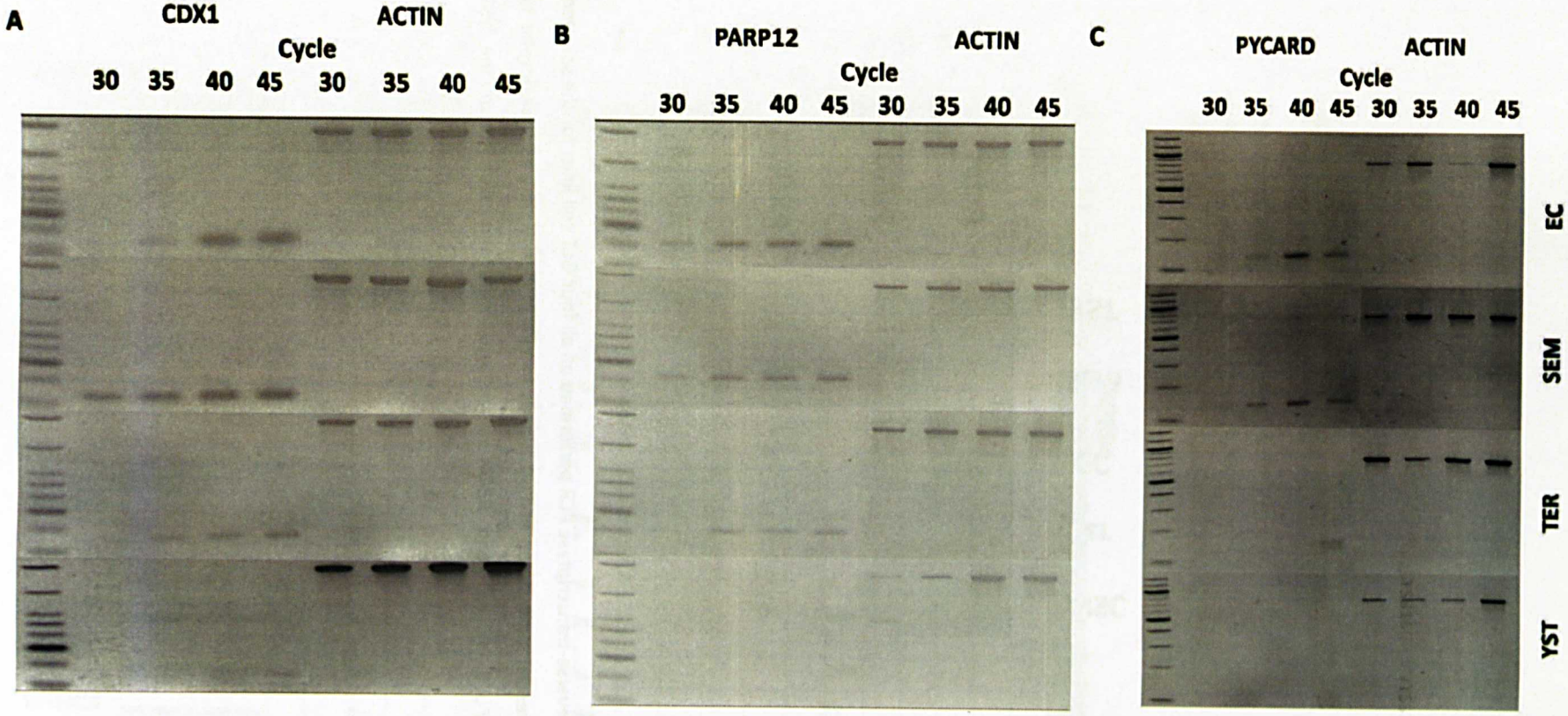


Figure 4.17: Semi-quantitative RT-PCR on A) *CDX1* B) *PARP12* and C) *PYCARD* showed a reduced expression of *CDX1* and a complete silencing of *PARP12* and *PYCARD* even after 45 cycles of PCR.

4.3. *Affymetrix array expression analysis*

In order to be able to determine the effect of methylation on gene regulation, we performed expression analysis on the four GCT cell lines using Affymetrix HumanGeneChip U133 Plus 2.0 arrays. This chip is an expansion from the previous Affymetrix HumanGeneChip U133 and covers an additional 6,500 genes to the previous coverage of 33,000 genes. This expression analysis was conducted at Nottingham Arabidopsis Stock Centre (NASC) in Sutton Bonnington, Nottingham. RNA from all four GCT cell lines was extracted. The eluted RNA was then analysed using a Bioanalyzer 2100 (Agilent). RNA which showed an RNA integrity number (RIN) of more than 9.0 (refer subsection 2.7.2) was selected for microarray, since this represents good-quality RNA.

Initial analysis of the data was performed using GeneSpring GX (Agilent Technologies) utilizing the software's default settings. By selecting these settings, all genes with a fold change of more than 2.0 are considered significant. This is in line with a previous study by Butte *et al.*(2001), which showed that Affymetrix gene expression data with a fold change measurement of more than 1.7 are reproducible and thus considered to be significant.

4.3.1. Correlation of methylation and expression data

Methylation of CpG islands (including those in the region near the transcription start site) has been considered to play a role in gene silencing. Thus, it is not surprising to see that many methylation studies have focused on CpG islands around the promoter regions. However, recent advancement in genome-wide methylation study has put these studies' findings into question since other gene regions/locations have also been found to be methylated and silenced (Irizarry et al., 2009, Brenet et al., 2012).

To understand further the correlation between methylation and expression in the GCT cell lines, we compared our Infinium methylation data to the Affymetrix expression array data. Our initial analysis was focused on the expression between the

YST and seminoma cell lines. Due to time constriction, initial analysis was performed only on genes that were methylated at the CpG islands and the CpG shores. We chose CpG islands since this was the location which showed the highest difference of methylation in the YST cell line as compared to the seminoma cell line. Furthermore our observation that the EC and teratoma cell lines displayed high differential methylation at all regions as compared to just CpG islands in the YST suggests that the methylation at CpG islands might be important in the YSTs.

On the other hand, we also observed a difference in the methylation level at the CpG shores between the YST and the seminoma cell lines albeit a smaller difference than at the CpG islands. However recently Irizarry *et al.* (2009) in their study conducted on colon cancer suggested that differential methylation at the CpG shores was more strongly correlated with gene expression as compared to the CpG islands. Since CpG shores methylation and its relationship to gene expression has not been mentioned in any previous GCT studies, we believed this was worth pursuing.

Out of 778 genes that were differentially methylated between seminoma and YST at the CpG islands, we obtained expression data for 67% of them (527 genes). Meanwhile expression data was obtained for 74% (337 out of 454 genes) of the differentially methylated genes at the CpG shores. This was not surprising given that the Infinium methylation arrays offered wider gene coverage of the genome as compared to the Affymetrix expression array. We also found that a gene could be represented by just one or more than one probe in the Affymetrix array panels. Therefore we categorized the expression data into three groups; one with no difference in expression observed, while the other two could either have differential expression in amajority of the probes (including genes with only one probe) or were only differentially expressed in a minority of the probes. In the YST cell line, the majority of genes (77.8% and 78%) that were represented in the expression analysis and hypermethylated at the CpG islands or the CpG shores, respectively, showed no difference in expression. Only 18.6% and 18.1% of genes that were hypermethylated at the CpG islands and CpG shores, respectively, showed lower expression at a majority of the probes (Figures 4.18A & B). Using Fisher's exact test, methylation at both CpG islands and CpG shores was found not to be associated with gene expression (p-value

of 0.9280). The top 30 genes that were hypermethylated at the CpG islands in the YST cell line and differentially expressed in the seminoma cell line are listed on Table 4.1 (a full list of genes is included in Appendix II).

Given that teratoma and EC cell lines displayed greater methylation than seminoma at all regions, we wondered whether in these cell lines either of the two regions (CpG islands or the CpG shores) could have a clearer association with gene expression. Initial analysis performed on the teratoma cell line still showed that the majority of genes differentially methylated at the CpG islands (82.7%) and the CpG shores (79.5%) were not differentially expressed. Only 16.1% and 14% of differentially methylated genes at the CpG islands and the CpG shores, respectively, showed differential expression at a majority of the probes (Figure4 4.19A & B). Again, using Fisher's exact test, methylation at both the CpG islands and the CpG shores turned out not to be associated with gene expression (p-value of 0.5962).

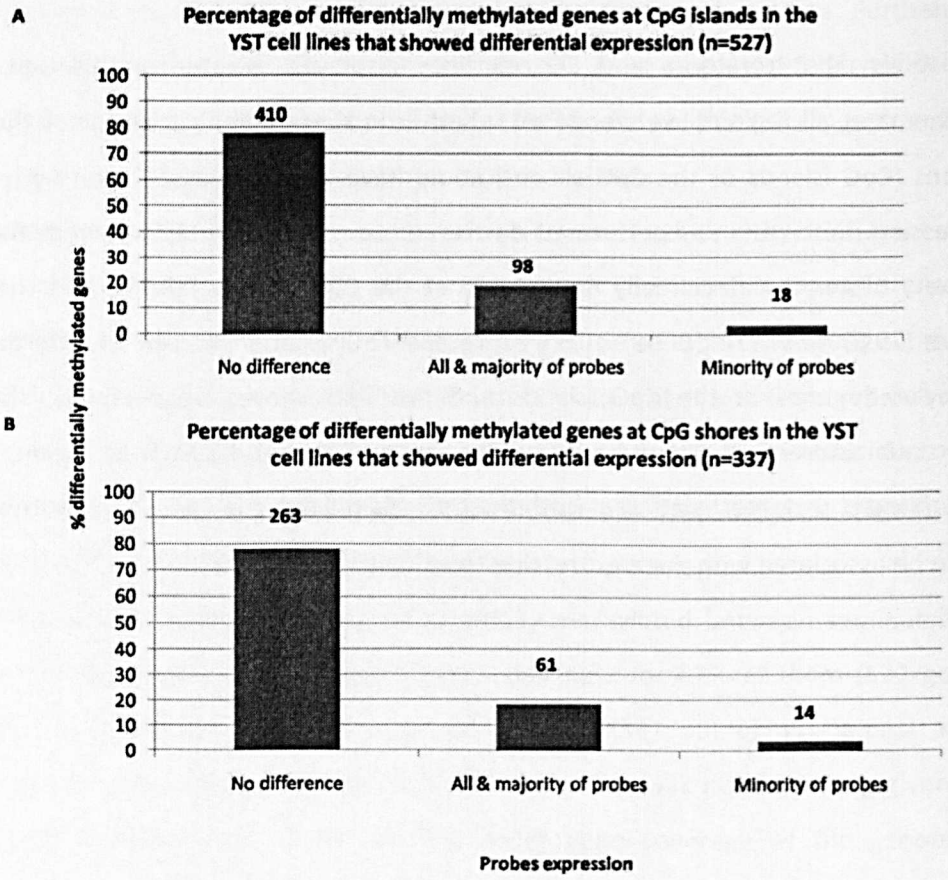


Figure 4.18: Graphs showing the percentage of differentially methylated genes at A) the CpG islands and B) the CpG shores in the YST cell lines for whicht we obtained expression data. Clearly a majority of the differentially methylated genes at both CpG islands and CpG shores did not show any difference in expression.

Genes	Infinium Methylation450K Array			Affymetrix gene expression
	Beta-value		Delta beta-value	Fold change (SEM/YST)
	SEM	YST		
TMEM179B	0.07988	0.96112	0.881231782	5.288
NAAA	0.09695	0.9664	0.869453578	4.216, 3.551, 0.938
RCSD1	0.11676	0.96193	0.845172125	4.492
LGALS3	0.0465	0.87019	0.823685457	27.934, 1.0
CYB5R2	0.1061	0.92785	0.82175694	17.977
PLEKHG4	0.08645	0.90034	0.813889264	7.164
C8orf42	0.11074	0.91336	0.8026243	26.467, 21.137, 13.038
TNFRSF10C	0.08396	0.87102	0.787060766	6.821, 2.124, 1.686, 1.035
DDX43	0.09841	0.87273	0.774321444	22.838
C13orf27	0.15784	0.93144	0.773596226	16.081
CXCL14	0.10936	0.88044	0.77108022	16.011, 10.608, 1.048
PYCARD	0.15634	0.92655	0.770201975	19.669
TRIM59	0.1343	0.89473	0.760432096	26.334, 2.112
AMPD3	0.17016	0.92856	0.758400967	2.07, 1.142
PARP12	0.07193	0.82972	0.757787533	4.293
ABHD14A	0.14316	0.89501	0.751853917	2.084
ECHDC2	0.1369	0.88179	0.74489879	16.349, 1.0
C8orf41	0.12148	0.86509	0.743610235	2.022
OVOL1	0.13545	0.87859	0.743140737	8.465, 2.08
C8orf84	0.21565	0.95544	0.739787541	3.005, 2.431, 1.0
HCG11	0.18361	0.91716	0.73355111	2.410, 1.674
HYLS1	0.15077	0.87805	0.727274774	15.174
PLD6	0.1701	0.8916	0.721497568	2.644
RBMXL2	0.13046	0.85041	0.719955922	43.16, 1.0
FABP5	0.10154	0.81806	0.716519285	32.377
MYD88	0.13919	0.84068	0.701490237	3.357
PRDM14	0.20302	0.8985	0.695481061	3.187
ACOT4	0.13438	0.81011	0.67572599	3.192
TTYH2	0.2548	0.92609	0.67129675	2.630, 1.117
ELMO3	0.26894	0.92462	0.65567793	2.188

Table 4.1: The top 30 genes that were differentially methylated in the YST cell line at the CpG islands and found to be differentially expressed. Using the default setting recommended by the Affymetrix gene expression array, expression with a fold change of more than 2.0 is considered significant.

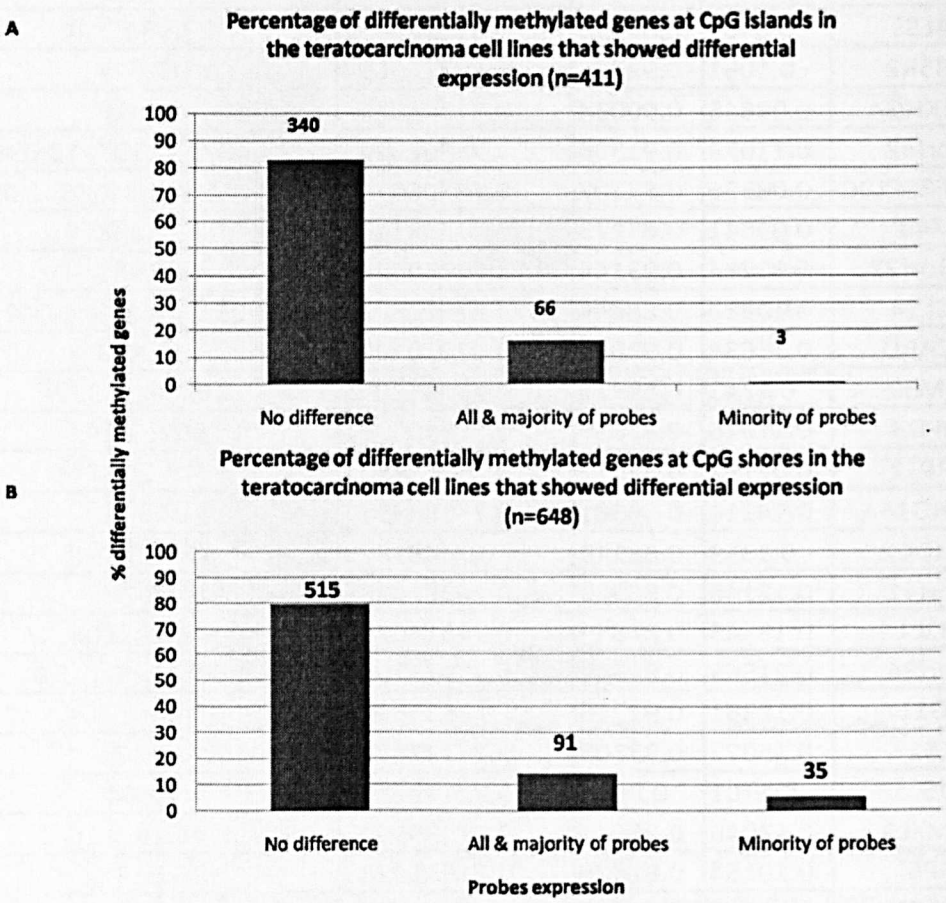


Figure 4.19: Graphs showing the percentage of differentially methylated genes at A) the CpG islands and B) the CpG shores in the teratoma cell lines for which we obtained expression data. Again, a majority of the differentially methylated genes at both CpG regions did not show any difference in expression.

4.4. Discussion

4.4.1. : Methylation profile of GCT cell lines

From the Infinium methylation array, we found non-seminomatous cell lines displayed a much higher methylation levels than the seminoma cell line which is in agreement with previous studies (Zhang et al., 2005, Smiraglia et al., 2002) although the levels we observed in both seminoma and non-seminomatous cell lines were much higher. We believe that this was due to the technique used and the fact that our coverage of genes was much wider than those studies.

We also showed that the EC and teratoma cell lines displayed a much higher methylation across the gene region as compared to the seminoma cell line. This was accompanied by a higher frequency of genes that were methylated as compared to seminoma. This suggests aberrant methylation features of these two cell lines. It is striking to see the similarity between these two cell lines even though EC is well known to share pluripotent characteristics while teratoma represent more differentiated cells (Oosterhuis and Looijenga, 2005). However, this could be explained by the origin of this teratoma cell line which was derived from a pluripotent stem cell population that can differentiate into all three germ layers and is able to give rise to teratoma (Andrews, 1984). Indeed, as mentioned earlier, the NT2D1 cell line used in this analysis may in fact be derived from an EC component of a teratoma.

Meanwhile, the YST cell line displayed a quite unique methylation profile as compared to the EC and teratoma cell lines. This is because in comparison to seminoma, YST only showed a differential methylation at the CpG islands and to a lesser extent in the shores. This might suggest that CpG island methylation is crucial in YST development. In addition to this, our observation totally contrasted with the finding by Irizarry *et al.* (2009) that the shores region was the most differentially methylated regions in colon cancer cell lines. However, this contrast in findings might be due to the different cell lines used and again implies that each cell line has its own methylation characteristics.

Nonetheless when we looked at the methylated genes in all four cell lines, we found that majority of the genes methylated in the seminoma cell line were also methylated in the other three cell lines. This similarity implies that EC, teratoma and YST could have arisen from seminoma that undergone aberrant methylation. Despite thegeneral idea that all germ cell tumours arise from carcinoma in situ (CIS), seminoma has been found to share similar characteristics with CIS in terms of histology (Sonne et al., 2009), methylation level (Netto et al., 2008) and gene expression (Almstrup et al., 2005). Therefore, aberrant methylation in seminoma could possibly give rise to the differentiated GCTs. This might explain why GCTs could have a mixture of seminoma and YST components(Looijenga, 2009) or how seminomatous GCTs could recur as YST after therapy (Wong et al., 2010). Furthermore, a study suggested that repression of *BLIMP1* as a consequence of inhibition of BMP/SMAD signalling in a seminoma cell line could cause seminoma differentiation into mixed non-seminomatous lineages (predominantly choriocarcinoma-like cells)(Nettersheim et al., 2011). This suggests that repression of *BLIMP1* by methylation might result in the same consequences. However, in our analysis, *BLIMP1* (also known as *PRDM1*) was equally expressed in all four GCT cell lines. Therefore, it is still inconclusive whether *BLIMP1* repression alone is sufficient to drive seminoma into non-seminomatous lineages.

4.4.2. Methylation and gene expression

By correlating methylation and expression array data in YST and teratoma cell lines, we found that neither methylation at the CpG islands nor methylation at the CpG shores had a strong influence on gene expression. Again, this finding is in contrast with the finding by Irizarry *et al.*(2009) which showed a strong correlation between CpG shores methylation and gene expression. This could mean that such an observation can be tumour- or cell-type specific. Indeed, methylation at CpG islands does not necessarily result in gene silencing as shown in the RT-PCR done on the selected six genes methylated at CpG islands in YST. This is not surprising since methylation is not the only factor that can contribute to transcription silencing. CpG islands have been known to harbour binding sites for transcription factors (Deaton and Bird, 2011). Studies have shown that human promoters, including those of housekeeping genes,

tend to have a transcription factor binding motif (Rozenberg et al., 2008, Landolin et al., 2010) with some of these factors have been shown to regulate methylation and gene transcription as well (Gebhard et al., 2010).

While many methylation studies have focused on CpG islands near the promoter region, other studies have also pointed out the possibility of methylation outside of this region that could also influence gene regulation (Eckhardt et al., 2006, Irizarry et al., 2009, Ball et al., 2009, Brenet et al., 2012). Indeed, our analysis of methylation across all gene locations showed that the highest methylation levels were in the gene body and the 3'UTR. Numerous reports have shown high gene-body methylation in fibroblast, induced pluripotent stem cell and even in the active X-chromosome (Ball et al., 2009, Hellman and Chess, 2007). However, this high methylation was associated with high gene expression suggesting that there might be a dual function of methylation (Ball et al., 2009). Further analysis still needs to be performed in order to find out whether the high gene-body methylation observed in our analysis correlates with gene expression.

The finding that other gene regions/locations could also be methylated and regulate gene transcription has sparked an interest in finding the right region/locations behind this observation. Brenet *et al.* (2012) in their study using the Sequence Tag Analysis of Methylation Patterns (STAMP) assay in human-derived leukemia cell lines showed that methylation at the 5'UTR and the first exon are important for transcriptional silencing. On the other hand, a study of liver cancer showed that methylation of just one CpG is enough to silence the *TTP* gene (Sohn et al., 2010) suggesting that gene silencing does not require methylation of a cluster of CpG islands to be silenced. Our method of answering the same question using the Infinium methylation array proved to be futile. Although Infinium reveals a clearer picture of methylation across the gene region as shown in our graphical representation, it does not really help in highlighting the crucial sites. Even though for one gene, *PARP12*, since its CpG islands was only methylated and silenced in the YST cell lines and not in the other three cell lines, this would suggest a role was played by the CpG islands methylation in *PARP12* silencing. However, this still needs to be validated and we

believe the only way to do that is by using a DNA methylation inhibitor such as 5-azadeoxycytidine and comparing the methylation profile with the non-treated cell lines.

4.4.3. Differentially methylated and expressed genes

We have identified 98 out of 528 hypermethylated genes in the YST cell line that showed lower expression as compared to the seminoma cell line. Using RT-PCR and semi-quantitative RT-PCR analysis on six of those hypermethylated genes, we further validated that three genes (*CDX1*, *PYCARD*, and *PARP12*) were expressed in the seminoma cell lines at a much higher level than the YST cell line. It is interesting that *PYCARD* in the YST cell line showed similar methylation and expression features as in the paediatric YST samples in our previous Golden Gate methylation array (Jeyapalan et al., 2011).

Caudal-related homeobox protein 1 (*CDX1*) is a transcription factor normally associated with the regulation of intestinal proliferation. In one study, overexpression of *Cdx1* in a rat intestinal epithelial cell line led to cell cycle arrest and reduced the cell proliferation (Lynch et al., 2000). The same observation was found in colon cancer cell lines (Lynch et al., 2003). *CDX1* expression was found to be reduced in colorectal carcinoma and colorectal cancer cell lines and this has been associated with promoter hypermethylation (Pilozzi et al., 2004, Suh et al., 2002, Wong et al., 2004). Therefore, hypermethylation and reduced expression of *CDX1* in the YST cell lines might be contributing to the aggressive nature of YSTs. Functional analysis of *CDX1* still needs to be performed to find out the exact role of *CDX1* silencing in the YST cell lines.

On the other hand, not much is known about the function of the poly (ADP-ribose) polymerase family, member 12 (*PARP12*). No study has been reported regarding its function in any type of cancer or even in normal human development although in cattle it has been associated with uterine receptivity during pregnancy (Bauersachs et al., 2006, Forde et al., 2012). Interestingly, *PARP12* has been found to have an alternative promoter (Rauch et al., 2009) in the B-cell methylome. Using a combination of the methylated CpG island recovery assay (MIRA) and whole-genome

tiling array, this group found four genes including *PARP12* that are expressed despite having promoter hypermethylation. They then mapped the transcription initiation site and found an unmethylated promoter in *PARP12* and two other genes which they believe is the promoter that controls gene expression. We did not determine whether such an alternative promoter does exist in the GCT cell lines, but from our analysis, promoter hypermethylation of *PARP12* correlates with the gene silencing.

There are still 95 genes that are hypermethylated and have reduced expression in the YST cell line that have not been validated by RT-PCR. Many of these genes represent genes with biological functions that are possibly related to tumour development. Therefore, future investigation of these genes is worth pursuing.

Korkola *et al.*(2005) in their analysis using Affymetrix microarrays, identified a list of genes uniquely expressed in each non-seminomatous GCT and suggested that these genes could be used as a signature marker for each cell line. Comparing their expression data on YST samples with our YST cell line, we found that six out of nine genes (*APOA2*, *BMP2*, *FOXA2*, *EOMES*, *CYP26A1*, and *CCKBR*) identified as uniquely expressed in YST by Korkola *et al.*(2005) were also highly expressed in the YST cell line in our analysis. We could not determine whether such expression is tumour-specific as we used a seminoma cell line as the control and not normal testis tissue as used by Korkola *et al.*(2005). However, all six genes were found to be highly expressed as compared to the other cell lines we analysed with most of them showing differential expression between 10 and 100 fold. This supports the possibility of using these genes as markers for YSTs.

Previously in the GoldenGate analysis, we showed that none of the paediatric seminoma samples showed any hypermethylation of *GSTP1* which was in contrast to a previous study (Koul et al., 2002). From the Infinium methylation analysis, we again found that *GSTP1* was not methylated (beta-value 0.0883) in the seminoma cell line. In addition to that, *SORBS1*, another gene previously found to be hypermethylated in seminoma samples (Lind et al., 2006), was also found to be not methylated in the seminoma cell line (beta-value 0.1034). However, since no normal control was

included in our Infinium arrays analysis, we could not determine whether these two genes methylation level were high enough to be considered as hypermethylated.

5. INHIBITION OF DNA METHYLATION AND ITS EFFECT ON GCT CELL LINES' BEHAVIOUR

5.1. Introduction

In the previous two chapters, we observed that both paediatric YSTs and non-seminomatous GCT cell lines were heavily methylated as compared to the seminomas. Given that non-seminomatous GCTs display genome-wide hypermethylation, they should in theory be able to respond to epigenetic therapy just like other tumours with a similar methylation feature.

Many epigenetic modifiers have been shown to contribute to the hypermethylation seen in cancer. Overexpression of DNA methyltransferase 3B (*DNMT3B*) and its splice variants has been found in lung, breast and embryonal carcinoma (Roll et al., 2008, Beaulieu et al., 2002, Gopalakrishnan et al., 2009, Van der Auwera et al., 2010). Meanwhile, *EZH2* and *SUZ12*, components of the Polycomb group were found to be overexpressed in prostate and pancreatic cancer (Chen et al., 2010, Karanikolas et al., 2010) and lymphoma (Martin-Perez et al., 2010) respectively.In order to identify the factor(s) behind the hypermethylation seen in our YST samples, expression analysis of these three modifiers was performed using Real-Time Quantitative PCR (RT-PCR).

5.2. Results

5.2.1. Factors contributing to YST hypermethylation

Quantitative PCR was carried out using a SYBR green kit and 100ng of the paediatric GCT cDNA sample. Samples were subjected to 40 cycles of quantitative PCR using a Stratagene MXPro 3005P machine. For relative quantification, we used one of the YST samples (#17) as a control (also known as a calibrator), which the expression of the gene of interest is compared to. Both tumour samples and calibrator signal were normalized with internal reference genes, in this case beta-actin and 18S RNA. These normalizers function as an internal control that eliminates any variability in terms of quantity and quality in the samples and the calibrator. Relative quantification of

expression was then calculated by taking the average fold change of the samples to the calibrator (Figure 5.1). Again, due to a limited amount of cDNA being available, we could only manage to do duplicate analysis of some of the samples.

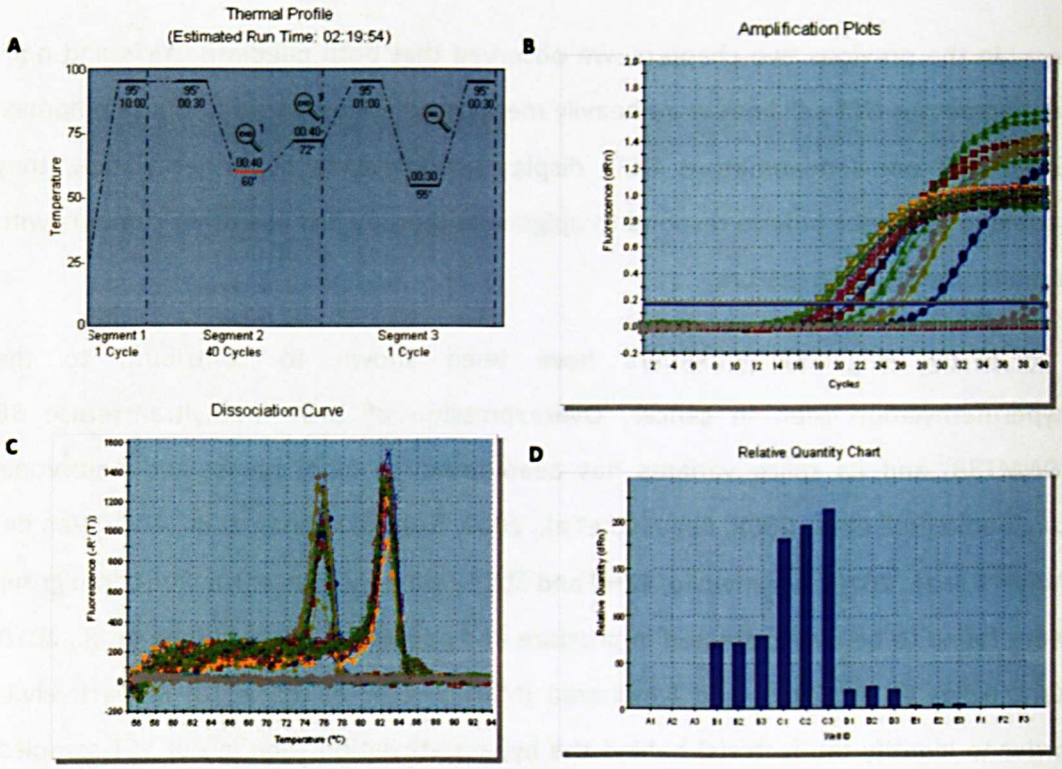


Figure 5.1: Representative diagrams of SYBR Green quantitative PCR consolidation data. A) Diagram showing the PCR cycle detail. B) Amplification plots which show that all samples were amplified at almost the same cycles and there was no amplification of the negative control. C) Dissociation curve which shows no irregular peaks suggesting the absence of contamination. D) Bar graph showing the relative expression quantification of the samples according to the calibrator.

From the average $\log_2$ fold change, we found that in relation to human neural stem cells, *DNMT3B* was more highly expressed in the majority of YST samples as compared to seminomas (Figure 5.2A) and this was significant (p-value of 0.035) when we grouped the samples according to their tumour type (Figure 5.2B). Meanwhile, we observed that both *EZH2* and *SUZ12* expression was similar in both tumour types (p-values of 0.556 and 0.5510 respectively) either as individual samples or as a group (Figures 5.3A and 5.3B). This suggests that *DNMT3B* might be a contributing factor towards the hypermethylation seen in YSTs.

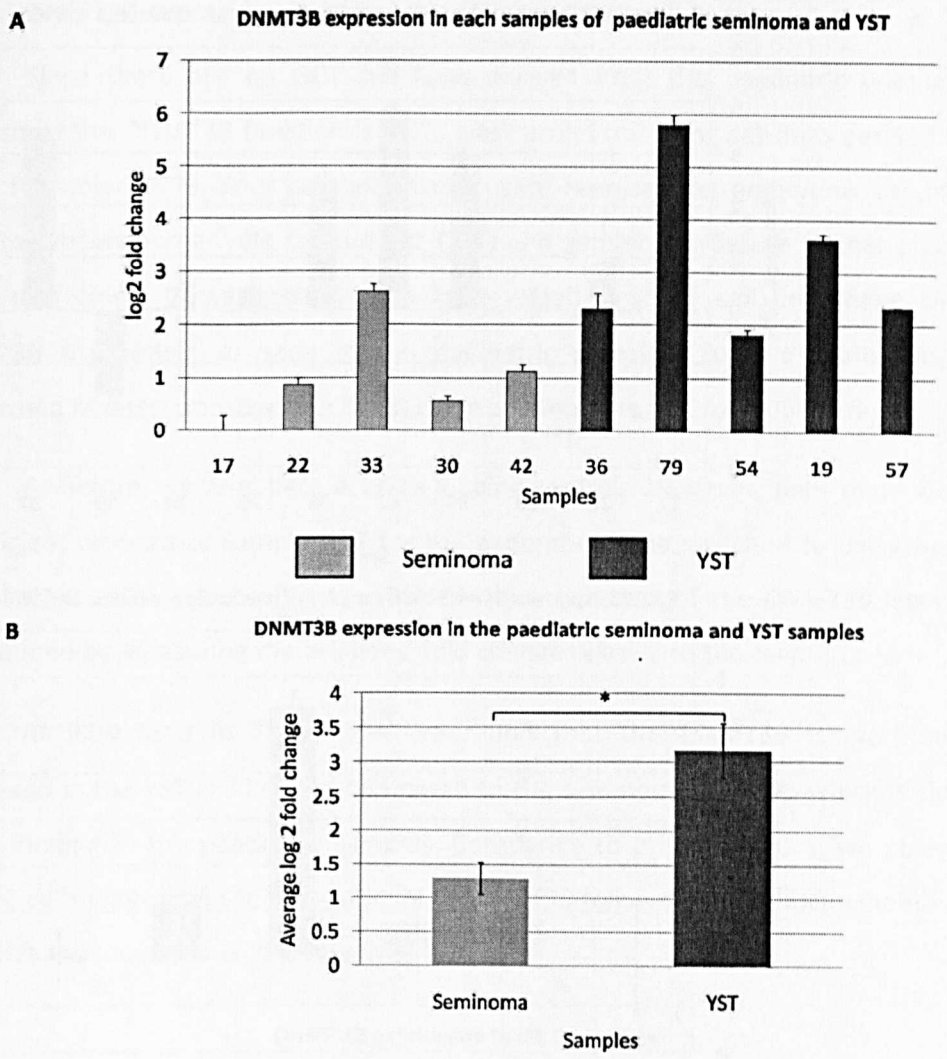


Figure 5.2: Bar graphs showing that YST samples had a higher level of *DNMT3B* expression than seminoma samples. *DNMT3B* expression levels in A) tumour samples which showed that YST samples displayed higher expression of *DNMT3B* than seminoma samples, and B) tumour group which showed significant difference of *DNMT3B* expression between seminoma and YST. * denotes p-value<0.05 using Mann-Whitney t-test. Error bars represent standard deviation.

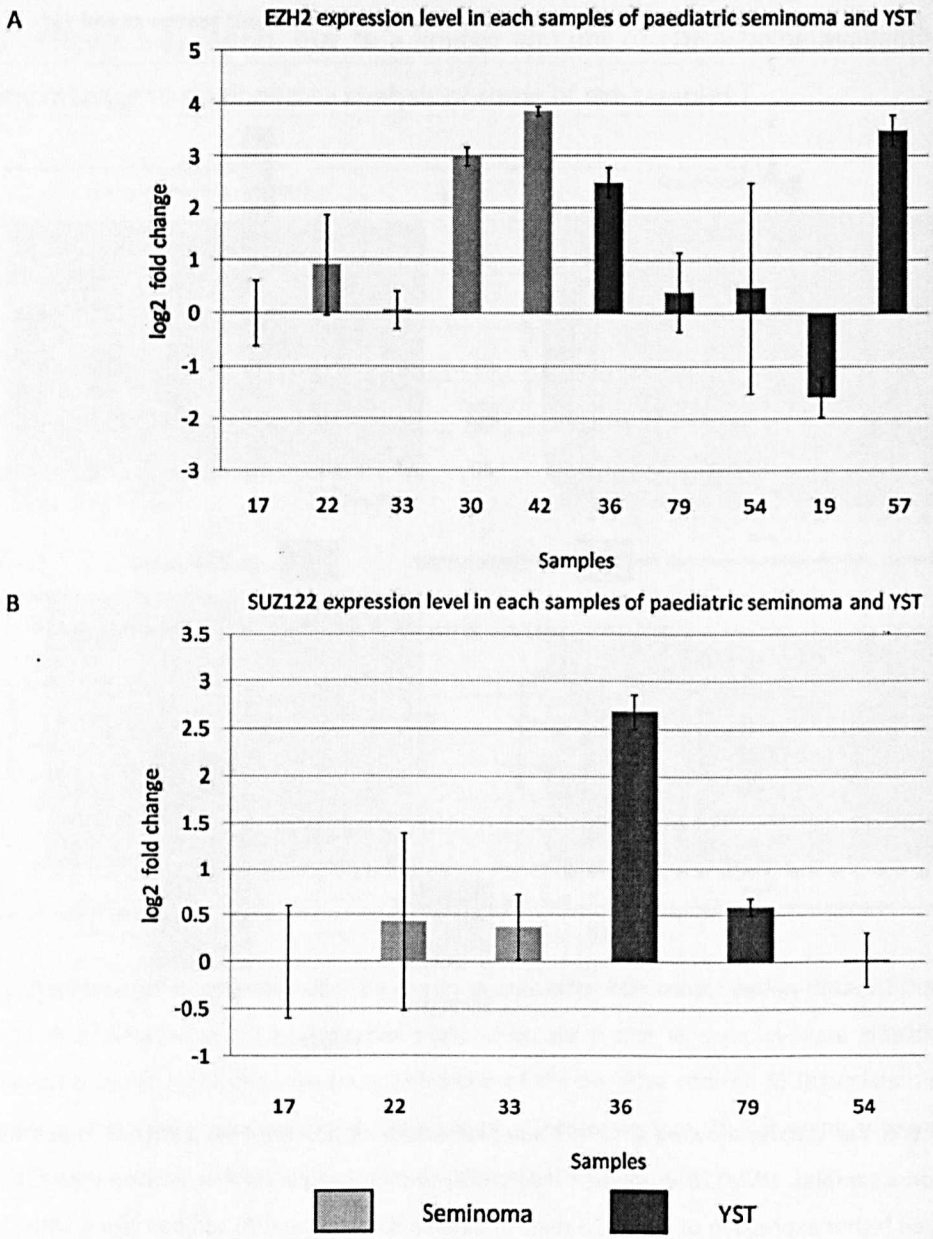


Figure 5.3: Expression levels of A) *EZH2* and B) *SUZ12* in seminoma and YST samples. Both YST and seminoma samples displayed almost similar levels of expression for both *EZH2* and *SUZ12*. Dark grey represents YSTs while lighter grey represents the seminomas. Error bars represent standard deviation.

5.2.2. DNMT3B expression in germ cell tumours (GCTs) cell lines

Since there are no GCT cell lines derived from the paediatric population, analysis of the DNMT3B function in GCTs was carried out using cell lines derived from adult testicular GCTs. Four cell lines were used, representing embryonal carcinoma (EC), teratocarcinoma, yolk sac tumour (YST) and seminoma. Before further analyses were performed, it was essential to know whether these cell lines have similar *DNMT3B* expression, as seen in the paediatric samples. To investigate this, we performed reverse-transcriptase QPCR using a Stratagene MxPro 3005P in duplicates.

As before, we used beta-actin as loading controls. However, since there was an insufficient amount of sample #17 for the experiment, we switched to using human neural stem cells as the calibrator. Relative quantification of the *DNMT3B* level was determined by measuring the cell lines'.fold change relative to the calibrator.

As illustrated in Figure 5.4, we found that the *DNMT3B* is more highly expressed in the YST cell lines as compared to the seminoma cell line, which is similar to the finding in the paediatric samples. Comparing to other cell lines, we observed that EC cells expressed the highest level of *DNMT3B* followed by teratocarcinoma and, YST, with the lowest level in seminoma.

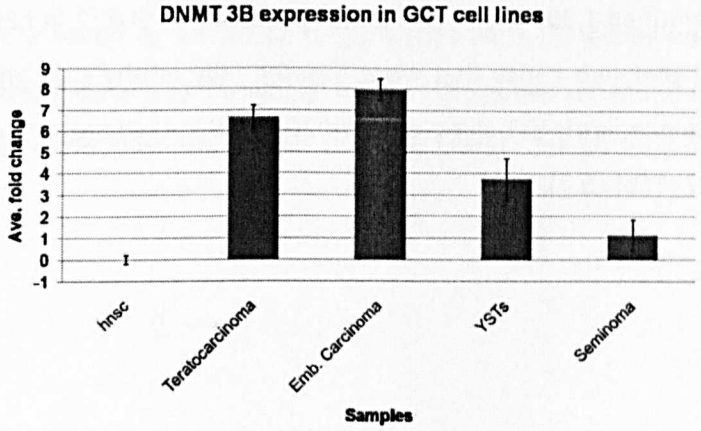


Figure 5.4: Bar graph showing the average log_2 fold change of *DNMT3B* expression in all four GCTs cell lines. Clearly non-seminomatous GCTs had a higher *DNMT3B* level than seminomatous GCTs, with embryonal carcinoma cell lines showing the highest level of *DNMT3B* expression. Error bars represent standard deviation.

5.2.3. Epigenetic modifiers' target gene among hypermethylated genes in YSTs

It seems, from the analysis above, that genes that are hypermethylated in both paediatric YST samples and the YST cell lines might be enriched for those targeted by DNMT3B. In order to determine whether there is any such bias,we looked at published articles related to the gene targets of DNMT3B, SUZ12 and EZH2. Choi *et al.*(2011), in a recent study, looked at DNMT target sites by stably transfecting HEK293T cells with plasmids containing DNMTs and their isoforms. Using the Illumina GoldenGate methylation array, they found 504 loci showed changes in methylation level after overexpressing each DNMT construct.

In order to compare our data to that of Choi *et al.*(2011), we first focused on those genes we found to be hypermethylated and differentially expressed in YSTs. From the eight differentially methylated and differentially expressed genes identified in the paediatric samples, we identified that three of them (*HLA-F, TFAP2C* and *HDAC9*) are targets of DNMT3B (Table 5.1). We next looked at studies performed in epithelial ovarian cancer (Li et al., 2012) and human embryonic stem cells (hESC) (Lee et al., 2006), which looked into the target genes of EZH2 and SUZ12 respectively. Li *et al.*(2012) identified 60 EZH2 target genes through chromatin immunoprecipitation and gene expression profile. Meanwhile, Lee *et al.*(2006), in their experiment using DNA microarrays, identified 1,893 annotated genes occupied by SUZ12 in hESC. Using these data, we found that two genes that were differentially methylated and differentially expressed in the paediatric YST samples (*TFAP2C* and *PYCARD*) are potential targets for SUZ12 and EZH2 (Table 5.1).

Genes	Epigenetic Modifier			
	DNMT3A	DNMT3B	SUZ12	EZH2
HLA-F	No	Yes	Yes	N/A
TFAP2C	Yes	Yes	Yes	Yes
CD2	No	No	N/A	N/A
ETV1	No	No	N/A	N/A
PYCARD	No	No	Yes	Yes
CASP8	No	No	N/A	N/A
HDAC9	Yes	Yes	N/A	N/A
EVI2A	No	No	N/A	N/A

Table 5.1: Table showing epigenetic modifiers' targets identified among the differentially expressed genes in the paediatric GCT cohort based on three previous studies by Choi et al (2011), Lee et al (2006) and Li et al (2012). Most of the differentially expressed genes in the paediatric GCT cohort turned out not to be a target for all three modifiers. N/A represents data that were not available from the references.

We then expanded our analysis to all 85 genes found to be hypermethylated in the YST samples. Using the same datasets as before, we found that eleven of the genes found to be hypermethylated in the YST samples are target genes for DNMT3B1 and only two are target genes for DNMT3A1. In addition to that, an additional 22 genes are target genes for both DNMT3B1 and DNMT3A1 (Figure 5.5). On the other hand,38 of these genes were found to be SUZ12 target genes while 27 genes were identified as EZH2 target genes (Figure 5.5). Strikingly, most of the genes are found to be the targets of more than one modifier with seven of them targets for all four modifiers (Figure 5.5).

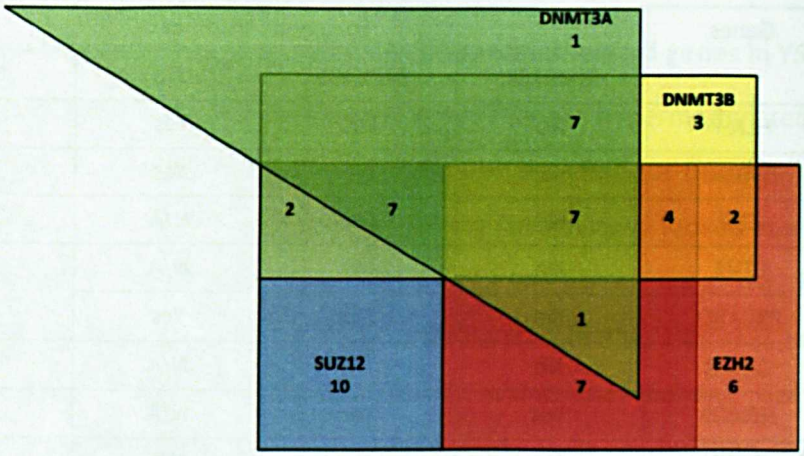


Figure 5.5: Diagram showing number of target genes for DNMT3A, DNMT3B, SUZ12 and EZH2 identified from our list of 85 genes hypermethylated in YSTs. None of the genes were exclusively a target of DNMT3B. Target genes were identified from previous studies (Choi et al., 2011, Lee et al., 2006, Li et al., 2012)

Applying the same analysis on the Infinium Methylation450K arrays data, we found only two out of 98 genes (*CDH1*, *WNT2B*) found to be differentially methylated and differentially expressed in the YST cell lines were previously identified DNMT3B targets (Table 5.2). We believed this small number might be due to the different array coverage used, because the Infinium methylation panel covers approximately 30 times more genes as compared to the Golden Gate methylation panel used by Choi *et al.*(2011). In contrast, 18 genes that were differentially methylated and differentially expressed in the YST cell line were identified as targets for EZH2 and one gene was found to be a target for SUZ12 (Table 5.2).

Genes	Epigenetic Modifier			
	DNMT3A	DNMT3B	SUZ12	EZH2
CDH1	No	Yes	N/A	N/A
WNT2B	No	Yes	N/A	N/A
RASD1	N/A	N/A	Yes	N/A
TTYH2	N/A	N/A	N/A	Yes
TMEM163	N/A	N/A	N/A	Yes
SHISA3	N/A	N/A	N/A	Yes
RCSD1	N/A	N/A	N/A	Yes
NPEPL1	N/A	N/A	N/A	Yes
MEIS1	N/A	N/A	N/A	Yes
MDFI	N/A	N/A	N/A	Yes
MAP7	N/A	N/A	N/A	Yes
HYLS1	N/A	N/A	N/A	Yes
GAL	N/A	N/A	N/A	Yes
FAM162B	N/A	N/A	N/A	Yes
FAM150B	N/A	N/A	N/A	Yes
DLX3	N/A	N/A	N/A	Yes
CYP11A1	N/A	N/A	N/A	Yes
C1QTNF4	N/A	N/A	N/A	Yes
AP1M2	N/A	N/A	N/A	Yes
ACOXL	N/A	N/A	N/A	Yes
ACADL	N/A	N/A	N/A	Yes

Table 5.2: Table showing epigenetic modifiers' targets identified among the differentially expressed genes in the YST cell line based on three previous studies by Choi et al (2011), Lee et al (2006) and Li et al (2012). It seems that none of the epigenetic modifiers share similar target genes, but this could be due to the different array coverage used in these methods as compared to the Infinium Methylation450K arrays that we used. N/A represents data that were not available from the references.

On the other hand, analysis performed on 1,195 genes found to be hypermethylated in the YST cell lines showed that only 11 genes werea target for DNMT3B while four genes were a target for DNMT3A. Only one gene, *HIC1*, was found to be a target for both DNMT3A and DNMT3B. Meanwhile, ten genes were found to be a target for SUZ12 with one gene, *PDGFRA*, also turned out to be a target for DNMT3B. In contrast, 170 genes were found to be a target for EZH2. Although it would seem that many more hypermethylated genes were targets for EZH2, we could not make a conclusive statement on the possible epigenetic modifiers behind YSTs' hypermethylation. This is because all three studies used different methods in identifying the target genes, and each method has a big difference in the number of genes covered in their analysis. Furthermore, all three studies were conducted on different cell lines or cancer cells; thus it seems likely that their findings are specific for particular cell types.

5.2.4. Expression analysis of differentially expressed genes in adult cell lines

As with DNMT3B, functional analysis on the differentially expressed genes in the paediatric population can only be assessed using cell lines that are entirely derived from adult testicular GCTs. Thus, it is crucial to determine those genes' expression in the cell lines. Again, we carried out reverse-transcriptase QPCR in duplicate to determine the expression of one of those genes, *CASP8*, in seminoma and YST cell lines. Using human neural stem cells as a calibrator and beta-actin as a normalizer, we found that *CASP8* is significantly more expressed in seminoma (Fig 5.6). By comparing both *DNMT3B* and *CASP8* expression, there seems to be an inverse correlation between them where high *DNMT3B* expression links with low *CASP8* expression.

To determine the expression of other differentially expressed genes in the cell lines, we decided to use the Affymetrix gene expression array. Out of eight differentially expressed genes in paediatric seminomas, only seven of them (*PYCARD*, *CASP8*, *HLA-F*, *TFAP2C*, *ETV1*, *EVI2A* and *HDAC9*) were included in the array. The expression levels of those genes in the seminoma and the YST cell lines were compared using Gene Spring software. All except *HLA-F* were successfully analysed and were expressed as a fold change. The initial results showed that four out of six genes

showed more than a two-fold change in expression. This result is summarized in Table 5.3.

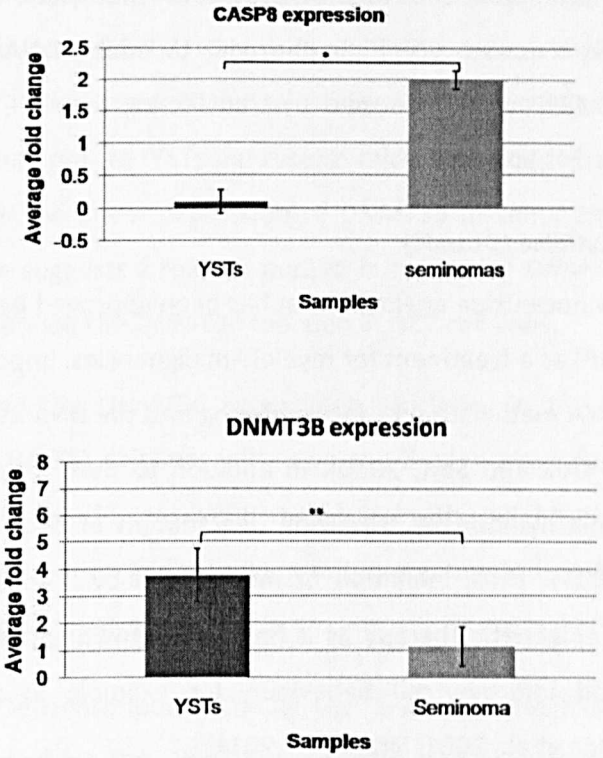


Figure 5.6: Bar graph showing the average fold change in the expression levels of *CASP8*, and *DNMT3B* in YST and seminoma cell lines. The *DNMT3B* expression level seems to correlate with their *CASP8* expression level. * denotes p-value <0.01 while ** denotes p-value <0.001 using Mann-Whitney t-test. Error bars represent standard deviation.

	Paediatric GCT	GCT cell lines
Genes	Ave. LODS value	Fold change in expression
CASP8	7.686	3.9, 2.1, 1.0
PYCARD	11.988	19.67
ETV1	8.93	0.57, 0.54, 0.45, 0.6, 0.22
TFAP2C	28.711	181.5, 36.0
HDAC9	9.563	21.5, 1.0, 1.0, 0.76
EVI2A	4.376	1

Table 5.3: Affymetrix expression array data of YST and seminoma cell lines on previously identified differentially expressed genes in the paediatric population. It clearly showed that most of the genes differentially expressed in the paediatric samples were also differentially expressed in the cell lines. LODS value of more than 4.0 and fold change of more than 2.0 is considered to be significant.

5.2.5. Inhibition of DNA methylation and DNMT3B

To further understand the function of DNA methylation and DNMT3B in GCT cell lines, we used two approaches to knockdown DNA methylation and DNMT3B. The first approach used 5-azadeoxycytidine (5-azadC) to inhibit DNA methylation while knockdown of *DNMT3B* was performed by transfecting the GCT cell lines with the microRNA-29b (mir-29b) plasmid.

5.2.5.1. *5-azadeoxycytidine (5-azadC)*

5-azadC is a nucleoside analogue that has been approved by the Food and Drug Administration (FDA) as a treatment for myeloid malignancies. Importantly, it has been shown to inhibit DNA methylation by incorporating into the DNA during DNA synthesis (Christman, 2002, Chik and Szyf, 2010). In addition to that, 5-azadC has also been shown to inhibit methylation by triggering degradation of DNA methyltransferases (Ghoshal et al., 2005). Thus, inhibition of methylation by 5-azadC proves to be an important tool in epigenetic therapy as it has been shown to be able to reactivate silenced genes and improve cell behaviour, for example in sensitizing cells to chemotherapy (Fulda et al., 2001, Siraj et al., 2011).

Among GCTs, previous studies using 5-azadC were only done in seminoma (Wermann et al., 2010) and teratocarcinoma cell lines (Beyrouthy et al., 2009) which used concentrations of 10 μm and between 100 and 1,000 nM respectively. Indeed from works performed by my colleague, Safiah Al-Hazmi, it was found that the teratoma cell line only showed toxicity towards 5-azadC at concentrations above 1 μm. However, the seminoma cell line was found to be able to tolerate up to 20 μm 5-azadC. By treating 3 X 10^4 cells with a range of 5-azadC concentrations, she also found that the EC cell line could only withstand up to 0.1 μM 5-azadC, while the YST cell line started to show signs of toxicity at 20 μM 5-azadC. Therefore, these concentrations were used as a guide for the assays on cisplatin sensitivity and the wound healing assay.

5.2.5.2. *MicroRNA 29b (mir29b)*

MicroRNA-29b (mir29b) has been found to be able to knockdown both *DNMT3A* and *DNMT3B*, and reactivate genes that were previously repressed in many tumour types (Fabbri et al., 2007, Garzon et al., 2009). Interestingly, members of the microRNA-29 family have been found to be significantly upregulated in paediatric seminomas as compared to YSTs in recent microRNA expression profiles which correlates well with the lower expression of *DNMT3B* in seminomas (Murray et al., 2010). This not only suggests a role for mir29b in regulating *DNMT3B*, but also offers us a means to manipulate the *DNMT3B* function in GCT cell lines.

To determine the *DNMT3B* knockdown efficiency in the YST cell lines, we transfected 1 - 2 X 10^6 YST cell lines with 3 μg and 7 μg of pLemiR plasmid containing mir29b. For the controls, YST cells were transfected with GFP. Mediums were changed 6 hours after electroporation and cells were left to recuperate for 48 hours after which cells were harvested for analysis.

Using reverse-transcriptase QPCR, the level of *DNMT3B* knockdown was determined by normalizing the signal to beta-actin, and calibration to human neural stem cells. We found that both transfection with three and seven micrograms of mir29b achieved significant knockdown of *DNMT3B* (Figures 5.7 A and B) with seven micrograms showing a much greater knockdown of up to an eight-fold change (Figure 5.7 B). Therefore, transfection with 7ug of mir29b was used in further experiments.

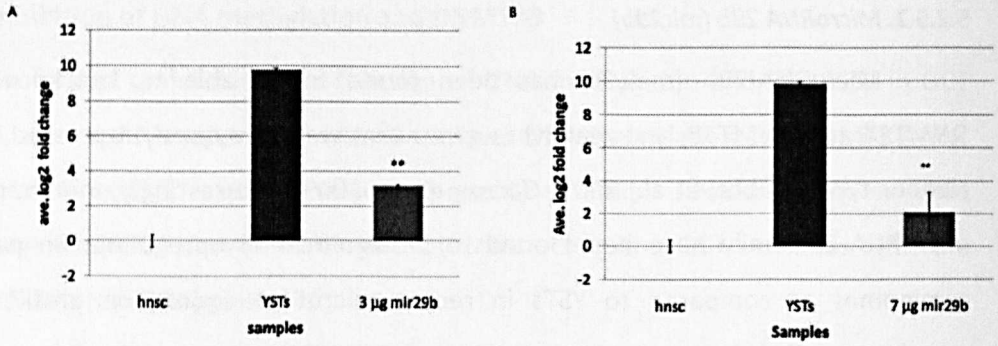


Figure 5.7: Bar graphs showing*DMT3B* expression level after transfection with A) 3ug and B) 7ug of mir29b. Both transfections significantly reduced *DNMT3B* expression level in the YST cell line as compared to the YST transfected with GFP. ** denotes p-value <0.001 using paired t-test. Error bars represent standard deviation.

5.2.6. : Cell line sensitivity to cisplatin after inhibition of DNA methylation and DNMT3B knockdown

Although germ cell tumours are considered to have a high cure rate, the cure rate decreases substantially in patients with a more malignant form or who have metastatic diseases (di Pietro et al., 2005). Cisplatin has been widely used to treat GCTs, but despite having a good response to cisplatin (Duale et al., 2007, di Pietro et al., 2005), resistance to this treatment has been reported in non-seminomatous GCTs (Houldsworth et al., 1998). Furthermore, a higher dosage of cisplatin to counteract this resistance has been associated with toxicity (Frazier et al., 2012, Travis et al., 2010).

DNA methylation has been implicated in cisplatin resistance, and the use of a DNA methylation inhibitor such as 5-azacytidine (5-aza) and its analogue, 5-azadeoxycytidine (5-azadC) has been shown to improve cell line and solid tumour sensitivity towards cisplatin (Wermann et al., 2010, Koberle et al., 2010). To determine whether inhibition of DNA methylation could improve the response of GCT cell lines towards cisplatin, we utilized a clonogenic survival assay. The clonogenic survival assay (also known as a colony formation assay) has been used to assess the ability of a single cell to form a colony after treatment with radiation or chemotherapy (Franken et al., 2006). We replicated this method to determine whether GCT cell lines showed

reduced resistance to cisplatin after those cell lines were exposed to the DNA methylation inhibitor, 5-azadC, or transfected with mir29b.

5.2.6.1. ***: Clonogenic survival assay in cells pre-treated with 5-azadC***

Cells were grown until they were 70% confluent before they were exposed to a range of 5-azadC concentrations for 24 hours. Cells were then trypsinized and 100 single cells were plated in each well of a six-well plate. Once cells had adhered to the well's surface, they were treated with 2.5 to 12.5 μM of cisplatin for four hours. The medium was then replaced with fresh medium without any drugs, and the cells were left for 7-10 days. Cells were then fixed with 4% paraformaldehyde for 20 minutes before they were stained with 0.5% crystal violet for five minutes (Figure 5.8). Under the microscope, viable cell colonies (defined as having an excess of approximately 50 cells) were counted.

Plating efficiency (PE), defined as the ratio of the number of colonies formed to the number of cells seeded, was calculated for each cell line, since each of them has a different plating efficiency.

$$PE = \frac{\text{Number of colonies formed}}{\text{Number of cells seeded}} \times 100$$

After determining the PE for each cell line, the number of colonies that survived after the treatment (defined as a colony with at least 50 cells), also called the surviving fraction (SF), was calculated as follows:

$$SF = \frac{\text{Number of colonies formed after treatment}}{\text{Number of cells seeded X PE}}$$

We found that only the EC cell line showed significant improvement in response towards cisplatin after 5-azadC treatment (Figure 5.9B, left panel) with more significant improvement seen in cells treated with a higher concentration of 5-azadC

(p-value <0.01 for 0.01 μM 5-azadC, p-value<0.001 for 0.05 and 0.1 μM 5-azadC). However, we also found that these concentrations of 5-azadC alone significantly reduced the number of colonies formed in the EC cell line (Figure 5.9B, right panel). Therefore, the observed improvement in response towards cisplatin in the EC cell line might be confounded by the toxicity of 5-azadC treatment. The same toxicity was observed in the teratoma (Figure 5.9A, right panel) and the seminoma cell lines (Figure 5.10A, right panel), although in both cases this toxicity did not seem to significantly influence the cell lines' response to cisplatin (Figures 5.9 A and 5.10A). Only the YST cell line was found not to be significantly affected by 5-azadC toxicity (Figure 5.10B, right panel), but again, no significant improvement in response to cisplatin was observed (Figure 5.10B left panel).

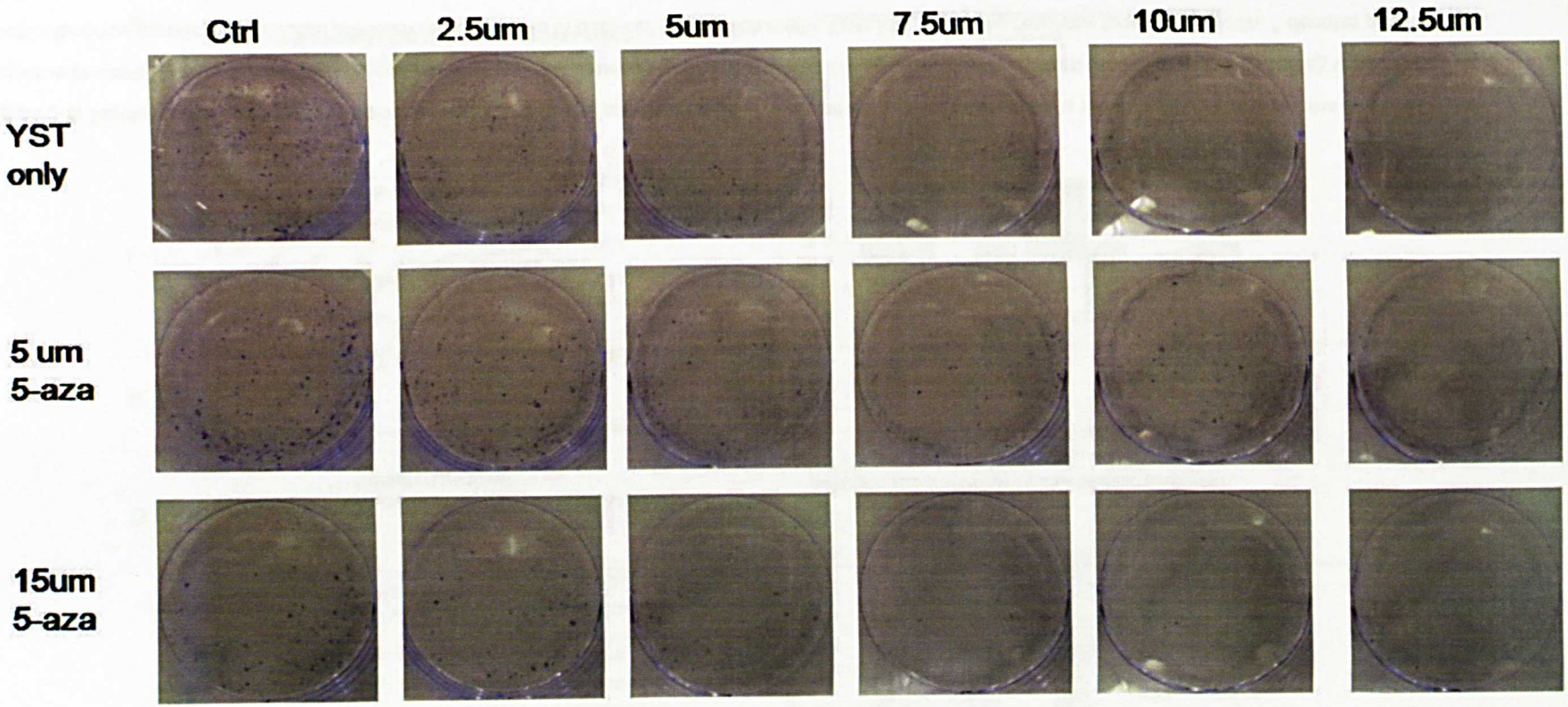


Figure 5.8: Representative diagram showing one of the plate replicates of the clonogenic survival assay done in 5-azadC pre-treated YST cell lines after cisplatin treatment.

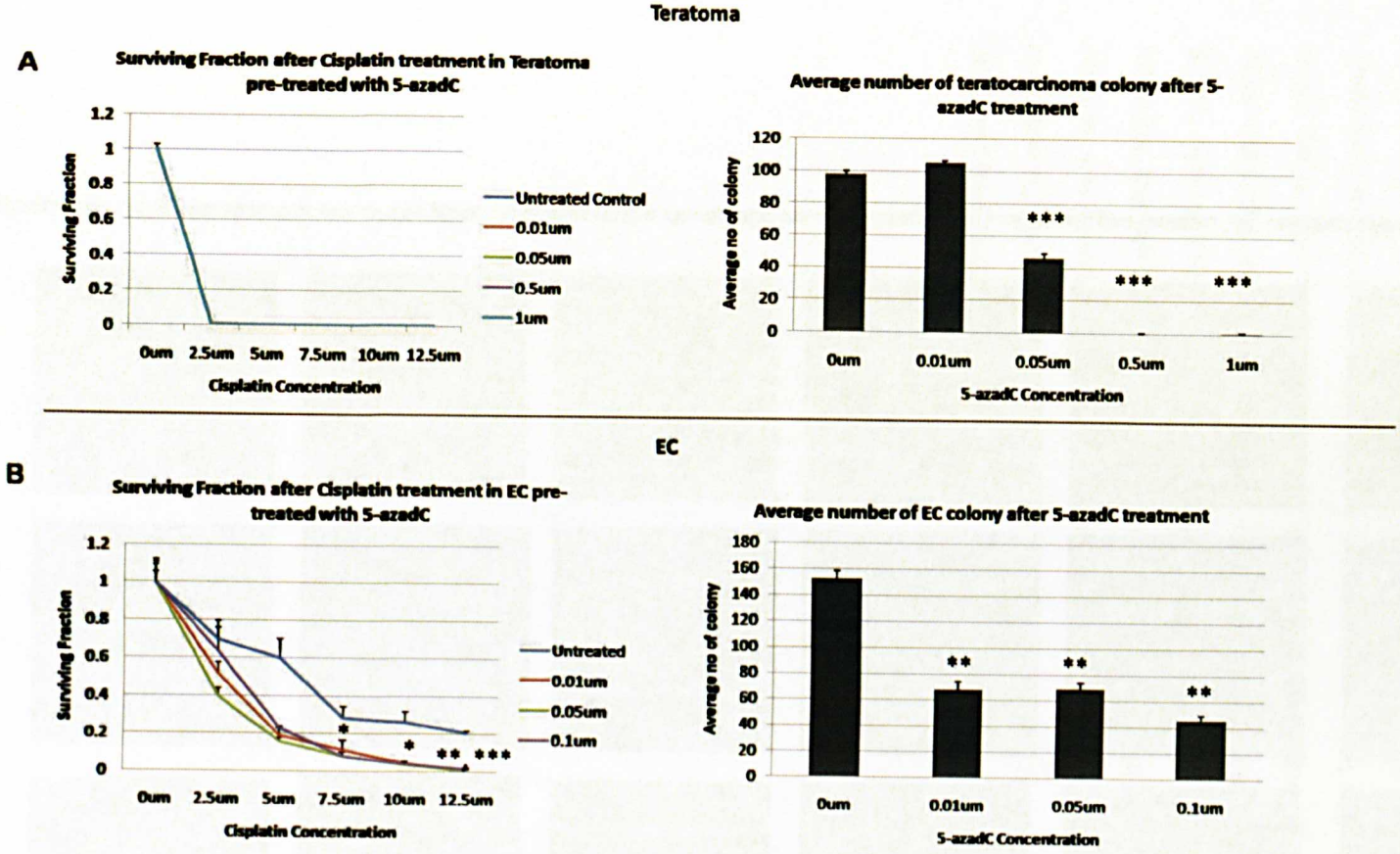


Figure 5.9: Surviving fraction after cisplatin treatment in 5-azadC pre-treated teratoma and EC cell lines. Line graphs showing A) teratocarcinoma and B) EC response to cisplatin after 5-azadC treatment, in which only EC showed significant changes in response. However, from the bar graphs (right side), we can see that 5-azadC alone has significantly reduced the number of colonies in both cell lines which might have influenced the response towards cisplatin. * denotes p-value<0.05, ** denotes p-value< 0.01, *** denotes p-value <0.001 using paired t-test. Error bars represent standard error of means.

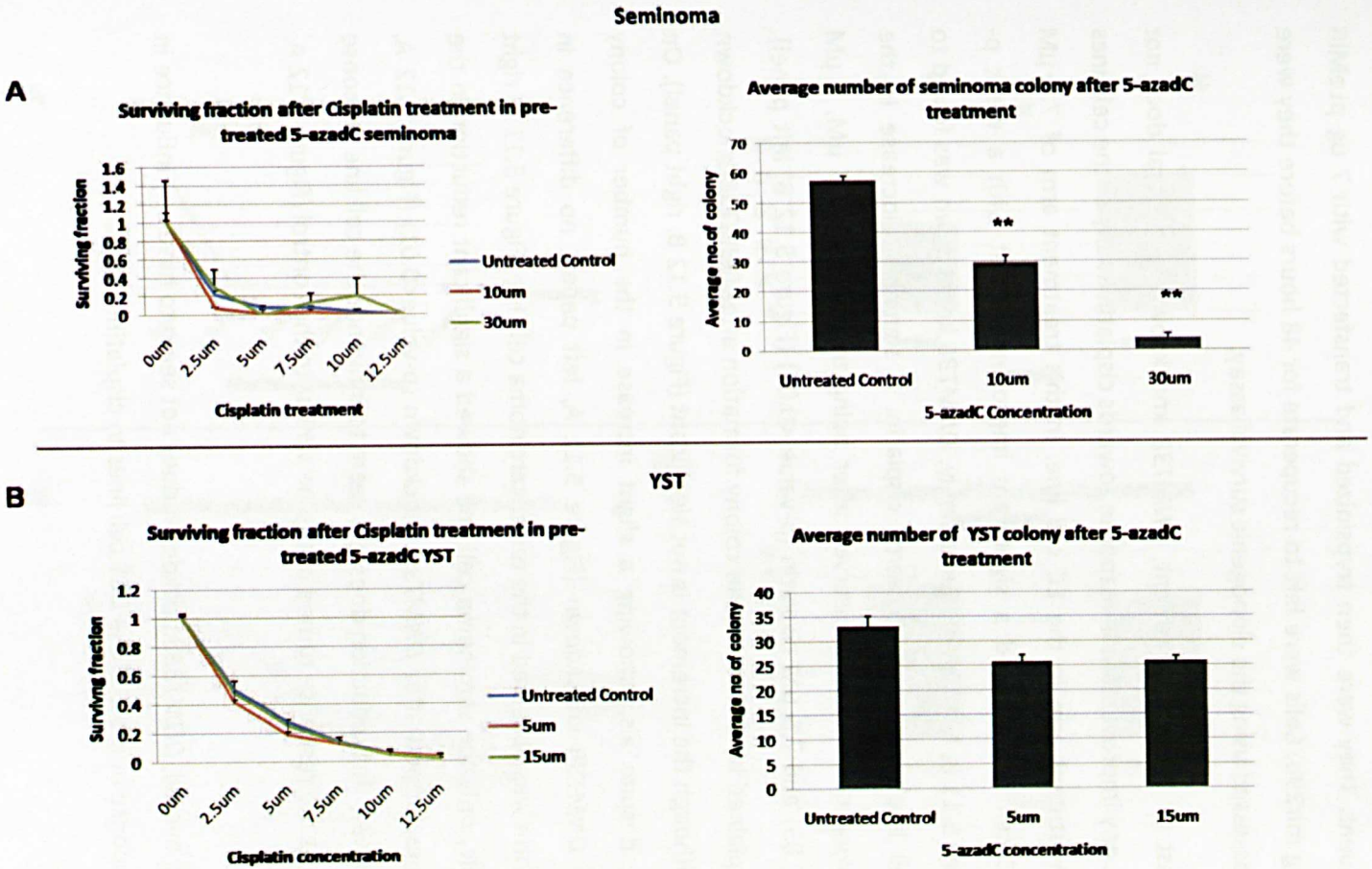


Figure 5.10: Surviving fraction after cisplatin treatment in 5-azadC pre-treated seminoma and YST cell lines. Line graphs showed that there are no significant changes in A) seminoma and B) YST response to cisplatin after 5-azadC treatment. From the bar graphs (right side), only seminoma showed a significant reduction in the number of colonies in 5-azadC only treatment. ** denotes p-value<0.01 using paired t-test. Error bars represent standard error of means.

5.2.6.2. : *Clonogenic survival assay after DNMT3B knockdown in the GCT cell lines*

We next tried to determine whether inhibition of *DNMT3B* is enough to improve the response of GCT cell lines to cisplatin. All four cell lines were grown until they were confluent. They were then trypsinized and transfected with 7 ug pLeMiR vector containing mir29b. Cells were left to recuperate for 48 hours before they were trypsinized and assessed using the clonogenic survival assay.

In contrast to 5-azadC treatment, *DNMT3B* knockdown in general does not seem to result in any improvement in response towards cisplatin in any of the cell lines except in one treatment arm in the EC cell line. In this treatment arm of 7.5 μM cisplatin, the EC cell line showed a significant improvement but with a weak p-value<0.05 (Figure 5.11 B, left panel). Surprisingly, *DNMT3B* knockdown was found to increase YST cell line's resistance towards cisplatin. A significant increase in the number of surviving colonies was observed after being treated with 2.5 μM, 5 μM (both p-value <0.05) and 7.5 μM cisplatin (p-value <0.01) (Figure 5.12 B, left panel). This might be explained by an increase in colony formation after DNMT3B knockdown in the cell line, although the increment is not significant (Figure 5.12 B, right panel). On the other hand, despite also showing a slight increase in the number of colony formations after *DNMT3B* knockdown (Figure 5.11 A, left panel), no difference in response to cisplatin was observed in the teratocarcinoma cell line (Figure 5.11 A, right panel). Meanwhile, only the seminoma cell line showed a significant reduction in the number of colonies formed after *DNMT3B* knockdown (p-value<0.01) (Figure 5.12 A, right panel). However, this reduction does not seem to influence the cell line response towards cisplatin as the response turned out to be similar to the control (Figure 5.12 A, left panel).

Therefore, overall, *DNMT3B* knockdown does not seem to have any influence in improving the response of most of the GCT cell lines to cisplatin.

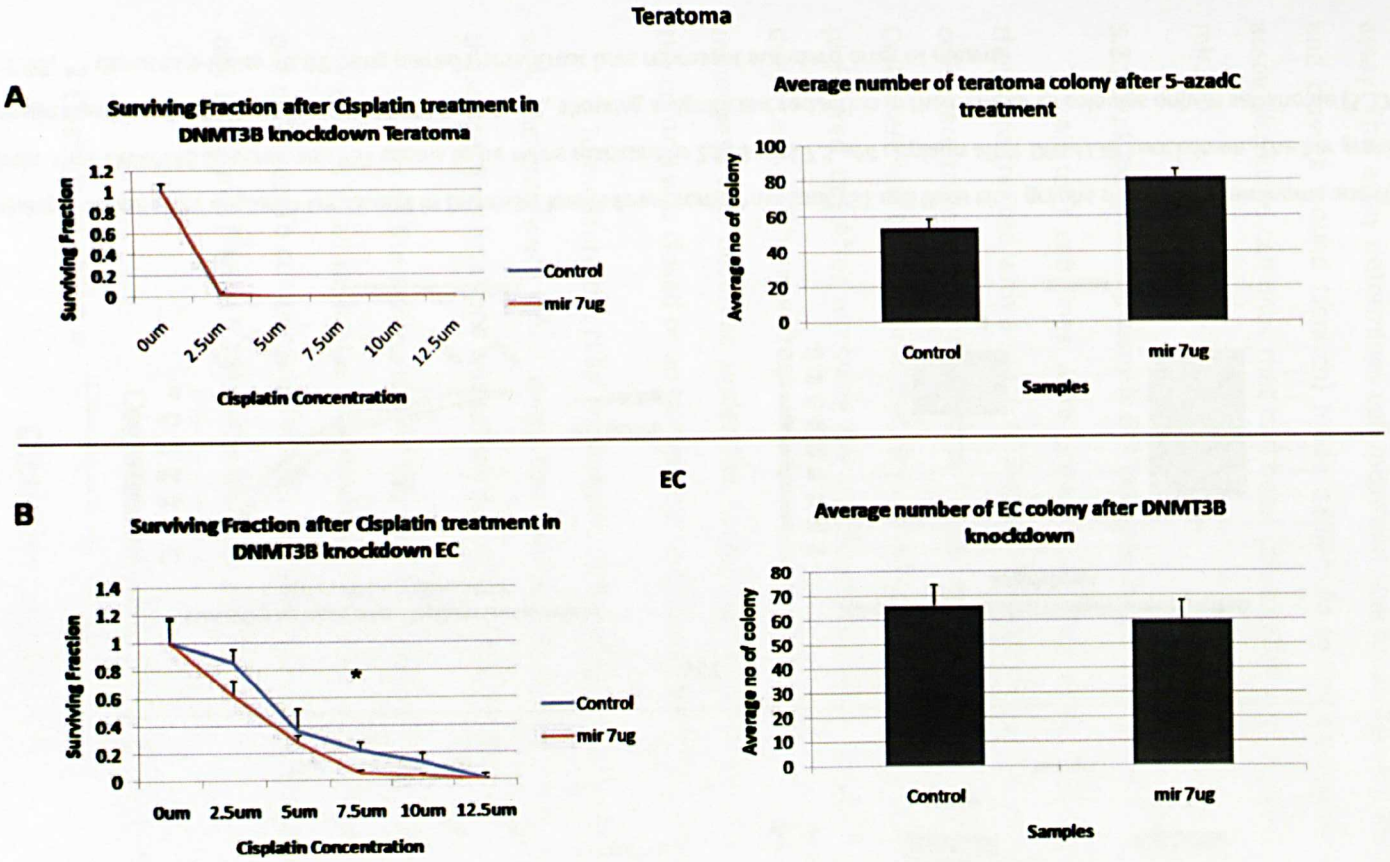


Figure 5.11: Surviving fraction after cisplatin treatment in *DNMT3B* knockdown teratoma and EC cell lines. Line graphs showing A) teratocarcinoma and B) EC cell lines response to cisplatin after *DNMT3B* knockdown. A significant improvement to cisplatin was only seen in the 7.5 um cisplatin treatment arm. The bar graphs (right side) show that *DNMT3B* knockdown does not have any effect on the number of colonies formed. * denotes p-value<0.05 using paired t-test. Error bars represent standard error of means.

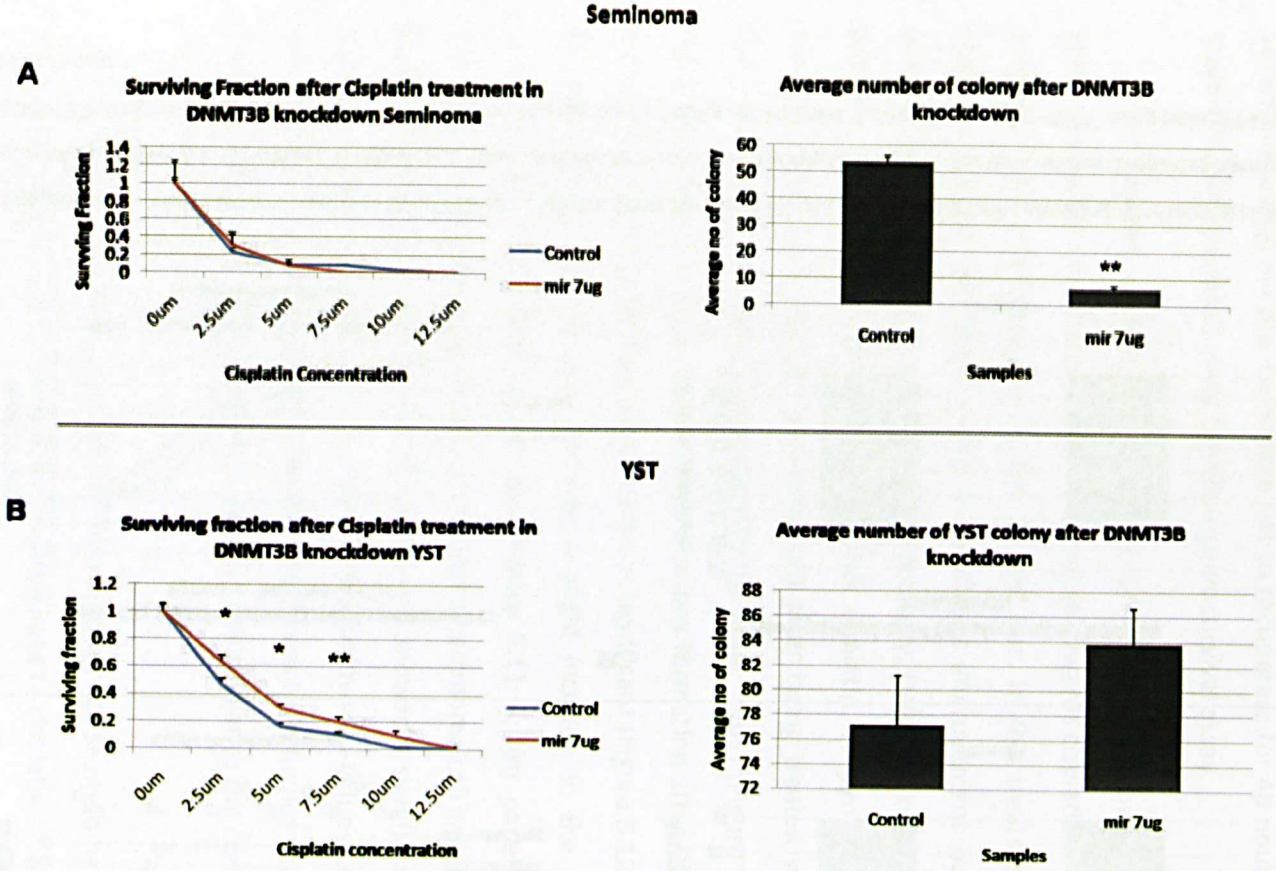


Figure 5.12: Surviving fraction after cisplatin treatment in *DNMT3B* knockdown seminoma and YST cell lines Line graphs showing A) seminoma and B) YST cell lines response to cisplatin after *DNMT3B* knockdown. YST seems to be more resistant to 2.5, 5 and 7.5 uM cisplatin after *DNMT3B* knockdown. The bar graph (right) represent the average number of colonies after *DNMT3B* knockdown, showing a significant reduction in the number of colonies only in seminoma (5.17 A, right). * denotes p-value<0.05, ** denotes p-value <0.01 using paired t-test. Error bars represent standard error of means.

5.2.7. : Cell migration in GCT cell lines after inhibition of DNA methylation

In order to analyze the role of DNA methylation, especially DNMT3B function, in GCT cell lines migration, we used a scratch assay, also known as a wound-healing assay. This assay determines cell migration rate by measuring the rate the cells move and close a wound (scratch) made earlier. As in the clonogenic survival assay, this assay also used cell lines that had been pre-treated with 5-azadC or transfected with mir29b.

5.2.7.1. : *Wound healing assay in GCT cell lines treated with 5-azadC*

All four cell lines were grown until they were 70% confluent, after which theywere treated with a range of 5-azadC concentrations for 24 hours. The next day, cells were trypsinized and plated at 1 X 10^5 densities into each well of a six-well plate. Cells were left to grow until they were confluent before a scratch was made using a 10 µl filtered tip. After removing the medium, cells were washed with PBS to remove dead cells. With new fresh medium added, cells were left in the incubator. Cell movement was observed under the microscope every day until either the wound in the control was closed or up to five days, whichever came first (Figure 5.13).

To measure the cells' movement, at least three locations were chosen for each well. Pictures were taken every day and the measurement of cell movement from these specified locations was calculated using ImageJ software.

One of the acknowledged drawbacks of this assay is the lack of uniformity of the scratch in all replicates (Hulkower and Herber, 2011) which we also observed in our cell lines. To compensate for this, we calculated the ratio between the distance on day 1 onwards (Figure 5.18) to the distance on day 0.

$$\text{Distance ratio} = \frac{\text{Distance on day 1 (2,3 etc)}}{\text{Distance on day 0}}$$

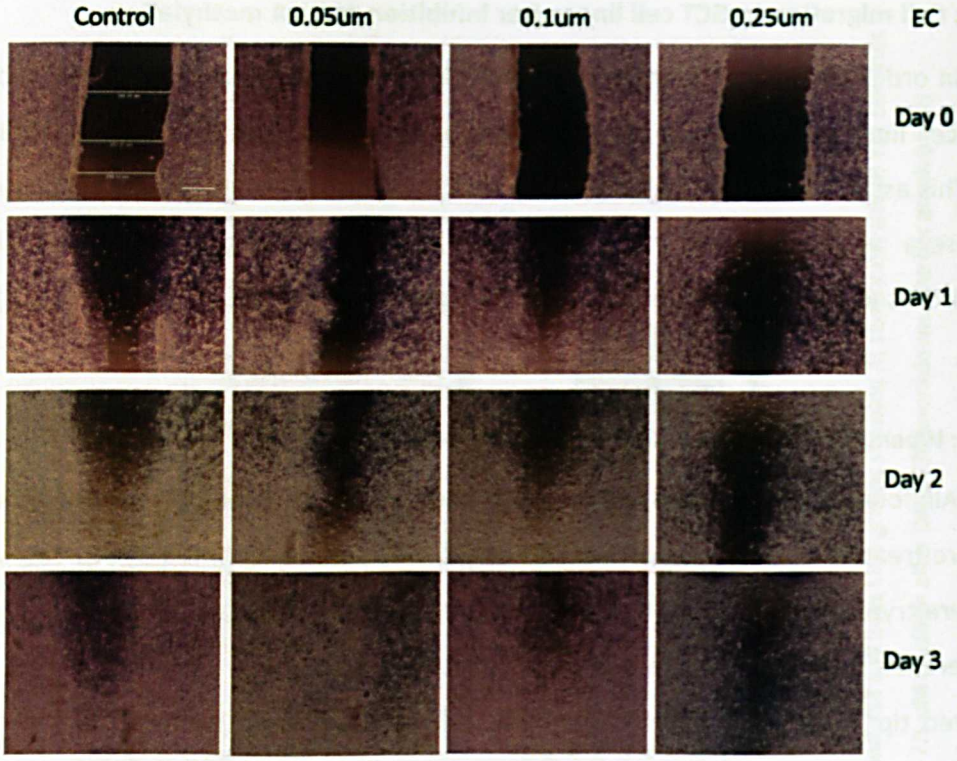


Figure 5.13: Representative diagram showing wound healing assay performed in EC cell lines. As shown in the control Day 0, three locations were choosen for measurement. Scale bars represent 100nm in length.

5-azadC treatment was found to have a significant impact on the migration ability of both the seminoma and the YST cell lines. In the seminoma cell line, **treatment with 30 µM and 50 µM 5-azadC significantly impaired the cell lines' ability to** close the wound (p-**value<0.01 for 30 µM and p-value<0.001 for 50 µM) (Figure 5.15** A). However by day 5, significant impairment in the migration ability only remained in **the cells treated with 50 µM 5**-azadC. Meanwhile, a significant impairment in the **migration ability in the YST cell line was observed in those cells treated with 2.5 µM (p-**value<0.01 on day 2 and p-**value<0.001 on day 3) and 5 µM (p**-values <0.001 on both day 2 and 3) (Figure 5.15 B). Given that the wound in the control healed on day 3, no further observations were made beyond day 3.

On the other hand, 5-azadC treatment did not have any striking impact on the migration ability of either the teratocarcinoma or the EC cell lines (Figures 5.14 A& B). **Although treatment with 0.75 µM 5**-azadC did show significant impairment in the

teratocarcinoma cell line (p-value < 0.01), this was restricted to day 2 of treatment. Beyond this day, no further significant impairment was observed.

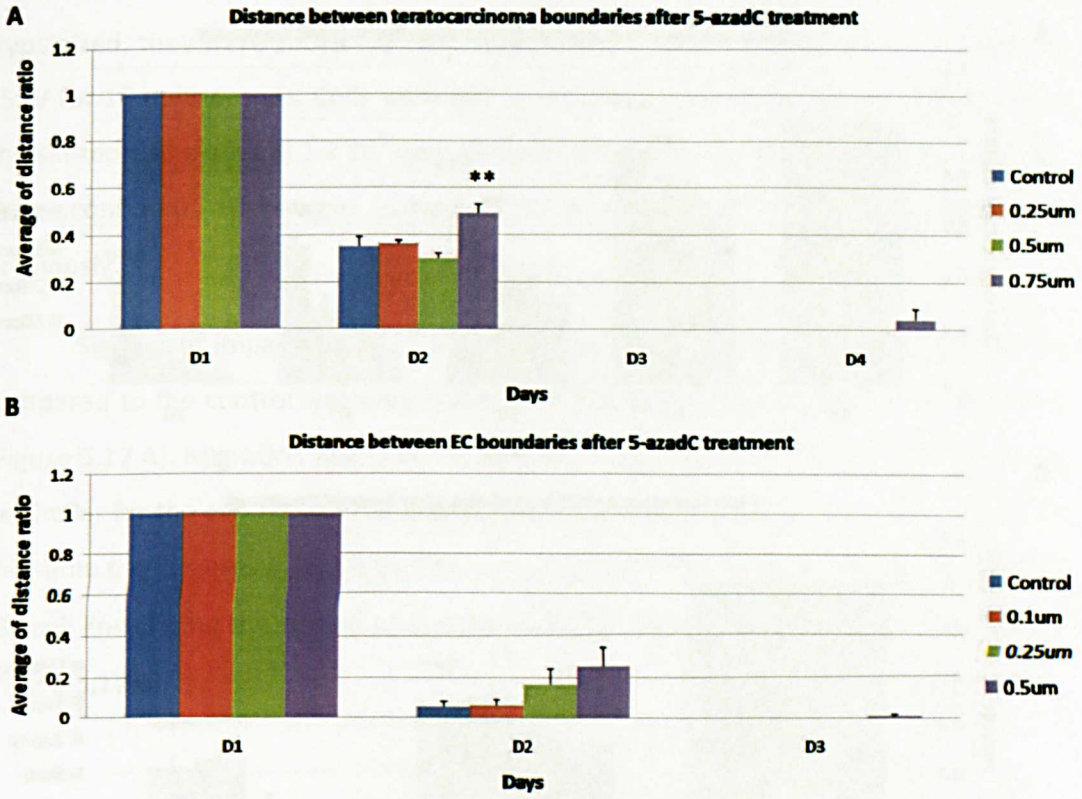


Figure 5.14: Wound-healing assay in 5-azadC pre-treated teratocarcinoma and EC cell lines. Bar graphs showing the average distance ratio between the control and 5-azadC treated A) teratocarcinoma and B) EC cell lines. Only 5-azadC treatment at 0.75 uM in teratocarcinoma showed significantly slower movement. However, on the final day there were no significant differences seen in both 5-azadC treated teratocarcinoma and EC cell lines.** denotes p-value<0.01 using paired t-test. Error bars represent standard error of means.

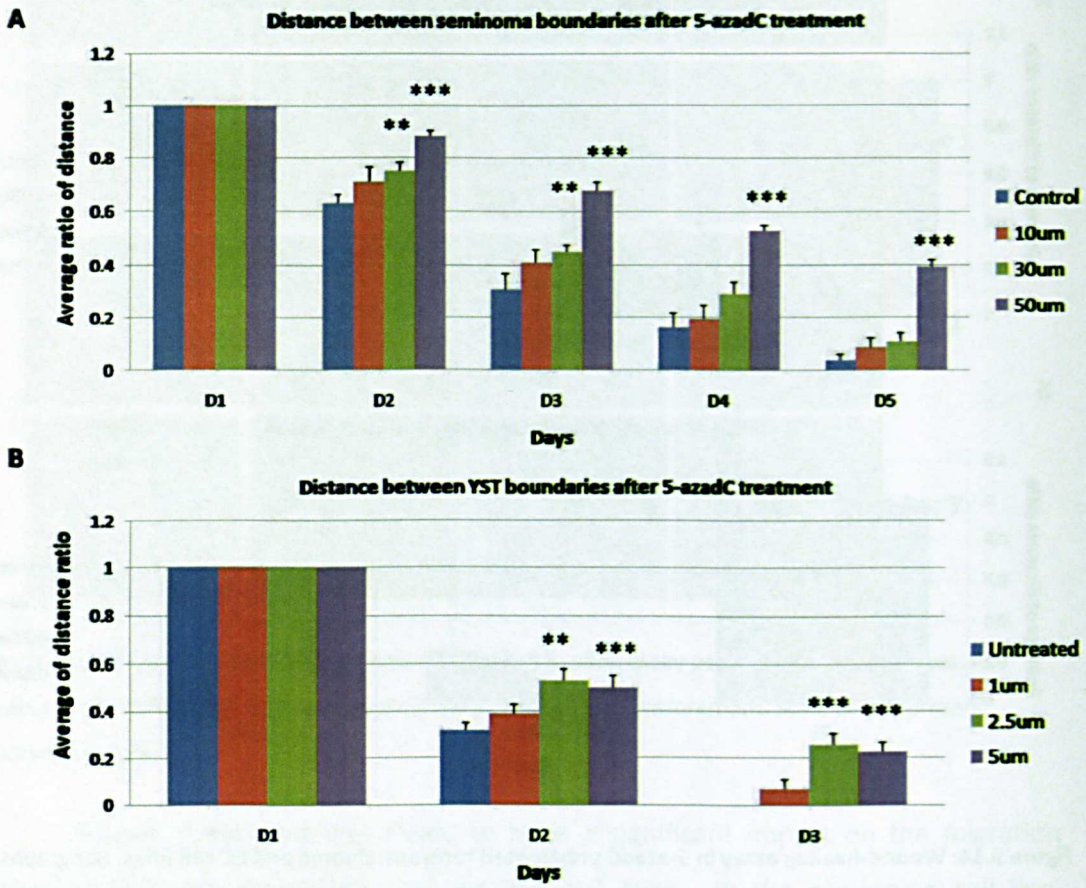


Figure 5.15: Wound-healing assay in 5-azadC pre-treated seminoma and YST cell lines. Bar graphs showing the average distance ratio between the control and 5-azadC treated A) seminoma and B) YST cell lines. Treatment at 30 and 50 uM **significantly reduced the seminoma cell line's movement.** However, this only remained significant until day 5 in 50 uM treatment. In the YST cell line, treatment at **both 2.5 and 5 uM significantly affected YST's movement until day 3 when the control had closed** up its wound.** denotes p-value<0.01 while *** denotes p-value<0.001 using paired t-test. Error bars represent standard error of means.

5.2.7.2.: *Wound healing assay in DNMT3B knockdown GCT cell lines*

We next asked whether DNMT3B knockdown alone is sufficient to affect GCT cell lines' movement. Cell lines were grown until they were confluent. Once trypsinized, they were subjected to transfection of 7 ug mir29b via electroporation at 250V for 10 milliseconds. Cells were left to recuperate for 48 hours before they were trypsinized and plated at 1 X 10^5 densities into each well of a six-well plate in triplicate. Once confluent, they were subjected to a wound-healing assay as described previously.

Significant impairment in the cells' ability to move and close up the wound as compared to the control was only observed in the seminoma cell line (p-value<0.001) (Figure 5.17 A). Migration ability in the EC and teratocarcinoma cell lines was found to be similar for the control and the knockdown, as both were able to close the wound on the same day (Figures 5.16 A& B). There was a slight delay in the DNMT3B knockdown YST cell line closing the wound as compared to the control, but this was not significant (Figure 5.17 B).

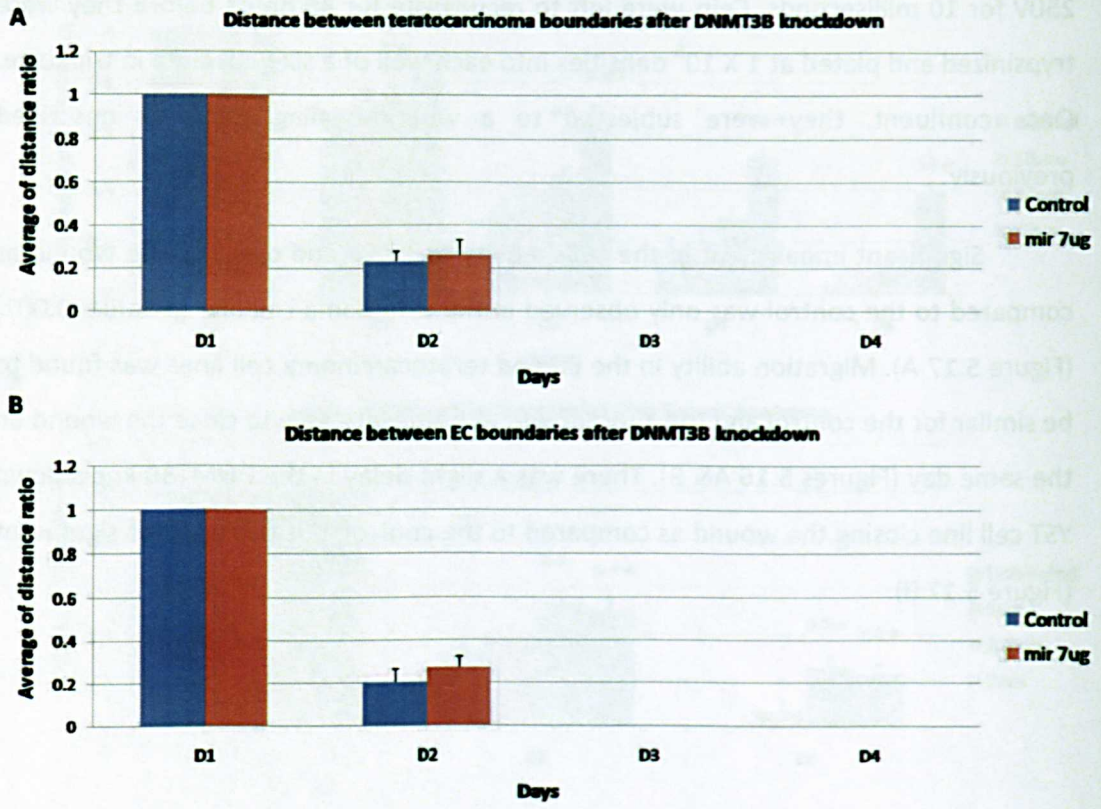


Figure 5.16:Wound-healing assay in DNMT3B knockdown teratoma and EC cell lines. Bar graphs showing the distance ratio of A) teratocarcinoma and B) EC cell lines' movement after DNMT3B knockdown. It is clear that DNMT3B knockdown does not have any impact on either cell lines' movement. Error bars represent standard error of means.

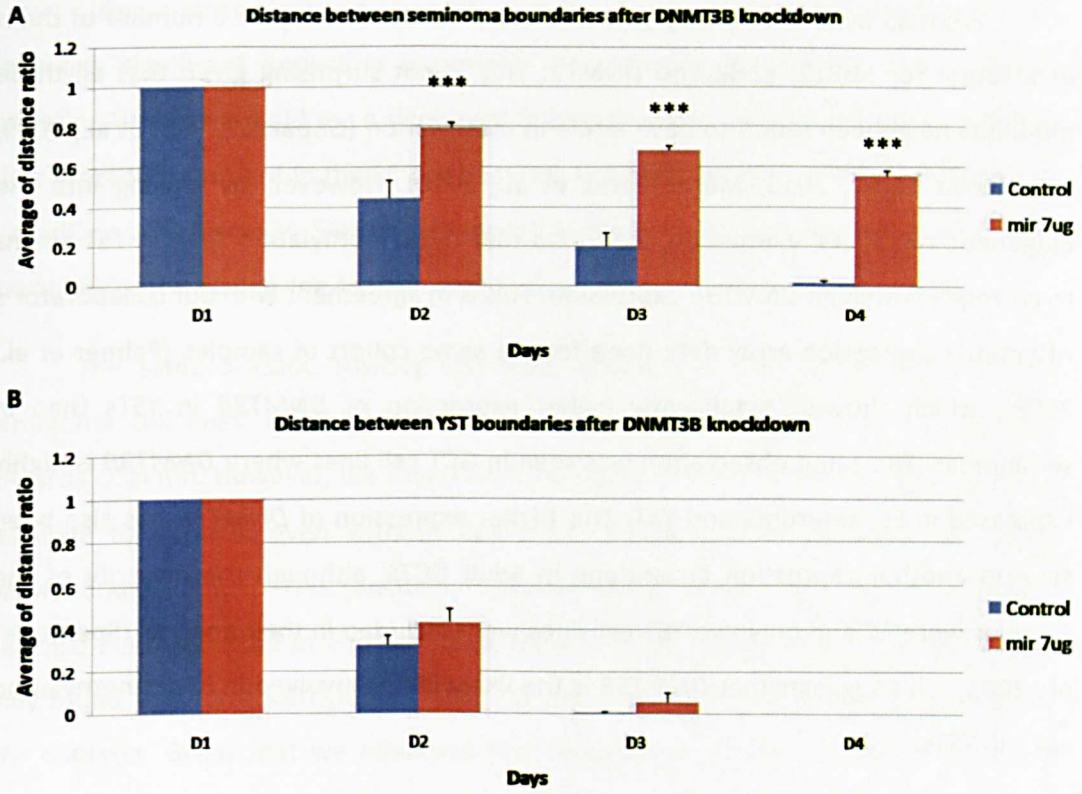


Figure 5.17: Wound-healing assay in DNMT3B knockdown seminoma and YST cell lines. Bar graphs showing the distance ratio of A) seminoma and B) YST cell lines' movement after DNMT3B knockdown. Only in seminoma did we observe a significant impairment in the cell's ability to move and close up the wound after DNMT3B knockdown. *** denotes p-value <0.001 using paired t-testand error bars represent standard error of mean.

5.3. Discussion

5.3.1. : DNMT3B as a contributing factor in YST hypermethylation

From 85 hypermethylated genes in YSTs, we identified quite a number of them as a target for SUZ12, EZH2 and DNMT3. This is not surprising given that all these modifiers have been found to have a role in methylation (Gopalakrishnan et al., 2009, Karanikolas et al., 2010, Martin-Perez et al., 2010). However, by looking into the epigenetic modifiers' expression, we found that hypermethylation seen in YST seems to correlate with high *DNMT3B* expression. This is in agreement with our collaborator's Affymetrix expression array data done for the same cohort of samples (Palmer et al., 2008), which showed significantly higher expression of *DNMT3B* in YSTs than in seminomas. The same observation was seen in GCT cell lines where *DNMT3B* is highly expressed in EC, teratoma and YST. This higher expression of *DNMT3B* has also been seen in another expression array done in adult GCTs, although the majority of the samples were EC and only two YST cell lines were included in their analysis (Sperger et al., 2003). This suggests that *DNMT3B* is the likely factor involved in hypermethylation seen in YSTs.

In one study comparing EZH2 and SUZ12 protein expression in 69 tumour tissue types, it was found that EZH2 was expressed in most of the tumours, while SUZ12 expression was restricted to a subset of tumours (Martin-Perez et al., 2010). In fact they found that tumours which showed SUZ12 expression were usually positive for EZH2 as well but not the other way around. Interestingly, seminoma, teratocarcinomas and embryonal carcinomas were among the tumours which display high expression of SUZ12. Although we did observe expression of *SUZ12* in both paediatric seminoma and YST samples, we did not see any significant difference between these two tumour types in *SUZ12* expression. However, this could be due to the small number of samples included in the assay, and furthermore we only managed to do duplicates on some of the samples.

5.3.2. Knockdown of DNA methyltransferase by 5-azadC and its effect on GCT cell lines' behaviour

5.3.2.1. *Cells' response towards cisplatin*

Using a clonogenic survival assay, we found that in general, 5-azadC does not have any significant impact in improving the response of GCT cell lines towards cisplatin. Although we did see a significant improvement at a higher dosage of cisplatin after 5-azadC treatment in the EC cell lines, we realised that this could be attributed to 5-azadC toxicity because 5-azadC treatment alone showed significant impairment in the ability of the EC cell line to form colonies.

The same 5-azadC toxicity was also observed in the teratocarcinoma and seminoma cell lines, although this did not have any influence on cells' response towards cisplatin. However, we found that the teratocarcinoma cell line was the most sensitive to cisplatin even without any prior treatment with 5-azadC or without *DNMT3B* knockdown. This sensitivity towards cisplatin in teratocarcinoma cell lines has also been reported in another study (Fenske et al., 2012). Using the MTT assay, they found that teratocarcinoma viability is reduced to 50% after treatment with 0.7 μM cisplatin. Given that we observed total impairment in teratocarcinoma colony-forming ability with 2.5 μM cisplatin, this might be due to the high dosage of cisplatin used. Adjustment to the cisplatin dosage maybe needed for future analysis.

Meanwhile, 5-azadC toxicity in the seminoma cell line leads to significant impairment in its ability to form colonies. However this does not result in significant improvement in its response to cisplatin. Treatments with both 2.5 μM cisplatin alone and 2.5 μM cisplatin in combination with 5-azadC caused more than a 50% reduction in the fraction of the seminoma cell line that survived. This is in contrast to the finding by Wermann *et al.*(2010), who showed a 50% reduction in cell survival after 12 μM cisplatin alone or 8 μM cisplatin in cells pre-treated with 5-azadC. This may be due to the different method and cell density used in the experiment.

Only the YST cell line did not show any toxicity from 5-azadC. This difference in observation between the YST and the other GCT cell lines might have several

explanations. First, the dosing of 5-azadC used in the experiment might be still low to produce any toxicity in the YST cell line. In this experiment we used the maximum concentration of 15μM of 5-azadC. Indeed previous work performed by my colleague, Safiah Al-Hazmi, showed that 5-azadC toxicity in the YST cell line was only visible after treatment with concentrations beyond 20 μM.

The other factor that might have influenced such observations is the proportion of replicating cells during 5-azadC treatment. 5-azadC needs to be incorporated into the DNA before it can form a covalent bond with DNA methyltransferase and cause DNMTs degradation (Stresemann and Lyko, 2008), thus cells need to be in the S phase to ensure optimal uptake of 5-azadC. Therefore, our method of treating the YST cell line with 5-azadC when they were 70% confluent might not provide enough replicating cells for 5-azadC uptake. However, using the same method seems to work with the other GCT cell lines although again this could be influenced by cells' doubling time. But since the seminoma cell line displayed longer doubling time than the YST cell line, we can rule out this possibility.

Lastly, the YST cell line might be resistant towards 5-azadC. Many studies have implicated many factors behind 5-azadC resistance. One such factor is the deficiencies in deoxycytidine kinase (*DCK*). Mutation of this *Dck* gene has been implicated in 5-azadC resistance in rat leukemic cell lines (Stegmann et al., 1995). Furthermore, low *DCK* expression was found in five cell lines known to be resistant to decitabine (Qin et al., 2009). Insertion of wild type *DCK* into one of these cell lines, HL60, was found to restore 5-azadC sensitivity. Although from the Affymetrix microarray data analysis, *DCK* was found to be highly expressed in the YST cell lines, we could not determine whether gene mutation was present in the YST cell lines. Other factors that have also been implicated in 5-azadC resistance are a low level of the nucleoside transporter, *hENT1* and *hENT2*(Qin et al., 2009) and a high level of cytidine deaminase (*CDA*) which inactivate cytidine analogues (Rius and Lyko, 2012). Although we could not get the expression data on the nucleoside transporter, we found that the YST cell line shares low expression of *CDA* with other GCT cell lines suggesting it could not have any

involvement in 5-azadC resistance in the YST cell line. Therefore, further analysis needs to be done to address this question.

Nonetheless, treatment with 5-azadC did not improve the response of the YST cell line towards cisplatin. As mentioned before, paediatric YST samples and the YST cell line showed reduced *PYCARD* and *CASP8* expression associated with promoter hypermethylation. Indeed, reduced susceptibility to cell death has been suggested as a mechanism of resistance to cisplatin in GCTs and other cancers (Kim et al., 2001, Fenske et al., 2012). Furthermore, reexpression of these genes has been shown to resensitize cancer cells to chemotherapy (Fulda et al., 2001, Ramachandran et al., 2011). However, we did not manage to determine whether our 5-azadC treatment did reactivate either one or both *CASP8* and *PYCARD*. Thus, it is possible that treatment with 5-azadC did not reactivate these apoptosis-related genes which might explain why no improvement in cisplatin response was observed in the YST cell line. Furthermore, Wu *et al.*(2010b), in their methylation analysis of four breast cancer cell lines, revealed the down-regulation of *CASP8* expression due to promoter hypermethylation. Interestingly, 5-azadC treatment could only caused *CASP8* demethylation in three out of the four cell lines analysed. This suggests that the effect of 5-azadC demethylation could be cell-type specific. It would be interesting to see whether the same observation could be seen in the GCT cell lines, which might explain the different effects of 5-azadC on each GCT cell line.

5.3.2.2. *GCT cell lines' migration ability*

In order to assess changes in the migration ability among the GCT cell lines after 5-azadC treatment, we resorted to using the wound-healing assay. Using this assay, we found that 5-azadC treatment leads to significant impairment in movement in the seminoma and YST cell lines. Neither teratocarcinoma nor EC cell lines showed this kind of impairment after 5-azadC treatment. A previous study performed on a choriocarcinoma cell line, BeWo, showed that 5-azadC treatment led to upregulation of E-cadherin (*CDH13*) and resulted in the impairment of cell migration (Rahnama et al., 2006). The same result was observed when forced expression of *CDH13* was performed on the same cell line. Although in our analysis there was no difference in

the methylation levels of *CDH13* in any of the four GCT cell lines, *CDH13* expression was found to be 10-fold and 13-fold higher in teratocarcinoma and EC cell lines respectively, as compared to the seminoma and YST cell lines. We did not analyze whether 5-azadC treatment caused reactivation of CDH13 expression in either the seminoma or the YST cell lines, which resulted in the migration impairment observed in these two cell lines. However, we are currently planning to do Affymetrix gene expression arrays on 5-azadC treated GCT cell lines. Hopefully this data will provide some explanation of whether *CDH13* or other genes might be involved in such observations.

5.3.3. Knockdown of DNMT3B by microRNA 29b and its effect on cell behaviour

5.3.3.1. *Cells' response to cisplatin*

Knockdown of DNMT3B does not seem to improve the response of any of the four GCT cell lines towards cisplatin. Surprisingly, DNMT3B knockdown was found to increase the resistance of the YST cell line towards cisplatin. This is in contrast to the finding that mir29b can up-regulate *p53* levels and induce apoptosis in a *p53*-dependent manner (Park et al., 2009a). Furthermore, in hepatocellular carcinoma, Mcl-1 and Bcl-2 (anti-apoptotic proteins) were found to be direct targets for the mir-29 family (Xiong et al., 2010). In addition to that, overexpression of the mir-29 family resulted in the release of cytochrome c, suggesting a role for the mir-29 family in apoptosis. One possible explanation behind the increased resistance observed in the YST cell lines is that the effect of the mir-29 family could be cell-type-specific. Indeed, previous miRNA expression microarrays performed on a cisplatin resistant breast cancer cell line, MCF7/CDDP, showed upregulation of the mir-29 family (Pogribny et al., 2010). However, this study did not show whether such upregulation was directly linked to cisplatin resistance. Therefore, it is still unclear whether overexpression of mir-29b could also lead to increased resistance.

5.3.3.2. *GCT cell lines' migration ability*

Only the seminoma cell line showed significant impairment in its movement after *DNMT3B* knockdown. Given that the seminoma cell line showed the lowest level of methylation, knockdown of *DNMT3B* might have caused further hypomethylation and caused this impairment. Indeed, *DNMT3B* knockdown by siRNA has been shown to reduce cell growth and migration of the prostate cancer cell line, PC3 (Yaqinuddin et al., 2008). However, why this observation is not seen in the other GCT cell lines is still unclear. It could be that *DNMT3B*knockdown alone is not enough to exert its demethylation potential. Indeed, a study that looked at promoter demethylation after knockdown of either *DNMT1*, *DNMT3A* or *DNMT3B* revealed that knockdown of all three DNMTs gives a higher degree of demethylation as compared to single DNMT knockdown (Chik and Szyf, 2010). Another possible explanation might be related to the knockdown efficacy. We observed, during the course of our experiments, that the knockdown reproducibility was inconsistent and we believe this might be due to plasmid degradation.A recent study showed that a HDAC inhibitor, AR-42, could also increase mir-29b level and downregulate mir-29b target genes such as *DNMT3B*(Mims et al., 2012). This might be another alternative approach to knockdown *DNMT3B* that can be tried in the future.

6. GENERAL DISCUSSION

6.1. *YST methylator phenotype*

Based on the data analysis performed on the paediatric GCT samples in this study, we showed that YSTs display hypermethylation at many of the gene promoter regions analysed. Further analysis performed in a YST cell line using a wider coverage methylation array, Infinium 450K, validated this finding. In addition to that, this high level of methylation displayed by the YST cell line was found to be predominantly restricted to CpG island regions. This finding seems to fit the criteria of CpG island methylator phenotype (CIMP) reported in many types of cancer in the past decade (Noushmehr et al., 2010, Weisenberger et al., 2006, Kusano et al., 2006, Marsit et al., 2006, Toyota et al., 1999). However none of the genes identified previously as consistent CIMP genes (*MINT1, MINT2, MINT31, CDKN2A, MLH1, CACNA1G, IGF2, NEUROG1, RUNX3, SOCS1*) (Issa, 2004, Weisenberger et al., 2006) were methylated in the YST cell lines, while only *RUNX3* was methylated in the YST paediatric samples. But, since these genes were identified from studies performed in colorectal cancer; this could mean that they were tissue specific. Instead, by correlating methylation and expression data, we have identified six and 98 possible candidate genes in the paediatric YST samples and the YST cell line respectively that were differentially methylated and showed differential gene expression. Further functional analysis is required before we can establish the function of these genes in YST development. In contrast to the YST cell line, the other two GCT cell lines, the teratoma and EC cell lines, showed different methylation at all regions investigated. This suggests that there might be a difference in the underlying mechanism disrupting methylation in the development of the different GCT subtypes.

We have identified *DNMT3B* as a possible factor behind the hypermethylation observed in YSTs and other non-seminomatous GCTs, which is consistent with other studies previously performed in GCTs. Indeed as mentioned before, *DNMT3B* has also been implicated in the hypermethylation seen in other types of tumour (Beaulieu et al., 2002, Gopalakrishnan et al., 2009, Roll et al., 2008, Van der Auwera et al., 2010). However in our assay, *DNMT3B* knockdown alone does not seem to improve the

response of non-seminomatous GCT cell lines to Cisplatin or reduce their migration ability. Although this still needs further analysis, we could not rule out that there might be other factors that may contribute to the hypermethylation seen in the YST cell lines.

Indeed, in recent years other possible candidates have been recognized to be able to contribute to DNA hypermethylation. All these candidates (the TET family, AID and the IDH family) are involved in the process of DNA demethylation. The ten-eleven translocation (TET) family was found to modify 5-methylcytosine (5-mC) into 5-hydroxymethylcytosine (5-hmC) in experiments performed in both human and mouse (Ito et al., 2010, Tahiliani et al., 2009)a(Figure 6.1). Early developmental studies performed in mouse showed that 5-mC was actively converted into 5-hmC in the male pronucleus upon zygotic development (Iqbal et al., 2011, Wossidlo et al., 2011). Given that *Tet3* was found to be abundant during this stage, it is believed that *Tet3* was responsible for 5-mC conversion into 5-hmC. Furthermore, knockdown of *Tet3* resulted in an increase of 5-mC, thus strengthening the role of *Tet3* in the demethylation process (Wossidlo et al., 2011). Indeed, loss of 5-hydroxymetylcytosine and *TET* mutation has been reported in many types of cancer (Kudo et al., 2012, Lian et al., 2012, Yang et al., 2012) which might implicate them in the hypermethylation previously observed in these cancers.

Meanwhile, activation-induced cytidine deaminase (AID) is involved in DNA demethylation by causing deamination of cytosine residues into uracil. This uracil will then be converted into thymine by DNA repair mechanism such as base excision repair (BER) or mismatch repair (MMR) (Figure 6.1). Although no direct involvement of *AID* and methylation in cancer has been reported, a developmental study has shown that the primordial germ cells (PGCs) of *Aid* mutant mice ($Aid^{-/-}$) display an increase of genome-wide hypermethylation of up to three times as compared to the wild-type (Popp et al., 2010). In another study, rapid demethylation of *POU5F1* and *NANOG* observed upon fusion of mouse ESC and human fibroblast was blocked when AID was knocked down with siRNA, which further supports the involvement of AID in demethylation (Bhutani et al., 2010). However, given that *Aid* mutant mice did not show any striking phenotype and the fact that some demethylation is still observed in

Aid mutant mice PGCs(Popp et al., 2010) suggest that AID may not be solely responsible for demethylation.

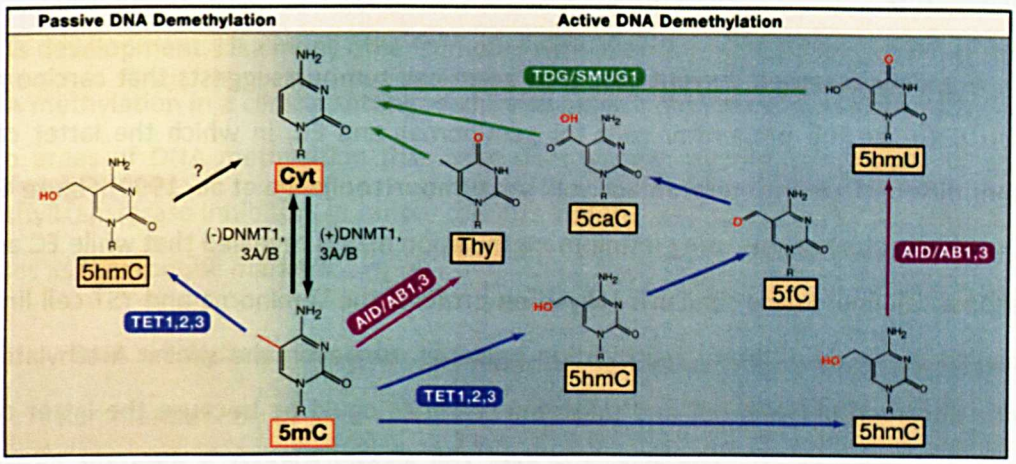


Figure 6.1: Diagram showing the process of DNA demethylation. Passive DNA demethylation occurs by loss of DNA methyltransferases (DNMTs) activity. Active DNA demethylation could occur by two means. The first is the conversion of 5-methylcytosine (5-mC) into 5-hydroxymethylcytosine (5-hmC) by ten eleven translocation (TET) family (blue). This 5-hmC could be further oxidized into 5-formylcytosine (5-fC) and 5-carboxycytosine (5-caC). This modification will then be converted into cytosine by DNA repair mechanism such as by thymine DNA glycosylase (TDG) or selective monofunctional uracil DNA glycosylase q (SMUG1) (green). The second process of DNA demethylation involves the deamination of 5-mC or 5-hmC into 5-methyluracil (5-mU) or 5-hydroxymethyluracil (5-hmU) by activation induced cytidine deaminase (AID) (purple). Again this modification will be repaired by TDG or SMUG1 into cytosine. Diagram adapted from Bhutani et al (2011)

In contrast to TET and AID, isocitrate dehydydrogenase (IDH) has an indirect role in the DNA demethylation process. IDH produces α-ketoglutarate (α-KG) by catalyzing the oxidative decarboxylation of isocitrate. However, when *IDH* was mutated, this activity was lost and replaced with a gain in the production of 2-hydroxyglutarate (2-HG) (Dang et al., 2009). This 2-HG was found to competitively inhibit the activity of α-KG-dependent dioxygenases which include the TET family demethylation activity. This will result in reduced TET enzymatic activity and leads to DNA hypermethylation. Indeed, *IDH* mutation has been reported in many types of cancer (Marcucci et al., 2010, Turcan et al., 2012, Wang et al., 2012, Yan et al., 2009) and was found to lead to a CpG island methylator phenotype (CIMP) in leukemia, glioblastoma and glioma (Figueroa et al., 2010a, Noushmehr et al., 2010, Turcan et al., 2012). However, to date

no IDH mutation has been reported in GCTs, but given the YSTs showed CIMP feature, it would be interesting to see whether YSTs harbour *IDH* mutation that could lead to such a feature.

A widely accepted current model of germ cell tumourssuggests that carcinoma in-situ (CIS) are the progenitor cells for seminomas and EC, in which the latter can further differentiate into teratomaor yolk sac tumour(Looijenga et al., 1999)(Figure 6.2 A). However, our analysis using Infinium methylation arrays revealed that while EC and teratoma cell lines share similar methylation profiles, the seminoma and YST cell lines displayed distinctly different methylation patterns. However, the similar methylation profile observed between EC and teratoma cell lines could be because the latter cell line is in fact an EC. Despite this, our data still clearly suggest a different lineage relationship for these GCT subtypes in which YST is in a distinctly different branch from EC and teratoma (Figure 6.2 B). Therefore, previous reports of seminomas recurring as YST or teratoma(Wong et al., 2010, Kamoshima et al., 2008), or teratoma recurring as seminoma or YST (Czirjak et al., 1992, Utsuki et al., 2007) might be due to either acquired or disruption of methylation in the tumour cells.

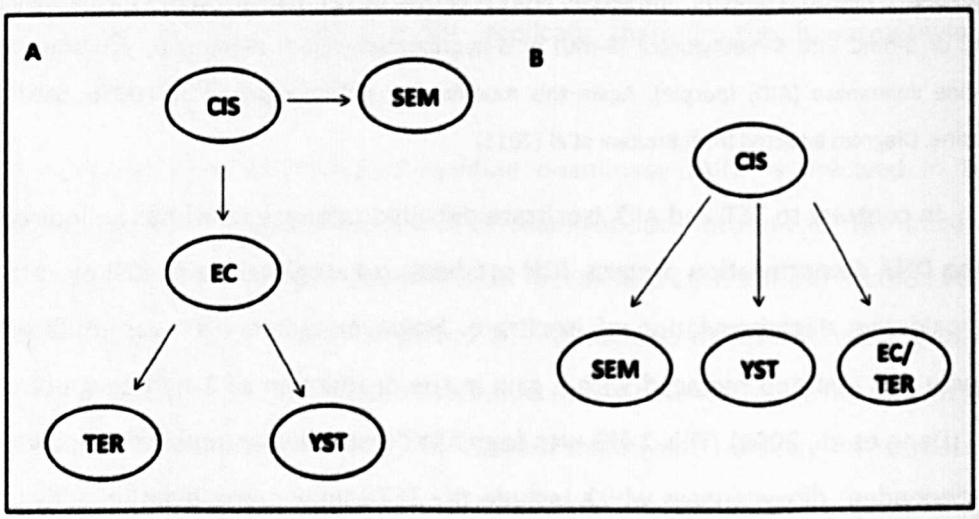


Figure 6.2: Model of GCT development. A) In the current model, carcinoma in-situ (CIS) is believed to be the progenitor cell for seminoma and EC. EC later could differentiate into teratoma or YST. B) Distinct methylation profile observed from our Infinium analysis suggests that each subtype of GCT have different lineages. Therefore, subsequent methylation in the CIS could give rise to any subtype of GCTs.

6.2. *Clinical application of DNA methylation*

From our studies, it is possible to postulate that DNA methylation plays a role in GCTs development. Like many other tumours with aberrant methylation, application of DNA methylation in a clinical setting might also benefit patients diagnosed with a GCT. Two areas of DNA methylation that have been widely studied are the use of DNA methyltransferase inhibitors in cancer therapy and the application of hypermethylated genes as therapeutic markers.

6.2.1. : DNA methylation in cancer therapy

As mentioned in the previous chapter, 5-azacytidine (5-aza) and 5-azadeoxycytidine (5-azadC) have been well recognized for their use in epigenetic therapy by inhibiting DNA methyltransferases. However, their early use in clinical trials has been limited by toxicity (Kantarjian et al., 2003, de Lima et al., 2003) which is believed to be due to high dosage of the drug used in these trials. Indeed, subsequent clinical trials that adopted lower doses of 5-aza or 5-azadC elicited a much better responses.

The better response seen at lower dose might be due to the interesting pharmacodynamics of both drugs. In studies performed in acute myeloid leukemia (AML) cell lines, both 5-aza and 5-azadC showed depletion of *DNMT1* level at low concentration (1 μM for 5-aza and 100 nM for 5-azadC) and hypomethylation of repeat elements at even lower concentration (300 nM for 5-aza and 30 nM for 5-azadC) (Hollenbach et al., 2010, Qin et al., 2007). Furthermore, these groups also showed that the hypomethylation effect of both drugs diminished at higher concentration (3-10 μM). This suggests that higher dosage of 5-aza and 5-azadC does not have any therapeutic value and may expose patients to toxicity.

Both 5-aza and 5-azadC have been clinically proven to benefit myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) patients. Clinical trials have shown that treatment with 5-aza or 5-azadC alone could benefit the patients as they require less red blood cell transfusion and showed improvement in outcome and overall response such as reduced duration of hospitalization and less severe side-effects as

compared to the conventional therapy (Fenaux et al., 2010, Fenaux et al., 2009, Lyons et al., 2009, Kantarjian et al., 2006, Kantarjian et al., 2007, Lubbert et al., 2008). Meanwhile, clinical trials in solid tumours are mostly performed with a combination of chemotherapy. There has been quite a mixed result from these trials. Schwartsmann *et al.*(2000) in their combination of 5-azadC and cisplatin trial in non-small cell lung carcinoma showed no improvement in the antitumor activity with two patient experiencing minor regressions. Recent trials using a much lower dosage (ranging from 10-90 mg/m^2) however have shown quite a promising result with almost 30% of patients showing partial response or stable disease for more than 4-6 months (Fang et al., 2010, George et al., 2010, Appleton et al., 2007, Pohlmann et al., 2002).

Meanwhile, trials that used 5-aza or 5-azadC in combination with HDAC inhibitors did not seem to produce a significant result. In one trial, addition of sodium phenylbutyrate to 5-aza only showed a small benefit but with lower toxicity in patients with refractory solid tumours (Lin et al., 2009). In addition to this, a trial conducted in acute myeloid leukemia showed that addition of valproic acid to 5-azadC therapy did not contribute to patient improvement (Blum et al., 2007). In fact addition of low dose valproic acid led to encephalopathy in three patients.

6.2.1.1. *Genome-wide demethylating effect*

One of the concerns of treatment using 5-aza or 5-azadC is that it might cause the activation of unwanted genes or genome instability. Indeed, a previous study in mouse NIH/3T3 fibroblast has revealed that demethylation at gene promoter regions only occured at low concentration (0.1-1 μM) (Lim et al., 2010). In contrast, demethylation of repetitive elements, minor satellite repeats and endogenous C-type viruses occured at a wider concentration range (0.1 – 5 μM) which overlaps those for gene promoter demethylation. Thus, there might be an increased risk of activating unwanted elements while aiming for promoter demethylation. In addition to that, studies performed in animal and cancer cells have shown that disruption of DNA methyltransferase has the tendency to cause genomic instability and even induced tumour formation (Gaudet et al., 2003, Karpf and Matsui, 2005).

Myelodysplastic syndrome (MDS) patients treated with 5-azadC was found to display demethylation of chromosome 1 satellite 2 sequences (Mund et al., 2005). Furthermore, in a clinical trial, patients treated with 5-azadC were found to have an increased level of demethylated *MAGE1A* after 8 days of treatment (Appleton et al., 2007). However, this level returned to its initial level after 22 days.

Although all these studies imply the risk associated with the demethylating agent, until now no study has been conducted to investigate whether such demethylation effects will expose patients to an adverse outcome in the long term. Furthermore, the animal study that showed hypomethylation induced tumour were performed in *Dnmt1* hypomorphic mice which continuously expressed a low level of *Dnmt1*. In contrast, the demethylation effect observed in the clinical trials were only transient with methylation level returning to initial level within a month. Whether such a short period of demethylation will have any prolonged effect in the patient still needs to be investigated.

6.2.1.2. *Stability issue*

Meanwhile, other studies also highlighted another problem in delivering 5-aza and 5-azadC. Both drugs are not stable at room temperature once dissolved in aqueous solution. In one study, the half-life of 5-aza and 5-azadC solution was found to be reduced to 7 hours and 21 hours, respectively at 37 °C (Stresemann and Lyko, 2008). Thus, it has been recommended that these drugs have to be administered within 15 minutes after reconstitution (Saba, 2007). Otherwise, they need to be prepared with cold infusion solution and stored at 2-8°C for a maximum of 7 hours. Therefore, careful planning among practitioners is crucial to ensure optimal drug delivery to the patient.

Another stability concern for these two drugs is their inactivation by cytidine deaminase. This enzyme catalyze deamination of (deoxy-)cytidine into (deoxy-)uridine and render inactivation of all cytidine analog drug which include both 5-aza and 5-azadC (Rius and Lyko, 2012). In one study using a mouse model of myeloid cancer, 5-azadC treatment managed to reduce the tumour burden in the spleen but not in the

liver (Ebrahem et al., 2012). This observation is believed to be underpinned by the high cytidine deaminase level found in the liver but not in the spleen. Furthermore, by combining tetrahydrouridine, a cytidine deaminase inhibitor, with low dose 5-azadC, a marked reduction of tumour burden was observed in the liver. This suggests the involvement of cytidine deaminase in 5-azadC efficacy.

6.2.1.3. *Development of new DNMTi*

Given the issues surrounding 5-aza and 5-azadC in clinical settings, efforts are being made to discover better and more tolerable demethylating agents. One of the promising agents is zebularine. This drug is more stable than 5-aza and 5-azadC since it can inhibit cytidine deaminase and has been shown to be well tolerated when given orally in a mouse model (Cheng et al., 2003). In contrast to the mouse model, zebularine oral bioavailability was found to be low when given to primates (Holleran et al., 2005). To date, no clinical trials have been reported for zebularine, thus its clinical value is still unclear. Another nucleoside analog beside zebularine that has been developed is 5,6-dihydroazacytidine (5,6-dihydroAza). However, despite showing hypomethylation activity in cells, its potency is somewhat less than 5-aza and this is believed to be due to its poor incorporation into the DNA (Foulks et al., 2012).

Since nucleoside analog incorporation into DNA has been blamed for its toxicity, researchers have tried to look into alternative demethylating agents in non-nucleoside analogs. Among the available drugs that have shown demethylation activity are the cardiovascular drugs, hydralazine and procainamide (Segura-Pacheco et al., 2003). However, since other study showed that this effect was not reproducible, the role of both hydralazine and procainamide as demethylating agents remains controversial (Chuang et al., 2005).

Other researchers have developed new derivatives of 5-aza as a way to improve drug uptake and escape its inactivation by cytidine deaminase. Using a short oligonucleotide containing an azapyrimidine called S110, Yoo *et al.*(2007) showed that this drug could inhibit DNA methylation. Although it still has similar aqueous stability and toxicity as 5-azadC, its main advantage is its ability to inhibit cytidine deaminase.

Another group has developed an elaidic acid derivative of 5-aza called CP-4200 (Brueckner et al., 2010). It was found that this modified form of 5-aza uses a different cellular uptake mechanism and was found to have higher efficacy than 5-aza when tested in human acute lymphatic leukemia cells. Despite this promising result, we still need to wait for clinical trial data to assess its clinical value.

6.2.2. : DNA methylation as therapeutic markers

Using DNA methylation as biomarker has gained a lot of interest due to many factors. First, DNA methylation appears to be an early event in tumour development and often occurs at higher number of genes as compared to the mutated genes. Therefore, it offers the potential for much higher detection sensitivity and might be helpful for diagnostic purposes. Furthermore with the advancement of technology, only a small amount of DNA is needed for analysis, thus eliminating the need to take multiple samples at one time. In addition, excess DNA can be stored for a long time due to the stability of DNA molecules. This proves to be useful when investigation of prognosis or drug response needs to be done since both new DNA samples (taken after therapy or in future follow-up) and the old samples can be analysed together, thus eliminating ambiguous results. Another interesting characteristic of DNA is that it is accessible from a variety of sources. Tumour DNA for instance can be released into the body fluid such as blood and urine and this will provide a non-invasive approach to epigenetic analysis (Heyn and Esteller, 2012). Therefore, this might provide an alternative approach to help physicians in their decision making. Some of these markers will be discussed further below.

6.2.2.1. *Diagnosis Markers*

Studies that showed the presence of methylation in early-onset cancer (Shim et al., 2003, Jin et al., 2007) suggest that DNA methylation could be used for early cancer detection or even to identify those at risk of developing cancer later in life. Indeed, a study showed that *p16* and/or *MGMT* genes were methylated in all sputum samples taken from patients diagnosed with squamus cell lung carcinoma (SCC) (Palmisano et al., 2000). Interestingly, in half of these patients, methylation of both genes was observed in sputum samples taken three years before they were diagnosed with SCC.

Methylations observed in the sputums were also found to be similar in most of the tumour samples taken from these patients. However, when expanding their study in 123 samples of high-risk and cancer-free population, they found that only two SCCs developed after two to six years. Even though the number was low, the author suggests that this might be because the timing of the tumour is variable and a longer follow-up period might uncover higher numbers of SCC in this population. Thus, methylation of *p16* and *MGMT* might still be useful in identifying a high risk population for SCC. Another marker investigated for lung cancer was *SHOX2*. Methylation of this gene was found to be helpful in lung cancer diagnosis in situations where no conclusive evidence from histology and cytology were found (Schmidt et al., 2010). Furthermore, this group also found that *SHOX2* methylation detected from bronchial aspirates could differentiate between malignant and benign lung cancer. Indeed, a kit detecting *SHOX2* methylation from bronchial fluid is now available for early detection of lung cancer (Mikeska et al., 2012). Besides bronchial aspirates, another group has also shown that *SHOX2* methylation could also be detected in the plasma of lung cancer patient with 90% specificity (Kneip et al., 2011).

In colorectal cancer (CRC), two genes (*SEPT9* and *VIM*) were shown to have potential in CRC diagnosis (deVos et al., 2009, Chen et al., 2005, Itzkowitz et al., 2008). In one study, detection of *SEPT9* methylation using plasma samples taken from CRC patients was found to be quite sensitive and specific (deVos et al., 2009). However its application in the early detection of CRC is not yet established. On the other hand, *VIM* methylation in the stool was found only in the CRC patients but not in normal tissue (Chen et al., 2005). A further study also found that *VIM* methylation was highly specific in CRC identification (Zou et al., 2011) by identifying more than 80% of CRCs with a specifity of 95%.

Meanwhile, *GSTP1* methylation was shown to be the most recognized marker for prostate cancer diagnosis. In fact, *GSTP1* is the only DNA methylation marker at the moment that has been validated in a large number of studies. Meta-analysis performed on 30 studies has recognized *GSTP1* as a marker for prostate cancer with a specificity of 95% (Van Neste et al., 2012). In addition to that, observation of GSTP1

methylation in the precursor lesion of prostate cancer suggests that it can also be used as an early detection marker (Nakayama et al., 2003). Meanwhile, this marker's specificity to prostate cancer was also able to overcome the problem faced when using serum prostate-specific antigen (PSA), which is the conventional marker used in diagnosing prostate cancer. This is because PSA could also be elevated in other prostate condition such as inflammation and benign prostate hyperplasia which could lead to false-positive result (Mikeska et al., 2012). One study has shown that combination of both PSA marker and *GSTP1* methylation could generate higher sensitivity of both markers in diagnosing prostate cancer (Sunami et al., 2009). Beside *GSTP1*, studies using combination of markers (*APC*, *RASSF1*, *PTGS2*, *MED15* and *MDR1*) has also been shown to increase the sensitivity and specificity of up to 100% in diagnosing prostate cancer (Bastian et al., 2005, Yegnasubramanian et al., 2004).

6.2.2.2. *Prognosis Marker*

Two types of DNA methylation profiling have been used to study the prognostic value of methylation biomarkers that involve either single-loci approaches or genome-wide profiling (Heyn & Esteller, 2012). Using a single-locus approach, elevated *PITX2* methylation has been found to correlate with disease recurrence with distant metastatis in node negative and estrogen receptor positive breast cancer patients treated with tamoxifen (Maier et al., 2007). In another study by the same group, it was later found that the same group of patients that have elevated *PITX2* methylation should be further treated with an adjuvant therapy since patients that were not treated with any adjuvant therapy had a higher risk of getting early metastatis and poor survival (Nimmrich et al., 2008). This clearly showed the usefulness of methylation biomarkers in guiding the physician in their treatment plan. Meanwhile, *PITX2* methylation was also found to be the strongest prognostic predictor in prostate cancer patients when compared with five other biomarkers (Weiss et al., 2009). In this study, it was found that prostate cancer patients that underwent prostatectomy had a higher chance of recurrence eight years after surgery if they displayed higher than the median *PITX2* methylation. This finding was further validated in a multicenter study by the same group (Banez et al., 2010) further validating *PITX2* methylation as a

prognostic marker in this group of prostate cancer patients. On the other hand, a study comparing 51 patients with non-small cell lung carcinoma (NSCLC) that had undergone resection and had a recurrence within 40 months with 116 patients treated with the same procedure but had no recurrence within the same period showed a distinct methylation signature (Brock et al., 2008). They found that methylation of four (*CDKN2A*, *CDH13*, *RASSF1A* and *APC*) out of seven genes analysed was associated with recurrence after surgery. However, further validation in larger samples is needed before these markers could be used in a clinical setting.

Although the single-locus approach provides a list of promising candidates, it has been restricted to certain genes relying on previous available data. To overcome this, genome-wide profiling has recently entered the foray in determining possible biomarker which may not have been identified before. In a study involving childhood acute lymphoblastic leukemia (ALL), methylation analysis of 406 genes were found to be able to discriminate between T-lineage ALL and B-cell precursor ALL patients (Milani et al., 2010). Furthermore, this group also identified 20 promising genes which can predict relapse in ALL. Meanwhile a study performed in 37 breast cancer patients using the Infinium HumanMethylation450K Array platform found two distinct groups with one showing a methylator phenotype (CIMP+) (Fang et al., 2011). Further analysis of three genes (*ALX4*, *ARHGEF7* and *RASGRF2*) was found to be predictive for CIMP+ groups. They found that methylation of these genes was associated with lower risk of metastatis and have a better survival rate as compared to the CIMP- group. The authors did not rule out that there might be other methylated genes that might be associated with poor outcome. However, their initial analysis of three genes previously associated with poor outcome (*CDKN2A*, *PTPRD* and *BRCA1*) was found to be not methylated in the CIMP+ groups. Thus, the authors suggest that methylation analysis of the three genes (*ALX4*, *ARHGEF7* and *RASGRF2*) might be useful in future metastasis and survival prediction in breast cancer patient.

6.2.2.3. *Markers of drug response*

The other possible contribution of methylation biomarkers is in predicting patient response towards certain therapies. This will help physician in tailoring therapy

to each patient. However, at the moment only methylation of the DNA repair gene, *MGMT*, has been well-studied and shown to be a promising tool to predict response towards alkylating agents in a clinical setting. In one study, glioma patients treated with cisplatin and carmustine were observed to have a significant increase in overall survival and progression-free survival if they showed methylation of *MGMT*(Esteller et al., 2000a). The same observation was made by the same group when a similar approach were applied in B-cell lymphoma patients treated with multiple drugs which included cyclophosphamide (Esteller et al., 2002). However, since both studies involves multiple drugs, it cannot be certain whether the survival observed could also be due to patient response to other drugs. Nonetheless, another study performed in glioblastoma patients clearly showed the potential of *MGMT* methylation as a response marker to alkylating agents (Hegi et al., 2005). In this study, glioblastoma patients were randomized to receive either radiotherapy alone or radiotherapy with temozolomide as an adjuvant therapy. They found that patients with *MGMT* methylation and receiving both radiotherapy and temozolomide had significantly higher median survival compared to those receiving only radiotherapy. On the other hand, no different in response were observed in both arms of treatment in patient with unmethylated *MGMT* suggesting no benefit in adding temozolomide to radiotherapy in this group of patient.

Beside *MGMT*, *BRCA1* methylation in breast cancer patients which display lack of expression of *ERBB2*, estrogen receptor and progesterone receptor was found be associated with a better response towards cisplatin (Silver et al., 2010). Meanwhile, methylation of *WRN* and *UGT1A1* were found to be associated with increased sensitivity towards irinotecan, a topoisomerase inhibitor, in CRC patient and CRC cell lines (Agrelo et al., 2006, Gagnon et al., 2006). However, unlike *MGMT*, further validation needs to be conducted before the true potential of these genes can be determined.

6.2.2.4. *Biomarkers in GCTs*

In comparison to other tumours, methylation biomarkers in GCTs are still in their infancy. Despite this, the contrast in the methylation pattern between seminoma and non-seminomas shown in our data and others might be useful in current clinical settings in distinguishing these two types of GCTs. However, for other clinical purposes mentioned earlier, there is still a lot more work needs to be done. Our data and others have identified a number of methylated genes that might be useful for these purposes, but these selected genes need to be further validated in a larger number of samples before they can be considered as promising biomarkers in GCTs.

Nevertheless, a recent study that used supernatant from testicular GCT (TGCT) patient serum may provide an insight into a less invasive approach to current technique that necessitates tissue biopsy. In this study that compared TGCT patients and healthy volunteers, CpG methylation was not only able to differentiate between healthy volunteers and TGCT patients, but could also identify TGCT patients in which conventional marker assessments were negative (Ellinger et al., 2009). Thus, this might offer a better and tolerable diagnostic tool. However, this group did not find any significant difference in methylation between seminomas and non-seminoma samples. This contrast to our findings and other previous data might be due to their methodology since they performed methylation analysis only on six genes (*APC, GSTP1, p14(ARF),p16(INK),PTGS2* and *RASSF1A*).

6.2.2.5. *Issues surrounding DNA methylation biomarkers*

Despite the potential of methylation biomarkers in a clinical setting, there are still unresolved issues going from its discovery to its implementation. First, most of the initial studies were performed in cell lines which are known to often differ in methylation levels as compared to primary tumours (Smiraglia et al., 2001). Thus, genes identified from this type of study need to be validated using patient samples in order to assess their reliability as a biomarker. As for those studies that used patient samples in their analysis, most of them involved a small number of samples. Therefore, validation needs to be performed in a larger set of samples and further validated using a different set of samples by different laboratories. This is to ensure the reliability of

the markers in its future usage in larger populations. In terms of GCTs, especially those involving paediatric populations, this seems to be the major hurdle since GCTs in children are quite rare, thus limiting the number of samples available for analysis. Nevertheless, our analysis on the paediatric GCT samples was among the first that incorporated both methylation and expression analysis. And of importance, through this analysis we have identified six genes as possible candidate genes involved in YST development.

Apart from the number of samples, available methylation data at the moment was performed using a wide range of techniques. Since each method investigated different region of CpG islands, conflicting result might occur. In addition to that, CpG island regions chosen in previous studies were based on the assumption that every CpG island region had a similar biological role which has been proven to be wrong (Everhard et al., 2009). In their study, Everhard and his group found that the methylation status of only two out of five CpG regions of *MGMT* analysed that correlated well with its expression and were found to be reliable as a surrogate markers for *MGMT* expression in the majority of glioblastoma patients. Indeed, a recent study also adds to the complexity in choosing regions of interest. In their study, Irizarry *et al.*observed that it was methylation at the CpG shores instead of CpG islands that correlated well with gene expression in colorectal cancer cell lines (Irizarry et al., 2009). Although the clinical value of CpG shore methylation is still unknown, this finding should make researchers cautious in their interpretation of data. Furthermore technical constraints, especially in designing PCR primers, might affect decisions in choosing the region of interest which could lead to selection of unreliable CpG regions. However, we believe we overcome this uncertainty in our analysis by using the GoldenGate and Infinium 450K methylation arrays that has been used and validated countless times in many types of cancer (Bibikova et al., 2011, Bady et al., 2012, Clark et al., 2012, Dedeurwaerder et al., 2011, Martin-Subero et al., 2009, McRonald et al., 2009).

As for GCTs, another technical issue that needs to be solved is the use of controls. Indeed, contrasting results have been blamed on the use of different types of control. Many studies resorted in using healthy testis samples as their controls. However, since testes contain many different type of cells, this might have an effect on the methylation level measurement. Others used carcinoma-in situ (CIS) samples as the controls by arguing that CIS is the precursor lesion of GCTs and thus supporting its use as a control. However, due to low availability of CIS and its inability to be grown as a cell culture, it was not possible to use CIS as a control in all studies. Furthermore, most of the time CIS is found adjacent to the tumour itself and there is the possibility of contamination of tumour cells in the CIS, thus reducing its reliability as a control. In our initial methylation study in the paediatric GCT samples, we found that seminoma methylation is similar to other somatic tissue such as the blood and buccal cells. Indeed, previous studies have also shown that seminomas shares similar methylation and gene expression characteristics with CIS (de Jong et al., 2008, Hoei-Hansen et al., 2005, Eckert et al., 2008). Therefore, we used seminoma cell lines as the control for the Infinium 450K methylation analysis.

6.3. Summary

In this project we set out to investigate the methylation profile of paediatric seminomas and yolk sac tumours. The availability of a new and wider coverage methylation array also prompted us to extend this investigation into GCT cell lines. This was used as a means to investigate the methylation profile at regions outside of the CpG islands which has never been reported before in GCTs. Furthermore, this study also aimed to identify genes repressed by DNA methylation and the possible factor(s) contributing to the hypermethylation features of the tumours.

With respect to the paediatric tumours analysed, seminomas and YSTs showed global hypomethylation of intergenic region, YSTs were found to be hypermethylated at many gene promoter regions as compared to the seminomas. Strikingly, many of these genes were found to be reproducibly hypermethylated in the YSTs, suggesting a methylated gene signature that might be useful as a therapeutic marker in the future. Interestingly only eight genes were found to be differentially methylated and

differentially expressed suggesting these were possible candidates in paediatric YST development. Meanwhile, methylation analysis performed in adult GCT cell lines also revealed that non-seminomatous GCT cell lines are more highly methylated than the seminoma cell line. Interestingly, different non-seminomatous GCT cell lines exhibited distinct methylation features. While the teratocarcinoma and EC cell lines displayed higher methylation than the seminoma cell line at all regions investigated, the YST cell line only showed higher methylation than the seminoma cell line at the CpG islands and to a lesser extent at the CpG shores. This suggests a different mechanism disrupting methylation in the different GCT subtypes. In addition, we observed only a weak correlation between methylation at the CpG islands or the CpG shores and gene expression. Nonetheless, from this correlation we further identified 98 possible candidate genes, potentially regulated by methylation that may influence YST developments, among which are *CDX1* and *PYCARD*.

References:

AGGER, K., CHRISTENSEN, J., CLOOS, P. A. & HELIN, K. (2008) The emerging functions of histone demethylases. *Curr Opin Genet Dev,* 18, 159-68.

AGRELO, R., CHENG, W. H., SETIEN, F., ROPERO, S., ESPADA, J., FRAGA, M. F., HERRANZ, M., PAZ, M. F., SANCHEZ-CESPEDES, M., ARTIGA, M. J., GUERRERO, D., CASTELLS, A., VON KOBBE, C., BOHR, V. A. & ESTELLER, M. (2006) Epigenetic inactivation of the premature aging Werner syndrome gene in human cancer. *Proc Natl Acad Sci U S A,* 103, 8822-7.

AGUIRRE, D., NIETO, K., LAZOS, M., PENA, Y. R., PALMA, I., KOFMAN-ALFARO, S. & QUEIPO, G. (2006) Extragonadal germ cell tumors are often associated with Klinefelter syndrome. *Hum Pathol,* 37, 477-80.

ALISCH, R. S., GARCIA-PEREZ, J. L., MUOTRI, A. R., GAGE, F. H. & MORAN, J. V. (2006) Unconventional translation of mammalian LINE-1 retrotransposons. *Genes Dev,* 20, 210-24.

ALMSTRUP, K., HOEI-HANSEN, C. E., NIELSEN, J. E., WIRKNER, U., ANSORGE, W., SKAKKEBAEK, N. E., RAJPERT-DE MEYTS, E. & LEFFERS, H. (2005) Genome-wide gene expression profiling of testicular carcinoma in situ progression into overt tumours. *Br J Cancer,* 92, 1934-41.

ALMSTRUP, K., HOEI-HANSEN, C. E., WIRKNER, U., BLAKE, J., SCHWAGER, C., ANSORGE, W., NIELSEN, J. E., SKAKKEBAEK, N. E., RAJPERT-DE MEYTS, E. & LEFFERS, H. (2004) Embryonic stem cell-like features of testicular carcinoma in situ revealed by genome-wide gene expression profiling. *Cancer Res,* 64, 4736-43.

ANDERSON, R., COPELAND, T. K., SCHOLER, H., HEASMAN, J. & WYLIE, C. (2000) The onset of germ cell migration in the mouse embryo. *Mech Dev,* 91, 61-8.

ANDREWS, P. W. (1984) Retinoic acid induces neuronal differentiation of a cloned human embryonal carcinoma cell line in vitro. *Dev Biol,* 103, 285-93.

ANG, P. W., LOH, M., LIEM, N., LIM, P. L., GRIEU, F., VAITHILINGAM, A., PLATELL, C., YONG, W. P., IACOPETTA, B. & SOONG, R. (2010) Comprehensive profiling of DNA methylation in colorectal cancer reveals subgroups with distinct clinicopathological and molecular features. *BMC Cancer,* 10, 227.

APPLETON, K., MACKAY, H. J., JUDSON, I., PLUMB, J. A., MCCORMICK, C., STRATHDEE, G., LEE, C., BARRETT, S., READE, S., JADAYEL, D., TANG, A., BELLENGER, K., MACKAY, L., SETANOIANS, A., SCHATZLEIN, A., TWELVES, C., KAYE, S. B. & BROWN, R. (2007) Phase I and pharmacodynamic trial of the DNA methyltransferase inhibitor decitabine and carboplatin in solid tumors. *J Clin Oncol,* 25, 4603-9.

ARA, T., NAKAMURA, Y., EGAWA, T., SUGIYAMA, T., ABE, K., KISHIMOTO, T., MATSUI, Y. & NAGASAWA, T. (2003) Impaired colonization of the gonads by primordial germ cells in mice lacking a chemokine, stromal cell-derived factor-1 (SDF-1). *Proc Natl Acad Sci U S A,* 100, 5319-23.

ARAI, E., CHIKU, S., MORI, T., GOTOH, M., NAKAGAWA, T., FUJIMOTO, H. & KANAI, Y. (2012) Single-CpG-resolution methylome analysis identifies clinicopathologically aggressive CpG island methylator phenotype clear cell renal cell carcinomas. *Carcinogenesis,* 33, 1487-93.

ARCECI, R. J., KING, A. A., SIMON, M. C., ORKIN, S. H. & WILSON, D. B. (1993) Mouse GATA-4: a retinoic acid-inducible GATA-binding transcription factor expressed in endodermally derived tissues and heart. *Mol Cell Biol,* 13, 2235-46.

ARORA, R. S., ALSTON, R. D., EDEN, T. O., GERACI, M. & BIRCH, J. M. (2012) Comparative incidence patterns and trends of gonadal and extragonadal germ cell tumors in England, 1979 to 2003. *Cancer.*

BABA, Y., HUTTENHOWER, C., NOSHO, K., TANAKA, N., SHIMA, K., HAZRA, A., SCHERNHAMMER, E. S., HUNTER, D. J., GIOVANNUCCI, E. L., FUCHS, C. S. & OGINO, S. (2010) Epigenomic diversity of colorectal cancer indicated by LINE-1 methylation in a database of 869 tumors. *Mol Cancer,* 9, 125.

BACOLLA, A., PRADHAN, S., LARSON, J. E., ROBERTS, R. J. & WELLS, R. D. (2001) Recombinant human DNA (cytosine-5) methyltransferase. III. Allosteric control, reaction order, and influence of plasmid topology and triplet repeat length on methylation of the fragile X CGG.CCG sequence. *J Biol Chem,* 276, 18605-13.

BADY, P., SCIUSCIO, D., DISERENS, A. C., BLOCH, J., VAN DEN BENT, M. J., MAROSI, C., DIETRICH, P. Y., WELLER, M., MARIANI, L., HEPPNER, F. L., MCDONALD, D. R., LACOMBE, D., STUPP, R., DELORENZI, M. & HEGI, M. E. (2012) MGMT methylation analysis of glioblastoma on the Infinium methylation BeadChip identifies two distinct CpG regions associated with gene silencing and outcome, yielding a prediction model for comparisons across datasets, tumor grades, and CIMP-status. *Acta Neuropathol,* 124, 547-60.

BAKER, J. A., BUCK, G. M., VENA, J. E. & MOYSICH, K. B. (2005) Fertility patterns prior to testicular cancer diagnosis. *Cancer Causes Control,* 16, 295-9.

BALL, M. P., LI, J. B., GAO, Y., LEE, J. H., LEPROUST, E. M., PARK, I. H., XIE, B., DALEY, G. Q. & CHURCH, G. M. (2009) Targeted and genome-scale strategies reveal gene-body methylation signatures in human cells. *Nat Biotechnol,* 27, 361-8.

BANEZ, L. L., SUN, L., VAN LEENDERS, G. J., WHEELER, T. M., BANGMA, C. H., FREEDLAND, S. J., ITTMANN, M. M., LARK, A. L., MADDEN, J. F., HARTMAN, A., WEISS, G. & CASTANOS-VELEZ, E. (2010) Multicenter clinical validation of PITX2 methylation as a prostate specific antigen recurrence predictor in patients with post-radical prostatectomy prostate cancer. *J Urol,* 184, 149-56.

BARSKI, A., CUDDAPAH, S., CUI, K., ROH, T. Y., SCHONES, D. E., WANG, Z., WEI, G., CHEPELEV, I. & ZHAO, K. (2007) High-resolution profiling of histone methylations in the human genome. *Cell,* 129, 823-37.

BARTHOLIN, L., MAGUER-SATTA, V., HAYETTE, S., MARTEL, S., GADOUX, M., CORBO, L., MAGAUD, J. P. & RIMOKH, R. (2002) Transcription activation of FLRG and follistatin by activin A, through Smad proteins, participates in a negative feedback loop to modulate activin A function. *Oncogene,* 21, 2227-35.

BARTOLOMEI, M. S. (2009) Genomic imprinting: employing and avoiding epigenetic processes. *Genes Dev,* 23, 2124-33.

BASTIAN, P. J., ELLINGER, J., WELLMANN, A., WERNERT, N., HEUKAMP, L. C., MULLER, S. C. & VON RUECKER, A. (2005) Diagnostic and prognostic information in prostate cancer with the help of a small set of hypermethylated gene loci. *Clin Cancer Res,* 11, 4037-43.

BAUERSACHS, S., ULBRICH, S. E., GROSS, K., SCHMIDT, S. E., MEYER, H. H., WENIGERKIND, H., VERMEHREN, M., SINOWATZ, F., BLUM, H. & WOLF, E. (2006) Embryo-induced transcriptome changes in bovine endometrium reveal species-specific and common molecular markers of uterine receptivity. *Reproduction,* 132, 319-31.

BEARD, C., LI, E. & JAENISCH, R. (1995) Loss of methylation activates Xist in somatic but not in embryonic cells. *Genes Dev,* 9, 2325-34.

BEAULIEU, N., MORIN, S., CHUTE, I. C., ROBERT, M. F., NGUYEN, H. & MACLEOD, A. R. (2002) An essential role for DNA methyltransferase DNMT3B in cancer cell survival. *J Biol Chem,* 277, 28176-81.

BERCHUCK, A., HERON, K. A., CARNEY, M. E., LANCASTER, J. M., FRASER, E. G., VINSON, V. L., DEFFENBAUGH, A. M., MIRON, A., MARKS, J. R., FUTREAL, P. A. & FRANK, T. S. (1998) Frequency of germline and somatic BRCA1 mutations in ovarian cancer. *Clin Cancer Res,* 4, 2433-7.

BESTOR, T. H. (2000) The DNA methyltransferases of mammals. *Hum Mol Genet,* 16, 2395-402.

BEYROUTHY, M. J., GARNER, K. M., HEVER, M. P., FREEMANTLE, S. J., EASTMAN, A., DMITROVSKY, E. & SPINELLA, M. J. (2009) High DNA methyltransferase 3B expression mediates 5-aza-deoxycytidine hypersensitivity in testicular germ cell tumors. *Cancer Res,* 69, 9360-6.

BHUTANI, N., BRADY, J. J., DAMIAN, M., SACCO, A., CORBEL, S. Y. & BLAU, H. M. (2010) Reprogramming towards pluripotency requires AID-dependent DNA demethylation. *Nature,* 463, 1042-7.

BHUTANI, N., BURNS, D. M. & BLAU, H. M. (2011) DNA demethylation dynamics. *Cell,* 146, 866-72.

BIBIKOVA, M., BARNES, B., TSAN, C., HO, V., KLOTZLE, B., LE, J. M., DELANO, D., ZHANG, L., SCHROTH, G. P., GUNDERSON, K. L., FAN, J. B. & SHEN, R. (2011) High density DNA methylation array with single CpG site resolution. *Genomics,* 98, 288-95.

BILLMIRE, D., VINOCUR, C., RESCORLA, F., COLOMBANI, P., CUSHING, B., HAWKINS, E., DAVIS, M., LONDON, W. B., LAUER, S. & GILLER, R. (2003) Malignant retroperitoneal and abdominal germ cell tumors: an intergroup study. *J Pediatr Surg,* 38, 315-8; discussion 315-8.

BILLMIRE, D., VINOCUR, C., RESCORLA, F., COLOMBANI, P., CUSHING, B., HAWKINS, E., LONDON, W. B., GILLER, R. & LAUER, S. (2001) Malignant mediastinal germ cell tumors: an intergroup study. *J Pediatr Surg,*.36, 18-24.

BLUM, W., KLISOVIC, R. B., HACKANSON, B., LIU, Z., LIU, S., DEVINE, H., VUKOSAVLJEVIC, T., HUYNH, L., LOZANSKI, G., KEFAUVER, C., PLASS, C., DEVINE, S. M., HEEREMA, N. A., MURGO, A., CHAN, K. K., GREVER, M. R., BYRD, J. C. & MARCUCCI, G. (2007) Phase I study of decitabine alone or in combination with valproic acid in acute myeloid leukemia. *J Clin Oncol,* 25, 3884-91.

BORGEL, J., GUIBERT, S., LI, Y., CHIBA, H., SCHUBELER, D., SASAKI, H., FORNE, T. & WEBER, M. (2010) Targets and dynamics of promoter DNA methylation during early mouse development. *Nat Genet,* 42, 1093-100.

BOUCHER, S. & BENNETT, S. A. (2003) Differential connexin expression, gap junction intercellular coupling, and hemichannel formation in NT2/D1 human neural progenitors and terminally differentiated hNT neurons. *J Neurosci Res,* 72, 393-404.

BRAIT, M., MALDONADO, L., BEGUM, S., LOYO, M., WEHLE, D., TAVORA, F. F., LOOIJENGA, L. H., KOWALSKI, J., ZHANG, Z., ROSENBAUM, E., HALACHMI, S., NETTO, G. J. & HOQUE, M. O. (2012) DNA methylation profiles delineate epigenetic heterogeneity in seminoma and non-seminoma. *Br J Cancer,* 106, 414-23.

BRENET, F., MOH, M., FUNK, P., FEIERSTEIN, E., VIALE, A. J., SOCCI, N. D. & SCANDURA, J. M. (2012) DNA methylation of the first exon is tightly linked to transcriptional silencing. *PLoS One,* 6, e14524.

BROCK, M. V., HOOKER, C. M., OTA-MACHIDA, E., HAN, Y., GUO, M., AMES, S., GLOCKNER, S., PIANTADOSI, S., GABRIELSON, E., PRIDHAM, G., PELOSKY, K., BELINSKY, S. A., YANG, S. C., BAYLIN, S. B. & HERMAN, J. G. (2008) DNA methylation markers and early recurrence in stage I lung cancer. *N Engl J Med,* 358, 1118-28.

BRUECKNER, B., RIUS, M., MARKELOVA, M. R., FICHTNER, I., HALS, P. A., SANDVOLD, M. L. & LYKO, F. (2010) Delivery of 5-azacytidine to human cancer cells by elaidic acid esterification increases therapeutic drug efficacy. *Mol Cancer Ther,* 9, 1256-64.

BRYCESON, Y. T., MARCH, M. E., LJUNGGREN, H. G. & LONG, E. O. (2006) Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. *Blood,* 107, 159-66.

BUCHBERG, A. M., BEDIGIAN, H. G., JENKINS, N. A. & COPELAND, N. G. (1990) Evi-2, a common integration site involved in murine myeloid leukemogenesis. *Mol Cell Biol,* 10, 4658-66.

BUSSEY, K. J., LAWCE, H. J., HIMOE, E., SHU, X. O., HEEREMA, N. A., PERLMAN, E. J., OLSON, S. B. & MAGENIS, R. E. (2001a) SNRPN methylation patterns in germ cell tumors as a reflection of primordial germ cell development. *Genes Chromosomes Cancer,* 32, 342-52.

BUSSEY, K. J., LAWCE, H. J., HIMOE, E., SHU, X. O., SUIJKERBUIJK, R. F., OLSON, S. B. & MAGENIS, R. E. (2001b) Chromosomes 1 and 12 abnormalities in pediatric germ cell tumors by interphase fluorescence in situ hybridization. *Cancer Genet Cytogenet,* 125, 112-8.

BUTTE, A. J., YE, J., HARING, H. U., STUMVOLL, M., WHITE, M. F. & KOHANE, I. S. (2001) Determining significant fold differences in gene expression analysis. *Pac Symp Biocomput,* 6-17.

CAIRNS, J. M., DUNNING, M. J., RITCHIE, M. E., RUSSELL, R. & LYNCH, A. G. (2008) BASH: a tool for managing BeadArray spatial artefacts. *Bioinformatics,* 24, 2921-2.

CARICASOLE, A., WARD-VAN OOSTWAARD, D., ZEINSTRA, L., VAN DEN EIJNDEN-VAN RAAIJ, A. & MUMMERY, C. (2000) Bone morphogenetic proteins (BMPs) induce epithelial differentiation of NT2D1 human embryonal carcinoma cells. *Int J Dev Biol,* 44, 443-50.

CEDAR, H. & BERGMAN, Y. (2009) Linking DNA methylation and histone modification: patterns and paradigms. *Nat Rev Genet,* 10, 295-304.

CHAUBERT, P., GUILLOU, L., KURT, A. M., BERTHOLET, M. M., METTHEZ, G., LEISINGER, H. J., BOSMAN, F. & SHAW, P. (1997) Frequent p16INK4 (MTS1) gene inactivation in testicular germ cell tumors. *Am J Pathol,* 151, 859-65.

CHEN, R. Z., PETTERSSON, U., BEARD, C., JACKSON-GRUSBY, L. & JAENISCH, R. (1998) DNA hypomethylation leads to elevated mutation rates. *Nature,* 395, 89-93.

CHEN, T., TSUJIMOTO, N. & LI, E. (2004) The PWWP domain of Dnmt3a and Dnmt3b is required for directing DNA methylation to the major satellite repeats at pericentric heterochromatin. *Mol Cell Biol,* 24, 9048-58.

CHEN, W. D., HAN, Z. J., SKOLETSKY, J., OLSON, J., SAH, J., MYEROFF, L., PLATZER, P., LU, S., DAWSON, D., WILLIS, J., PRETLOW, T. P., LUTTERBAUGH, J., KASTURI, L., WILLSON, J. K., RAO, J. S., SHUBER, A. & MARKOWITZ, S. D. (2005) Detection in fecal DNA of colon cancer-specific methylation of the nonexpressed vimentin gene. *J Natl Cancer Inst,* 97, 1124-32.

CHEN, W. G., CHANG, Q., LIN, Y., MEISSNER, A., WEST, A. E., GRIFFITH, E. C., JAENISCH, R. & GREENBERG, M. E. (2003) Derepression of BDNF transcription involves calcium-dependent phosphorylation of MeCP2. *Science,* 302, 885-9.

CHEN, Y., XIE, D., YIN LI, W., MAN CHEUNG, C., YAO, H., CHAN, C. Y., XU, F. P., LIU, Y. H., SUNG, J. J. & KUNG, H. F. (2010) RNAi targeting EZH2 inhibits tumor growth and liver metastasis of pancreatic cancer in vivo. *Cancer Lett,* 297, 109-16.

CHENG, J. C., MATSEN, C. B., GONZALES, F. A., YE, W., GREER, S., MARQUEZ, V. E., JONES, P. A. & SELKER, E. U. (2003) Inhibition of DNA methylation and reactivation of silenced genes by zebularine. *J Natl Cancer Inst,* 95, 399-409.

CHENG, X. & BLUMENTHAL, R. M. (2008) Mammalian DNA methyltransferases: a structural perspective. *Structure,* 16, 341-50.

CHEUNG, H. H., LEE, T. L., RENNERT, O. M. & CHAN, W. Y. (2009) DNA methylation of cancer genome. *Birth Defects Res C Embryo Today,* 87, 335-50.

CHI, P., CHEN, Y., ZHANG, L., GUO, X., WONGVIPAT, J., SHAMU, T., FLETCHER, J. A., DEWELL, S., MAKI, R. G., ZHENG, D., ANTONESCU, C. R., ALLIS, C. D. & SAWYERS, C. L. (2010) ETV1 is a lineage survival factor that cooperates with KIT in gastrointestinal stromal tumours. *Nature,* 467, 849-53.

CHIK, F. & SZYF, M. (2010) Effects of specific DNMT gene depletion on cancer cell transformation and breast cancer cell invasion; toward selective DNMT inhibitors. *Carcinogenesis,* 32, 224-32.

CHOI, S. H., HEO, K., BYUN, H. M., AN, W., LU, W. & YANG, A. S. (2011) Identification of preferential target sites for human DNA methyltransferases. *Nucleic Acids Res,* 39, 104-18.

CHRISTENSEN, J., AGGER, K., CLOOS, P. A., PASINI, D., ROSE, S., SENNELS, L., RAPPSILBER, J., HANSEN, K. H., SALCINI, A. E. & HELIN, K. (2007) RBP2 belongs to a family of demethylases, specific for tri-and dimethylated lysine 4 on histone 3. *Cell,* 128, 1063-76.

CHRISTMAN, J. K. (2002) 5-Azacytidine and 5-aza-2'-deoxycytidine as inhibitors of DNA methylation: mechanistic studies and their implications for cancer therapy. *Oncogene,* 21, 5483-95.

CHRISTOPH, F., KEMPKENSTEFFEN, C., WEIKERT, S., KRAUSE, H., SCHOSTAK, M., MILLER, K. & SCHRADER, M. (2007) Frequent epigenetic inactivation of p53 target genes in seminomatous and nonseminomatous germ cell tumors. *Cancer Lett,* 247, 137-42.

CHUANG, J. C., YOO, C. B., KWAN, J. M., LI, T. W., LIANG, G., YANG, A. S. & JONES, P. A. (2005) Comparison of biological effects of non-nucleoside DNA methylation inhibitors versus 5-aza-2'-deoxycytidine. *Mol Cancer Ther,* 4, 1515-20.

CHUANG, L. S., IAN, H. L, KOH, T. W., NG, H. H., XU, G. & LI, B. F. (1997) Human DNA-(cytosine-5) methyltransferase-PCNA complex as a target for p21WAF1. *Science,* 277, 1996-2000.

CIRIO, M. C., MARTEL, J., MANN, M., TOPPINGS, M., BARTOLOMEI, M., TRASLER, J. & CHAILLET, J. R. (2008) DNA methyltransferase 1o functions during preimplantation development to preclude a profound level of epigenetic variation. *Dev Biol,* 324, 139-50.

CLARK, C., PALTA, P., JOYCE, C. J., SCOTT, C., GRUNDBERG, E., DELOUKAS, P., PALOTIE, A. & COFFEY, A. J. (2012) A Comparison of the Whole Genome Approach of MeDIP-Seq to the Targeted Approach of the Infinium HumanMethylation450 BeadChip((R)) for Methylome Profiling. *PLoS One,* 7, e50233.

CRUICKSHANKS, H. A. & TUFARELLI, C. (2009) Isolation of cancer-specific chimeric transcripts induced by hypomethylation of the LINE-1 antisense promoter. *Genomics,* 94, 397-406.

CZIRJAK, S., PASZTOR, E., SLOWIK, F. & SZEIFERT, G. (1992) Third ventricle germinoma after total removal of intrasellar teratoma. Case report. *J Neurosurg,* 77, 643-7.

DAHM, L., KLUGMANN, F., GONZALEZ-ALGABA, A. & REUSS, B. (2010) Tamoxifen and raloxifene modulate gap junction coupling during early phases of retinoic acid-dependent neuronal differentiation of NTera2/D1 cells. *Cell Biol Toxicol,* 26, 579-91.

DANG, L., WHITE, D. W., GROSS, S., BENNETT, B. D., BITTINGER, M. A., DRIGGERS, E. M., FANTIN, V. R., JANG, H. G., JIN, S., KEENAN, M. C., MARKS, K. M., PRINS, R. M., WARD, P. S., YEN, K. E., LIAU, L. M., RABINOWITZ, J. D., CANTLEY, L. C., THOMPSON, C. B., VANDER HEIDEN, M. G. & SU, S. M. (2009) Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. *Nature,* 462, 739-44.

DANIEL, F. I., CHERUBINI, K., YURGEL, L. S., DE FIGUEIREDO, M. A. & SALUM, F. G. (2011) The role of epigenetic transcription repression and DNA methyltransferases in cancer. *Cancer,* 117, 677-87.

DAS, P. M., RAMACHANDRAN, K., VANWERT, J., FERDINAND, L., GOPISETTY, G., REIS, I. M. & SINGAL, R. (2006) Methylation mediated silencing of TMS1/ASC gene in prostate cancer. *Mol Cancer,* 5, 28.

DASKALOS, A., NIKOLAIDIS, G., XINARIANOS, G., SAVVARI, P., CASSIDY, A., ZAKOPOULOU, R., KOTSINAS, A., GORGOULIS, V., FIELD, J. K. & LILOGLOU, T. (2009) Hypomethylation of retrotransposable elements correlates with genomic instability in non-small cell lung cancer. *Int J Cancer,* 124, 81-7.

DE GOUVEIA BRAZAO, C. A., PIERIK, F. H., OOSTERHUIS, J. W., DOHLE, G. R., LOOIJENGA, L. H. & WEBER, R. F. (2004) Bilateral testicular microlithiasis predicts the presence of the precursor of testicular germ cell tumors in subfertile men. *J Urol,* 171, 158-60.

DE JONG, J., STOOP, H., GILLIS, A. J. M., R.J.H.L.M., V. G., G.J.M., V. D. G., M.DE, B., R., H., P.T.K., S., R.A., A., J.W., O. & LOOIJENGA, L. H. J. (2008) Differential expression of SOX17 and SOX2 in germ cells and stem cells has biological and clinical implications. *J Pathol,* 215, 21-30.

DE LIMA, M., RAVANDI, F., SHAHJAHAN, M., ANDERSSON, B., COURIEL, D., DONATO, M., KHOURI, I., GAJEWSKI, J., VAN BESIEN, K., CHAMPLIN, R., GIRALT, S. & KANTARJIAN, H. (2003) Long-term follow-up of a phase I study of high-dose decitabine, busulfan, and cyclophosphamide plus allogeneic transplantation for the treatment of patients with leukemias. *Cancer,* 97, 1242-7.

DE SMET, C. & LORIOT, A. (2010) DNA hypomethylation in cancer: Epigenetic scars of a neoplastic journey. *Epigenetics,* 5.

DE VRIES, J. F., WAMMES, L. J., JEDEMA, I., VAN DREUNEN, L., NIJMEIJER, B. A., HEEMSKERK, M. H., WILLEMZE, R., FALKENBURG, J. H. & BARGE, R. M. (2007) Involvement of caspase-8 in chemotherapy-induced apoptosis of patient derived leukemia cell lines independent of the death receptor pathway and downstream from mitochondria. *Apoptosis,* 12, 181-93.

DEATON, A. M. & BIRD, A. (2011) CpG islands and the regulation of transcription. *Genes Dev,* 25, 1010-22.

DEDEURWAERDER, S., DEFRANCE, M., CALONNE, E., DENIS, H., SOTIRIOU, C. & FUKS, F. (2011) Evaluation of the Infinium Methylation 450K technology. *Epigenomics,* 3, 771-84.

DEININGER, P. L. & BATZER, M. A. (1999) Alu repeats and human disease. *Mol Genet Metab,* 67, 183-93.

DEVOS, T., TETZNER, R., MODEL, F., WEISS, G., SCHUSTER, M., DISTLER, J., STEIGER, K. V., GRUTZMANN, R., PILARSKY, C., HABERMANN, J. K., FLESHNER, P. R., OUBRE, B. M., DAY, R., SLEDZIEWSKI, A. Z. & LOFTON-DAY, C. (2009) Circulating methylated SEPT9 DNA in plasma is a biomarker for colorectal cancer. *Clin Chem,* 55, 1337-46.

DI CROCE, L., RAKER, V. A., CORSARO, M., FAZI, F., FANELLI, M., FARETTA, M., FUKS, F., LO COCO, F., KOUZARIDES, T., NERVI, C., MINUCCI, S. & PELICCI, P. G. (2002) Methyltransferase recruitment and DNA hypermethylation of target promoters by an oncogenic transcription factor. *Science,* 295, 1079-82.

DI PIETRO, A., VRIES, E. G. E. D., GIETEMA, J. A., SPIERINGS, D. C. J. & DE JONG, S. (2005) Testicular germ cell tumours: The paradigm of chemo-sensitive solid tumours. *The International Journal of Biochemistry & Cell Biology,* 37, 2437-2456.

DOBROVIC, A. & SIMPFENDORFER, D. (1997) Methylation of the BRCA1 gene in sporadic breast cancer. *Cancer Res,* 57, 3347-50.

DODGE, J. E., RAMSAHOYE, B. H., WO, Z. G., OKANO, M. & LI, E. (2002) De novo methylation of MMLV provirus in embryonic stem cells: CpG versus non-CpG methylation. *Gene,* 289, 41-8.

DONOVAN, P. J. (1998) The germ cell--the mother of all stem cells. *Int J Dev Biol,* 42, 1043-50.

DORIA-ROSE, V. P., BIGGS, M. L. & WEISS, N. S. (2005) Subfertility and the risk of testicular germ cell tumors (United States). *Cancer Causes Control,* 16, 651-6.

DUALE, N., LINDEMAN, B., KOMADA, M., OLSEN, A. K., ANDREASSEN, A., SODERLUND, E. J. & BRUNBORG, G. (2007) Molecular portrait of cisplatin induced response in human testis cancer cell lines based on gene expression profiles. *Mol Cancer,* 6, 53.

DUNNING, M. J., SMITH, M. L., RITCHIE, M. E. & TAVARE, S. (2007) beadarray: R classes and methods for Illumina bead-based data. *Bioinformatics,* 23, 2183-4.

EBRAHEM, Q., MAHFOUZ, R. Z., NG, K. P. & SAUNTHARARAJAH, Y. (2012) High cytidine deaminase expression in the liver provides sanctuary for cancer cells from decitabine treatment effects. *Oncotarget,* 3, 1137-45.

ECHEVARRIA, M. E., FANGUSARO, J. & GOLDMAN, S. (2008) Pediatric central nervous system germ cell tumors: a review. *Oncologist,* 13, 690-9.

ECKERT, D., BIERMANN, K., NETTERSHEIM, D., GILLIS, A. J., STEGER, K., JACK, H. M., MULLER, A. M., LOOIJENGA, L. H. & SCHORLE, H. (2008) Expression of BLIMP1/PRMT5 and concurrent histone H2A/H4 arginine 3 dimethylation in fetal germ cells, CIS/IGCNU and germ cell tumors. *BMC Dev Biol,* 8, 106.

ECKHARDT, F., LEWIN, J., CORTESE, R., RAKYAN, V. K., ATTWOOD, J., BURGER, M., BURTON, J., COX, T. V., DAVIES, R., DOWN, T. A., HAEFLIGER, C., HORTON, R., HOWE, K., JACKSON, D. K., KUNDE, J., KOENIG, C., LIDDLE, J., NIBLETT, D., OTTO, T., PETTETT, R., SEEMANN, S., THOMPSON, C., WEST, T., ROGERS, J., OLEK, A., BERLIN, K. & BECK, S. (2006) DNA methylation profiling of human chromosomes 6, 20 and 22. *Nat Genet,* 38, 1378-85.

EDEN, A., GAUDET, F., WAGHMARE, A. & JAENISCH, R. (2003) Chromosomal instability and tumors promoted by DNA hypomethylation. *Science,* 300, 455.

EHRLICH, M., JIANG, G., FIALA, E., DOME, J. S., YU, M. C., LONG, T. I., YOUN, B., SOHN, O. S., WIDSCHWENDTER, M., TOMLINSON, G. E., CHINTAGUMPALA, M., CHAMPAGNE, M., PARHAM, D., LIANG, G., MALIK, K. & LAIRD, P. W. (2002) Hypomethylation and hypermethylation of DNA in Wilms tumors. *Oncogene,* 21, 6694-702.

EHRLICH, M., WOODS, C. B., YU, M. C., DUBEAU, L., YANG, F., CAMPAN, M., WEISENBERGER, D. J., LONG, T., YOUN, B., FIALA, E. S. & LAIRD, P. W. (2006) Quantitative analysis of associations between DNA hypermethylation, hypomethylation, and DNMT RNA levels in ovarian tumors. *Oncogene,* 25, 2636-45.

ELLINGER, J., ALBERS, P., PERABO, F. G., MULLER, S. C., VON RUECKER, A. & BASTIAN, P. J. (2009) CpG island hypermethylation of cell-free circulating serum DNA in patients with testicular cancer. *J Urol,* 182, 324-9.

ESTECIO, M. R., GHARIBYAN, V., SHEN, L., IBRAHIM, A. E., DOSHI, K., HE, R., JELINEK, J., YANG, A. S., YAN, P. S., HUANG, T. H., TAJARA, E. H. & ISSA, J. P. (2007) LINE-1 hypomethylation in cancer is highly variable and inversely correlated with microsatellite instability. *PLoS One.,* 2, e399.

ESTELLER, M., GAIDANO, G., GOODMAN, S. N., ZAGONEL, V., CAPELLO, D., BOTTO, B., ROSSI, D., GLOGHINI, A., VITOLO, U., CARBONE, A., BAYLIN, S. B. & HERMAN, J. G. (2002) Hypermethylation of the DNA repair gene O(6)-methylguanine DNA methyltransferase and survival of patients with diffuse large B-cell lymphoma. *J Natl Cancer Inst,* 94, 26-32.

ESTELLER, M., GARCIA-FONCILLAS, J., ANDION, E., GOODMAN, S. N., HIDALGO, O. F., VANACLOCHA, V., BAYLIN, S. B. & HERMAN, J. G. (2000a) Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. *N Engl J Med,* 343, 1350-4.

ESTELLER, M., RISQUES, R. A., TOYOTA, M., CAPELLA, G., MORENO, V., PEINADO, M. A., BAYLIN, S. B. & HERMAN, J. G. (2001) Promoter hypermethylation of the DNA repair gene O(6)-methylguanine-DNA methyltransferase is associated with the presence of G:C to A:T transition mutations in p53 in human colorectal tumorigenesis. *Cancer Res,* 61, 4689-92.

ESTELLER, M., SILVA, J. M., DOMINGUEZ, G., BONILLA, F., MATIAS-GUIU, X., LERMA, E., BUSSAGLIA, E., PRAT, J., HARKES, I. C., REPASKY, E. A., GABRIELSON, E., SCHUTTE, M., BAYLIN, S. B. & HERMAN, J. G. (2000b) Promoter hypermethylation and BRCA1 inactivation in sporadic breast and ovarian tumors. *J Natl Cancer Inst,* 92, 564-9.

ESTELLER, M., TOYOTA, M., SANCHEZ-CESPEDES, M., CAPELLA, G., PEINADO, M. A., WATKINS, D. N., ISSA, J. P., SIDRANSKY, D., BAYLIN, S. B. & HERMAN, J. G. (2000c) Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation is associated with G to A mutations in K-ras in colorectal tumorigenesis. *Cancer Res,* 60, 2368-71.

EVERHARD, S., TOST, J., EL ABDALAOUI, H., CRINIERE, E., BUSATO, F., MARIE, Y., GUT, I. G., SANSON, M., MOKHTARI, K., LAIGLE-DONADEY, F., HOANG-XUAN, K., DELATTRE, J. Y. & THILLET, J. (2009) Identification of regions correlating MGMT promoter methylation and gene expression in glioblastomas. *Neuro Oncol,* 11, 348-56.

FABBRI, M., GARZON, R., CIMMINO, A., LIU, Z., ZANESI, N., CALLEGARI, E., LIU, S., ALDER, H., COSTINEAN, S., FERNANDEZ-CYMERING, C., VOLINIA, S., GULER, G., MORRISON, C. D., CHAN, K. K., MARCUCCI, G., CALIN, G. A., HUEBNER, K. & CROCE, C. M. (2007) MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B. *Proc Natl Acad Sci U S A,* 104, 15805-10.

FANG, F., BALCH, C., SCHILDER, J., BREEN, T., ZHANG, S., SHEN, C., LI, L., KULESAVAGE, C., SNYDER, A. J., NEPHEW, K. P. & MATEI, D. E. (2010) A phase 1 and pharmacodynamic study of decitabine in combination with carboplatin in patients with recurrent, platinum-resistant, epithelial ovarian cancer. *Cancer,* 116, 4043-53.

FANG, F., TURCAN, S., RIMNER, A., KAUFMAN, A., GIRI, D., MORRIS, L. G., SHEN, R., SESHAN, V., MO, Q., HEGUY, A., BAYLIN, S. B., AHUJA, N., VIALE, A., MASSAGUE, J., NORTON, L., VAHDAT, L. T., MOYNAHAN, M. E. & CHAN, T. A. (2011) Breast cancer methylomes establish an epigenomic foundation for metastasis. *Sci Transl Med,* 3, 75ra25.

FANG, J. Y., ZHU, S. S., XIAO, S. D., JIANG, S. J., SHI, Y., CHEN, X. Y., ZHOU, X. M. & QIAN, L. F. (1996) Studies on the hypomethylation of c-myc, c-Ha-ras oncogenes and histopathological changes in human gastric carcinoma. *J Gastroenterol Hepatol,* 11, 1079-82.

FAULKNER, G. J., KIMURA, Y., DAUB, C. O., WANI, S., PLESSY, C., IRVINE, K. M., SCHRODER, K., CLOONAN, N., STEPTOE, A. L., LASSMANN, T., WAKI, K., HORNIG, N., ARAKAWA, T., TAKAHASHI, H., KAWAI, J., FORREST, A. R., SUZUKI, H., HAYASHIZAKI, Y., HUME, D. A., ORLANDO, V., GRIMMOND, S. M. & CARNINCI, P. (2009) The regulated retrotransposon transcriptome of mammalian cells. *Nat Genet,* 41, 563-71.

FENAUX, P., GATTERMANN, N., SEYMOUR, J. F., HELLSTROM-LINDBERG, E., MUFTI, G. J., DUEHRSEN, U., GORE, S. D., RAMOS, F., BEYNE-RAUZY, O., LIST, A., MCKENZIE, D., BACKSTROM, J. & BEACH, C. L. (2010) Prolonged survival with improved tolerability in higher-risk myelodysplastic syndromes: azacitidine compared with low dose ara-C. *Br J Haematol,* 149, 244-9.

FENAUX, P., MUFTI, G. J., HELLSTROM-LINDBERG, E., SANTINI, V., FINELLI, C., GIAGOUNIDIS, A., SCHOCH, R., GATTERMANN, N., SANZ, G., LIST, A., GORE, S. D., SEYMOUR, J. F., BENNETT, J. M., BYRD, J., BACKSTROM, J., ZIMMERMAN, L., MCKENZIE, D., BEACH, C. & SILVERMAN, L. R. (2009) Efficacy of azacitidine compared with that of conventional care regimens in the treatment of higher-risk myelodysplastic syndromes: a randomised, open-label, phase III study. *Lancet Oncol,* 10, 223-32.

FENSKE, A. E., GLAESENER, S., BOKEMEYER, C., THOMALE, J., DAHM-DAPHI, J., HONECKER, F. & DARTSCH, D. C. (2012) Cisplatin resistance induced in germ cell tumour cells is due to reduced susceptibility towards cell death but not to altered DNA damage induction or repair. *Cancer Lett,* 324, 171-8.

FERGUSON-SMITH, A. C. (2011) Genomic imprinting: the emergence of an epigenetic paradigm. *Nat Rev Genet,* 12, 565-75.

FERLIN, A., TESSARI, A., GANZ, F., MARCHINA, E., BARLATI, S., GAROLLA, A., ENGL, B. & FORESTA, C. (2005) Association of partial AZFc region deletions with spermatogenic impairment and male infertility. *J Med Genet,* 42, 209-13.

FERNANDEZ, A. F., ASSENOV, Y., MARTIN-SUBERO, J. I., BALINT, B., SIEBERT, R., TANIGUCHI, H., YAMAMOTO, H., HIDALGO, M., TAN, A. C., GALM, O., FERRER, I., SANCHEZ-CESPEDES, M., VILLANUEVA, A., CARMONA, J., SANCHEZ-MUT, J. V., BERDASCO, M., MORENO, V., CAPELLA, G., MONK, D., BALLESTAR, E., ROPERO, S., MARTINEZ, R., SANCHEZ-CARBAYO, M., PROSPER, F., AGIRRE, X., FRAGA, M. F., GRANA, O., PEREZ-JURADO, L., MORA, J., PUIG, S., PRAT, J., BADIMON, L., PUCA, A. A., MELTZER, S. J., LENGAUER, T., BRIDGEWATER, J., BOCK, C. & ESTELLER, M. (2011) A DNA methylation fingerprint of 1628 human samples. *Genome Res,* 22, 407-19.

FIGUEROA, M. E., ABDEL-WAHAB, O., LU, C., WARD, P. S., PATEL, J., SHIH, A., LI, Y., BHAGWAT, N., VASANTHAKUMAR, A., FERNANDEZ, H. F., TALLMAN, M. S., SUN, Z., WOLNIAK, K., PEETERS, J. K., LIU, W., CHOE, S. E., FANTIN, V. R., PAIETTA, E., LOWENBERG, B., LICHT, J. D., GODLEY, L. A., DELWEL, R., VALK, P. J., THOMPSON, C. B., LEVINE, R. L. & MELNICK, A. (2010a) Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. *Cancer Cell,* 18, 553-67.

FIGUEROA, M. E., LUGTHART, S., LI, Y., ERPELINCK-VERSCHUEREN, C., DENG, X., CHRISTOS, P. J., SCHIFANO, E., BOOTH, J., VAN PUTTEN, W., SKRABANEK, L., CAMPAGNE, F., MAZUMDAR, M., GREALLY, J. M., VALK, P. J., LOWENBERG, B., DELWEL, R. & MELNICK, A. (2010b) DNA methylation signatures identify biologically distinct subtypes in acute myeloid leukemia. *Cancer Cell,* 17, 13-27.

FISCHLE, W., DEQUIEDT, F., HENDZEL, M. J., GUENTHER, M. G., LAZAR, M. A., VOELTER, W. & VERDIN, E. (2002) Enzymatic activity associated with class II HDACs is dependent on a multiprotein complex containing HDAC3 and SMRT/N-CoR. *Mol Cell,* 9, 45-57.

FISHER, J. S., MACPHERSON, S., MARCHETTI, N. & SHARPE, R. M. (2003) Human 'testicular dysgenesis syndrome': a possible model using in-utero exposure of the rat to dibutyl phthalate. *Hum Reprod,* 18, 1383-94.

FORDE, N., DUFFY, G. B., MCGETTIGAN, P. A., BROWNE, J. A., MEHTA, J. P., KELLY, A. K., MANSOURI-ATTIA, N., SANDRA, O., LOFTUS, B. J., CROWE, M. A., FAIR, T., ROCHE, J. F., LONERGAN, P. & EVANS, A. C. (2012) Evidence for an early endometrial response to pregnancy in cattle: both dependent upon and independent of interferon tau. *Physiol Genomics,* 44, 799-810.

FORMAN, D., OLIVER, R. T., BRETT, A. R., MARSH, S. G., MOSES, J. H., BODMER, J. G., CHILVERS, C. E. & PIKE, M. C. (1992) Familial testicular cancer: a report of the UK family register, estimation of risk and an HLA class 1 sib-pair analysis. *Br J Cancer,* 65, 255-62.

FOULKS, J. M., PARNELL, K. M., NIX, R. N., CHAU, S., SWIERCZEK, K., SAUNDERS, M., WRIGHT, K., HENDRICKSON, T. F., HO, K. K., MCCULLAR, M. V. & KANNER, S. B. (2012) Epigenetic drug discovery: targeting DNA methyltransferases. *J Biomol Screen,* 17, 2-17.

FRAGA, M. F., BALLESTAR, E., MONTOYA, G., TAYSAVANG, P., WADE, P. A. & ESTELLER, M. (2003) The affinity of different MBD proteins for a specific methylated locus depends on their intrinsic binding properties. *Nucleic Acids Res,* 31, 1765-74.

FRANKEN, N. A., RODERMOND, H. M., STAP, J., HAVEMAN, J. & VAN BREE, C. (2006) Clonogenic assay of cells in vitro. *Nat Protoc,* 1, 2315-9.

FRAZIER, A. L., WELDON, C. & AMATRUDA, J. (2012) Fetal and neonatal germ cell tumors. *Semin Fetal Neonatal Med,* 17, 222-30.

FROMMER, M., MCDONALD, L. E., MILLAR, D. S., COLLIS, C. M., WATT, F., GRIGG, G. W., MOLLOY, P. L. & PAUL, C. L. (1992) A genomic sequencing protocol that yields a

positive display of 5-methylcytosine residues in individual DNA strands. *Proc Natl Acad Sci U S A,* 89, 1827-31.

FUKS, F., HURD, P. J., WOLF, D., NAN, X., BIRD, A. P. & KOUZARIDES, T. (2003) The methyl-CpG-binding protein MeCP2 links DNA methylation to histone methylation. *J Biol Chem,* 278, 4035-40.

FULDA, S. (2009) Caspase-8 in cancer biology and therapy. *Cancer Lett,* 281, 128-33.

FULDA, S., KUFER, M. U., MEYER, E., VAN VALEN, F., DOCKHORN-DWORNICZAK, B. & DEBATIN, K. M. (2001) Sensitization for death receptor- or drug-induced apoptosis by re-expression of caspase-8 through demethylation or gene transfer. *Oncogene,* 20, 5865-77.

FURUKAWA, S., HARUTA, M., ARAI, Y., HONDA, S., OHSHIMA, J., SUGAWARA, W., KAGEYAMA, Y., HIGASHI, Y., NISHIDA, K., TSUNEMATSU, Y., NAKADATE, H., ISHII, M. & KANEKO, Y. (2009) Yolk sac tumor but not seminoma or teratoma is associated with abnormal epigenetic reprogramming pathway and shows frequent hypermethylation of various tumor suppressor genes. *Cancer Sci,* 100, 698-708.

FUSTINO, N., RAKHEJA, D., ATEEK, C. S., NEUMANN, J. C. & AMATRUDA, J. F. (2011) Bone morphogenetic protein signalling activity distinguishes histological subsets of paediatric germ cell tumours. *Int J Androl,* 34, e218-33.

FUTREAL, P. A., LIU, Q., SHATTUCK-EIDENS, D., COCHRAN, C., HARSHMAN, K., TAVTIGIAN, S., BENNETT, L. M., HAUGEN-STRANO, A., SWENSEN, J., MIKI, Y. & ET AL. (1994) BRCA1 mutations in primary breast and ovarian carcinomas. *Science,* 266, 120-2.

GAGNON, J. F., BERNARD, O., VILLENEUVE, L., TETU, B. & GUILLEMETTE, C. (2006) Irinotecan inactivation is modulated by epigenetic silencing of UGT1A1 in colon cancer. *Clin Cancer Res,* 12, 1850-8.

GARDINER-GARDEN, M. & FROMMER, M. (1987) CpG islands in vertebrate genomes. *J Mol Biol,* 196, 261-82.

GARZON, R., LIU, S., FABBRI, M., LIU, Z., HEAPHY, C. E., CALLEGARI, E., SCHWIND, S., PANG, J., YU, J., MUTHUSAMY, N., HAVELANGE, V., VOLINIA, S., BLUM, W., RUSH, L. J., PERROTTI, D., ANDREEFF, M., BLOOMFIELD, C. D., BYRD, J. C., CHAN, K., WU, L. C., CROCE, C. M. & MARCUCCI, G. (2009) MicroRNA-29b induces global DNA hypomethylation and tumor suppressor gene reexpression in acute myeloid leukemia by targeting directly DNMT3A and 3B and indirectly DNMT1. *Blood,* 113, 6411-8.

GASI, D., VAN DER KORPUT, H. A., DOUBEN, H. C., DE KLEIN, A., DE RIDDER, C. M., VAN WEERDEN, W. M. & TRAPMAN, J. (2011) Overexpression of full-length ETV1 transcripts in clinical prostate cancer due to gene translocation. *PLoS One,* 6, e16332.

GAUDET, F., HODGSON, J. G., EDEN, A., JACKSON-GRUSBY, L., DAUSMAN, J., GRAY, J. W., LEONHARDT, H. & JAENISCH, R. (2003) Induction of tumors in mice by genomic hypomethylation. *Science,* 300, 489-92.

GEBHARD, C., BENNER, C., EHRICH, M., SCHWARZFISCHER, L., SCHILLING, E., KLUG, M., DIETMAIER, W., THIEDE, C., HOLLER, E., ANDREESEN, R. & REHLI, M. (2010) General transcription factor binding at CpG islands in normal cells correlates with resistance to de novo DNA methylation in cancer cells. *Cancer Res,* 70, 1398-407.

GEORGE, R. E., LAHTI, J. M., ADAMSON, P. C., ZHU, K., FINKELSTEIN, D., INGLE, A. M., REID, J. M., KRAILO, M., NEUBERG, D., BLANEY, S. M. & DILLER, L. (2010) Phase I study of decitabine with doxorubicin and cyclophosphamide in children with neuroblastoma and other solid tumors: a Children's Oncology Group study. *Pediatr Blood Cancer,* 55, 629-38.

GHOSHAL, K., DATTA, J., MAJUMDER, S., BAI, S., KUTAY, H., MOTIWALA, T. & JACOB, S. T. (2005) 5-Aza-deoxycytidine induces selective degradation of DNA methyltransferase 1

by a proteasomal pathway that requires the KEN box, bromo-adjacent homology domain, and nuclear localization signal. *Mol Cell Biol,* 25, 4727-41.

GICQUEL, C., ROSSIGNOL, S., CABROL, S., HOUANG, M., STEUNOU, V., BARBU, V., DANTON, F., THIBAUD, N., LE MERRER, M., BURGLEN, L., BERTRAND, A. M., NETCHINE, I. & LE BOUC, Y. (2005) Epimutation of the telomeric imprinting center region on chromosome 11p15 in Silver-Russell syndrome. *Nat Genet,* 37, 1003-7.

GILBERT, D., RAPLEY, E. & SHIPLEY, J. (2011) Testicular germ cell tumours: predisposition genes and the male germ cell niche. *Nat,* 11, 278-88. Epub 2011 Mar 17.

GOBEL, U., SCHNEIDER, D. T., CALAMINUS, G., HAAS, R. J., SCHMIDT, P. & HARMS, D. (2000) Germ-cell tumors in childhood and adolescence. GPOH MAKEI and the MAHO study groups. *Ann Oncol,* 11, 263-71.

GOEDERT, J. J., SAUTER, M. E., JACOBSON, L. P., VESSELLA, R. L., HILGARTNER, M. W., LEITMAN, S. F., FRASER, M. C. & MUELLER-LANTZSCH, N. G. (1999) High prevalence of antibodies against HERV-K10 in patients with testicular cancer but not with AIDS. *Cancer Epidemiol Biomarkers Prev,* 8, 293-6.

GOODIER, J. L. & KAZAZIAN, H. H., JR. (2008) Retrotransposons revisited: the restraint and rehabilitation of parasites. *Cell,* 135, 23-35.

GOPALAKRISHNAN, S., VAN EMBURGH, B. O., SHAN, J., SU, Z., FIELDS, C. R., VIEWEG, J., HAMAZAKI, T., SCHWARTZ, P. H., TERADA, N. & ROBERTSON, K. D. (2009) A novel DNMT3B splice variant expressed in tumor and pluripotent cells modulates genomic DNA methylation patterns and displays altered DNA binding. *Mol Cancer Res,* 7, 1622-34.

GOWHER, H. & JELTSCH, A. (2002) Molecular enzymology of the catalytic domains of the Dnmt3a and Dnmt3b DNA methyltransferases. *J Biol Chem,* 277, 20409-14.

GRADY, W. M., WILLIS, J., GUILFORD, P. J., DUNBIER, A. K., TORO, T. T., LYNCH, H., WIESNER, G., FERGUSON, K., ENG, C., PARK, J. G., KIM, S. J. & MARKOWITZ, S. (2000) Methylation of the CDH1 promoter as the second genetic hit in hereditary diffuse gastric cancer. *Nat Genet,* 26, 16-7.

GU, Y., RUNYAN, C., SHOEMAKER, A., SURANI, A. & WYLIE, C. (2009) Steel factor controls primordial germ cell survival and motility from the time of their specification in the allantois, and provides a continuous niche throughout their migration. *Development,* 136, 1295-303.

GUIBERT, S., FORNE, T. & WEBER, M. (2012) Global profiling of DNA methylation erasure in mouse primordial germ cells. *Genome Res,* 22, 633-41.

HACKETT, J. A., ZYLICZ, J. J. & SURANI, M. A. (2012) Parallel mechanisms of epigenetic reprogramming in the germline. *Trends Genet,* 28, 164-74.

HAJKOVA, P., ERHARDT, S., LANE, N., HAAF, T., EL-MAARRI, O., REIK, W., WALTER, J. & SURANI, M. A. (2002) Epigenetic reprogramming in mouse primordial germ cells. *Mech Dev,* 117, 15-23.

HAN, H., CORTEZ, C. C., YANG, X., NICHOLS, P. W., JONES, P. A. & LIANG, G. (2012) DNA methylation directly silences genes with non-CpG island promoters and establishes a nucleosome occupied promoter. *Hum Mol Genet,* 20, 4299-310.

HANSEN, K. H. & HELIN, K. (2009) Epigenetic inheritance through self-recruitment of the polycomb repressive complex 2. *Epigenetics,* 4, 133-8.

HARA, K., KANAI-AZUMA, M., UEMURA, M., SHITARA, H., TAYA, C., YONEKAWA, H., KAWAKAMI, H., TSUNEKAWA, N., KUROHMARU, M. & KANAI, Y. (2009) Evidence for crucial role of hindgut expansion in directing proper migration of primordial germ cells in mouse early embryogenesis. *Dev Biol,* 330, 427-39.

HASLE, H., JACOBSEN, B. B., ASSCHENFELDT, P. & ANDERSEN, K. (1992) Mediastinal germ cell tumour associated with Klinefelter syndrome. A report of case and review of the literature. *Eur J Pediatr,* 151, 735-9.

HASLE, H., MELLEMGAARD, A., NIELSEN, J. & HANSEN, J. (1995) Cancer incidence in men with Klinefelter syndrome. *Br J Cancer,* 71, 416-20.

HATA, K., OKANO, M., LEI, H. & LI, E. (2002) Dnmt3L cooperates with the Dnmt3 family of de novo DNA methyltransferases to establish maternal imprints in mice. *Development,* 129, 1983-93.

HAYATSU, H. (2008) The bisulfite genomic sequencing used in the analysis of epigenetic states, a technique in the emerging environmental genotoxicology research. *Mutat Res,* 659, 77-82.

HEGI, M. E., DISERENS, A. C., GORLIA, T., HAMOU, M. F., DE TRIBOLET, N., WELLER, M., KROS, J. M., HAINFELLNER, J. A., MASON, W., MARIANI, L., BROMBERG, J. E., HAU, P., MIRIMANOFF, R. O., CAIRNCROSS, J. G., JANZER, R. C. & STUPP, R. (2005) MGMT gene silencing and benefit from temozolomide in glioblastoma. *N Engl J Med,* 352, 997-1003.

HELLMAN, A. & CHESS, A. (2007) Gene body-specific methylation on the active X chromosome. *Science,* 315, 1141-3.

HEMMINKI, K. & CHEN, B. (2006) Familial risks in testicular cancer as aetiological clues. *Int J Androl,* 29, 205-10.

HEMMINKI, K. & LI, X. (2002) Cancer risks in second-generation immigrants to Sweden. *Int J Cancer,* 99, 229-37.

HERMANN, A., GOWHER, H. & JELTSCH, A. (2004) Biochemistry and biology of mammalian DNA methyltransferases. *Cell Mol Life Sci,* 61, 2571-87.

HEYN, H. & ESTELLER, M. (2012) DNA methylation profiling in the clinic: applications and challenges. *Nat Rev Genet,* 13, 679-92.

HOEI-HANSEN, C. E., ALMSTRUP, K., NIELSEN, J. E., BRASK SONNE, S., GRAEM, N., SKAKKEBAEK, N. E., LEFFERS, H. & RAJPERT-DE MEYTS, E. (2005) Stem cell pluripotency factor NANOG is expressed in human fetal gonocytes, testicular carcinoma in situ and germ cell tumours. *Histopathology,* 47, 48-56.

HOEI-HANSEN, C. E., NIELSEN, J. E., ALMSTRUP, K., HANSEN, M. A., SKAKKEBAEK, N. E., RAJPERT-DEMEYTS, E. & LEFFERS, H. (2004) Identification of genes differentially expressed in testes containing carcinoma in situ. *Mol Hum Reprod,* 10, 423-31.

HOLLENBACH, P. W., NGUYEN, A. N., BRADY, H., WILLIAMS, M., NING, Y., RICHARD, N., KRUSHEL, L., AUKERMAN, S. L., HEISE, C. & MACBETH, K. J. (2010) A comparison of azacitidine and decitabine activities in acute myeloid leukemia cell lines. *PLoS One,* 5, e9001.

HOLLERAN, J. L., PARISE, R. A., JOSEPH, E., EISEMAN, J. L., COVEY, J. M., GLAZE, E. R., LYUBIMOV, A. V., CHEN, Y. F., D'ARGENIO, D. Z. & EGORIN, M. J. (2005) Plasma pharmacokinetics, oral bioavailability, and interspecies scaling of the DNA methyltransferase inhibitor, zebularine. *Clin Cancer Res,* 11, 3862-8.

HONECKER, F., WERMANN, H., MAYER, F., GILLIS, A. J., STOOP, H., VAN GURP, R. J., OECHSLE, K., STEYERBERG, E., HARTMANN, J. T., DINJENS, W. N., OOSTERHUIS, J. W., BOKEMEYER, C. & LOOIJENGA, L. H. (2009) Microsatellite instability, mismatch repair deficiency, and BRAF mutation in treatment-resistant germ cell tumors. *J Clin Oncol,* 27, 2129-36.

HONORIO, S., AGATHANGGELOU, A., WERNERT, N., ROTHE, M., MAHER, E. R. & LATIF, F. (2003) Frequent epigenetic inactivation of the RASSF1A tumour suppressor gene in testicular tumours and distinct methylation profiles of seminoma and nonseminoma testicular germ cell tumours. *Oncogene,* 22, 461-6.

HORSTHEMKE, B. & WAGSTAFF, J. (2008) Mechanisms of imprinting of the Prader-Willi/Angelman region. *Am J Med Genet A,* 146A, 2041-52.

HORTON, Z., SCHLATTER, M. & SCHULTZ, S. (2007) Pediatric germ cell tumors. *Surg Oncol,* 16, 205-13.

HOULDSWORTH, J., REUTER, V., BOSL, G. J. & CHAGANTI, R. S. (1997) Aberrant expression of cyclin D2 is an early event in human male germ cell tumorigenesis. *Cell Growth Differ,* 8, 293-9.

HOULDSWORTH, J., XIAO, H., MURTY, V. V., CHEN, W., RAY, B., REUTER, V. E., BOSL, G. J. & CHAGANTI, R. S. (1998) Human male germ cell tumor resistance to cisplatin is linked to TP53 gene mutation. *Oncogene,* 16, 2345-9.

HOUSHDARAN, S., HAWLEY, S., PALMER, C., CAMPAN, M., OLSEN, M. N., VENTURA, A. P., KNUDSEN, B. S., DRESCHER, C. W., URBAN, N. D., BROWN, P. O. & LAIRD, P. W. (2010) DNA methylation profiles of ovarian epithelial carcinoma tumors and cell lines. *PLoS One,* 5, e9359.

HOWARD, G., EIGES, R., GAUDET, F., JAENISCH, R. & EDEN, A. (2008) Activation and transposition of endogenous retroviral elements in hypomethylation induced tumors in mice. *Oncogene,* 27, 404-8.

HSIEH, C. L. (2005) The de novo methylation activity of Dnmt3a is distinctly different than that of Dnmt1. *BMC Biochem,* 6, 6.

HULKOWER, K. I. & HERBER, R. L. (2011) Cell Migration and Invasion Assays as Tools for Drug Discovery. *Pharmaceutics,* 3, 107-124.

INAI, H., KAWAI, K., MORISHITA, Y., NAGATA, M., NOGUCHI, M. & AKAZA, H. (2005) Retroperitoneal extragonadal germ cell tumor in a patient with Klinefelter's syndrome. *Int J Urol,* 12, 765-7.

IQBAL, K., JIN, S. G., PFEIFER, G. P. & SZABO, P. E. (2011) Reprogramming of the paternal genome upon fertilization involves genome-wide oxidation of 5-methylcytosine. *Proc Natl Acad Sci U S A,* 108, 3642-7.

IRIZARRY, R. A., LADD-ACOSTA, C., WEN, B., WU, Z., MONTANO, C., ONYANGO, P., CUI, H., GABO, K., RONGIONE, M., WEBSTER, M., JI, H., POTASH, J. B., SABUNCIYAN, S. & FEINBERG, A. P. (2009) The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores. *Nat Genet,* 41, 178-86.

ISSA, J. P. (2004) CpG island methylator phenotype in cancer. *Nat Rev Cancer,* 4, 988-93.

ITMAN, C., MENDIS, S., BARAKAT, B. & LOVELAND, K. L. (2006) All in the family: TGF-beta family action in testis development. *Reproduction,* 132, 233-46.

ITO, S., D'ALESSIO, A. C., TARANOVA, O. V., HONG, K., SOWERS, L. C. & ZHANG, Y. (2010) Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification. *Nature,* 466, 1129-33.

ITZKOWITZ, S., BRAND, R., JANDORF, L., DURKEE, K., MILLHOLLAND, J., RABENECK, L., SCHROY, P. C., 3RD, SONTAG, S., JOHNSON, D., MARKOWITZ, S., PASZAT, L. & BERGER, B. M. (2008) A simplified, noninvasive stool DNA test for colorectal cancer detection. *Am J Gastroenterol,* 103, 2862-70.

JACKSON, K., YU, M. C., ARAKAWA, K., FIALA, E., YOUN, B., FIEGL, H., MULLER-HOLZNER, E., WIDSCHWENDTER, M. & EHRLICH, M. (2004) DNA hypomethylation is prevalent even in low-grade breast cancers. *Cancer Biol Ther,* 3, 1225-31.

JACOBSEN, G. K., HENRIKSEN, O. B. & VON DER MAASE, H. (1981) Carcinoma in situ of testicular tissue adjacent to malignant germ-cell tumors: a study of 105 cases. *Cancer,* 47, 2660-2.

JANE-VALBUENA, J., WIDLUND, H. R., PERNER, S., JOHNSON, L. A., DIBNER, A. C., LIN, W. M., BAKER, A. C., NAZARIAN, R. M., VIJAYENDRAN, K. G., SELLERS, W. R., HAHN, W. C.,

DUNCAN, L. M., RUBIN, M. A., FISHER, D. E. & GARRAWAY, L. A. (2010) An oncogenic role for ETV1 in melanoma. *Cancer Res,* 70, 2075-84.

JENSEN, T. K., VIERULA, M., HJOLLUND, N. H., SAARANEN, M., SCHEIKE, T., SAARIKOSKI, S., SUOMINEN, J., KEISKI, A., TOPPARI, J. & SKAKKEBAEK, N. E. (2000) Semen quality among Danish and Finnish men attempting to conceive. The Danish First Pregnancy Planner Study Team. *Eur J Endocrinol,* 142, 47-52.

JEYAPALAN, J. N., NOOR, D. A., LEE, S. H., TAN, C. L., APPLEBY, V. A., KILDAY, J. P., PALMER, R. D., SCHWALBE, E. C., CLIFFORD, S. C., WALKER, D. A., MURRAY, M. J., COLEMAN, N., NICHOLSON, J. C. & SCOTTING, P. J. (2011) Methylator phenotype of malignant germ cell tumours in children identifies strong candidates for chemotherapy resistance. *Br J Cancer,* 105, 575-85.

JIA, D., JURKOWSKA, R. Z., ZHANG, X., JELTSCH, A. & CHENG, X. (2007) Structure of Dnmt3a bound to Dnmt3L suggests a model for de novo DNA methylation. *Nature,* 449, 248-51.

JIANG, Y., LANGLEY, B., LUBIN, F. D., RENTHAL, W., WOOD, M. A., YASUI, D. H., KUMAR, A., NESTLER, E. J., AKBARIAN, S. & BECKEL-MITCHENER, A. C. (2008) Epigenetics in the nervous system. *J Neurosci,* 28, 11753-9.

JIN, Z., MORI, Y., YANG, J., SATO, F., ITO, T., CHENG, Y., PAUN, B., HAMILTON, J. P., KAN, T., OLARU, A., DAVID, S., AGARWAL, R., ABRAHAM, J. M., BEER, D., MONTGOMERY, E. & MELTZER, S. J. (2007) Hypermethylation of the nel-like 1 gene is a common and early event and is associated with poor prognosis in early-stage esophageal adenocarcinoma. *Oncogene,* 26, 6332-40.

JOHNSON, K. J., ROSS, J. A., POYNTER, J. N., LINABERY, A. M., ROBISON, L. L. & SHU, X. O. (2009) Paediatric germ cell tumours and congenital abnormalities: a Children's Oncology Group study. *Br J Cancer,* 101, 518-21.

JORGENSEN, N., CARLSEN, E., NERMOEN, I., PUNAB, M., SUOMINEN, J., ANDERSEN, A. G., ANDERSSON, A. M., HAUGEN, T. B., HORTE, A., JENSEN, T. K., MAGNUS, O., PETERSEN, J. H., VIERULA, M., TOPPARI, J. & SKAKKEBAEK, N. E. (2002) East-West gradient in semen quality in the Nordic-Baltic area: a study of men from the general population in Denmark, Norway, Estonia and Finland. *Hum Reprod,* 17, 2199-208.

KAIDO, T., SASAOKA, Y., HASHIMOTO, H. & TAIRA, K. (2003) De novo germinoma in the brain in association with Klinefelter's syndrome: case report and review of the literature. *Surg Neurol,* 60, 553-8; discussion 559.

KAMAKURA, Y., HASEGAWA, M., MINAMOTO, T., YAMASHITA, J. & FUJISAWA, H. (2006) C-kit gene mutation: common and widely distributed in intracranial germinomas. *J Neurosurg,* 104, 173-80.

KAMOSHIMA, Y., SAWAMURA, Y., IWASAKI, M., IWASAKI, Y. & SUGIYAMA, K. (2008) Metachronous mature teratoma in the corpus callosum occurring 12 years after a pineal germinoma. *J Neurosurg,* 109, 126-129.

KANEDA, A. & FEINBERG, A. P. (2005) Loss of imprinting of IGF2: a common epigenetic modifier of intestinal tumor risk. *Cancer Res,* 65, 11236-40.

KANEDA, M., OKANO, M., HATA, K., SADO, T., TSUJIMOTO, N., LI, E. & SASAKI, H. (2004) Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting. *Nature,* 429, 900-3.

KANTARJIAN, H., ISSA, J. P., ROSENFELD, C. S., BENNETT, J. M., ALBITAR, M., DIPERSIO, J., KLIMEK, V., SLACK, J., DE CASTRO, C., RAVANDI, F., HELMER, R., 3RD, SHEN, L., NIMER, S. D., LEAVITT, R., RAZA, A. & SABA, H. (2006) Decitabine improves patient outcomes in myelodysplastic syndromes: results of a phase III randomized study. *Cancer,* 106, 1794-803.

KANTARJIAN, H., OKI, Y., GARCIA-MANERO, G., HUANG, X., O'BRIEN, S., CORTES, J., FADERL, S., BUESO-RAMOS, C., RAVANDI, F., ESTROV, Z., FERRAJOLI, A., WIERDA, W., SHAN, J.,

DAVIS, J., GILES, F., SABA, H. I. & ISSA, J. P. (2007) Results of a randomized study of 3 schedules of low-dose decitabine in higher-risk myelodysplastic syndrome and chronic myelomonocytic leukemia. *Blood,* 109, 52-7.

KANTARJIAN, H. M., O'BRIEN, S., CORTES, J., GILES, F. J., FADERL, S., ISSA, J. P., GARCIA-MANERO, G., RIOS, M. B., SHAN, J., ANDREEFF, M., KEATING, M. & TALPAZ, M. (2003) Results of decitabine (5-aza-2'deoxycytidine) therapy in 130 patients with chronic myelogenous leukemia. *Cancer,* 98, 522-8.

KARANIKOLAS, B. D., FIGUEIREDO, M. L. & WU, L. (2010) Comprehensive evaluation of the role of EZH2 in the growth, invasion, and aggression of a panel of prostate cancer cell lines. *Prostate,* 70, 675-88.

KARPF, A. R. & MATSUI, S. (2005) Genetic disruption of cytosine DNA methyltransferase enzymes induces chromosomal instability in human cancer cells. *Cancer Res,* 65, 8635-9.

KATO, N., TAMURA, G., FUKASE, M., SHIBUYA, H. & MOTOYAMA, T. (2003) Hypermethylation of the RUNX3 gene promoter in testicular yolk sac tumor of infants. *Am J Pathol,* 163, 387-91.

KATO, Y., KANEDA, M., HATA, K., KUMAKI, K., HISANO, M., KOHARA, Y., OKANO, M., LI, E., NOZAKI, M. & SASAKI, H. (2007) Role of the Dnmt3 family in de novo methylation of imprinted and repetitive sequences during male germ cell development in the mouse. *Hum Mol Genet,* 16, 2272-80.

KAWAKAMI, T., OKAMOTO, K., KATAOKA, A., KOIZUMI, S., IWAKI, H., SUGIHARA, H., REEVE, A. E., OGAWA, O. & OKADA, Y. (2003a) Multipoint methylation analysis indicates a distinctive epigenetic phenotype among testicular germ cell tumors and testicular malignant lymphomas. *Genes Chromosomes Cancer,* 38, 97-101.

KAWAKAMI, T., OKAMOTO, K., SUGIHARA, H., HATTORI, T., REEVE, A. E., OGAWA, O. & OKADA, Y. (2003b) The roles of supernumerical X chromosomes and XIST expression in testicular germ cell tumors. *J Urol,* 169, 1546-52.

KEMMER, K., CORLESS, C. L., FLETCHER, J. A., MCGREEVEY, L., HALEY, A., GRIFFITH, D., CUMMINGS, O. W., WAIT, C., TOWN, A. & HEINRICH, M. C. (2004) KIT mutations are common in testicular seminomas. *Am J Pathol,* 164, 305-13.

KEMPKENSTEFFEN, C., CHRISTOPH, F., WEIKERT, S., KRAUSE, H., KOLLERMANN, J., SCHOSTAK, M., MILLER, K. & SCHRADER, M. (2006) Epigenetic silencing of the putative tumor suppressor gene testisin in testicular germ cell tumors. *J Cancer Res Clin Oncol,* 132, 765-70.

KESHET, E., LYMAN, S. D., WILLIAMS, D. E., ANDERSON, D. M., JENKINS, N. A., COPELAND, N. G. & PARADA, L. F. (1991) Embryonic RNA expression patterns of the c-kit receptor and its cognate ligand suggest multiple functional roles in mouse development. *EMBO J,* 10, 2425-35.

KIM, J. B., ZAEHRES, H., ARAUZO-BRAVO, M. J. & SCHOLER, H. R. (2009) Generation of induced pluripotent stem cells from neural stem cells. *Nat Protoc,* 4, 1464-70.

KIM, K. H., CHOI, J. S., KIM, I. J., KU, J. L. & PARK, J. G. (2006) Promoter hypomethylation and reactivation of MAGE-A1 and MAGE-A3 genes in colorectal cancer cell lines and cancer tissues. *World J Gastroenterol,* 12, 5651-7.

KIM, P. K., MAHIDHARA, R. & SEOL, D. W. (2001) The role of caspase-8 in resistance to cancer chemotherapy. *Drug Resist Updat,* 4, 293-6.

KIM, S. H., BAE, S. I., LEE, H. S. & KIM, W. H. (2003) Alteration of O6-methylguanine-DNA methyltransferase in colorectal neoplasms in sporadic and familial adenomatous polyposis patients. *Mol Carcinog,* 37, 32-8.

KLOSE, R. J. & BIRD, A. P. (2006) Genomic DNA methylation: the mark and its mediators. *Trends Biochem Sci,* 31, 89-97.

KLOSE, R. J., SARRAF, S. A., SCHMIEDEBERG, L., MCDERMOTT, S. M., STANCHEVA, I. & BIRD, A. P. (2005) DNA binding selectivity of MeCP2 due to a requirement for A/T sequences adjacent to methyl-CpG. *Mol Cell,* 19, 667-78.

KNEIP, C., SCHMIDT, B., SEEGEBARTH, A., WEICKMANN, S., FLEISCHHACKER, M., LIEBENBERG, V., FIELD, J. K. & DIETRICH, D. (2011) SHOX2 DNA methylation is a biomarker for the diagnosis of lung cancer in plasma. *J Thorac Oncol,* 6, 1632-8.

KNUDSON, A. G. (2001) Two genetic hits (more or less) to cancer. *Nat Rev Cancer,* 1, 157-62.

KOBERLE, B., TOMICIC, M. T., USANOVA, S. & KAINA, B. (2010) Cisplatin resistance: preclinical findings and clinical implications. *Biochim Biophys Acta,* 1806, 172-82.

KOHDA, M., HOSHIYA, H., KATOH, M., TANAKA, I., MASUDA, R., TAKEMURA, T., FUJIWARA, M. & OSHIMURA, M. (2001) Frequent loss of imprinting of IGF2 and MEST in lung adenocarcinoma. *Mol Carcinog,* 31, 184-91.

KORKOLA, J. E., HOULDSWORTH, J., CHADALAVADA, R. S., OLSHEN, A. B., DOBRZYNSKI, D., REUTER, V. E., BOSL, G. J. & CHAGANTI, R. S. (2006) Down-regulation of stem cell genes, including those in a 200-kb gene cluster at 12p13.31, is associated with in vivo differentiation of human male germ cell tumors. *Cancer Res,* 66, 820-7.

KORKOLA, J. E., HOULDSWORTH, J., DOBRZYNSKI, D., OLSHEN, A. B., REUTER, V. E., BOSL, G. J. & CHAGANTI, R. S. (2005) Gene expression-based classification of nonseminomatous male germ cell tumors. *Oncogene,* 24, 5101-7.

KOUL, S., HOULDSWORTH, J., MANSUKHANI, M. M., DONADIO, A., MCKIERNAN, J. M., REUTER, V. E., BOSL, G. J., CHAGANTI, R. S. & MURTY, V. V. (2002) Characteristic promoter hypermethylation signatures in male germ cell tumors. *Mol Cancer,* 1, 8.

KOUL, S., MCKIERNAN, J. M., NARAYAN, G., HOULDSWORTH, J., BACIK, J., DOBRZYNSKI, D. L., ASSAAD, A. M., MANSUKHANI, M., REUTER, V. E., BOSL, G. J., CHAGANTI, R. S. & MURTY, V. V. (2004) Role of promoter hypermethylation in Cisplatin treatment response of male germ cell tumors. *Mol Cancer,* 3, 16.

KRAGGERUD, S. M., SKOTHEIM, R. I., SZYMANSKA, J., EKNAES, M., FOSSA, S. D., STENWIG, A. E., PELTOMAKI, P. & LOTHE, R. A. (2002) Genome profiles of familial/bilateral and sporadic testicular germ cell tumors. *Genes Chromosomes Cancer,* 34, 168-74.

KRISTENSEN, D. M., SONNE, S. B., OTTESEN, A. M., PERRETT, R. M., NIELSEN, J. E., ALMSTRUP, K., SKAKKEBAEK, N. E., LEFFERS, H. & RAJPERT-DE MEYTS, E. (2008) Origin of pluripotent germ cell tumours: the role of microenvironment during embryonic development. *Mol Cell Endocrinol,* 288, 111-8.

KUDO, Y., TATEISHI, K., YAMAMOTO, K., YAMAMOTO, S., ASAOKA, Y., IJICHI, H., NAGAE, G., YOSHIDA, H., ABURATANI, H. & KOIKE, K. (2012) Loss of 5-hydroxymethylcytosine is accompanied with malignant cellular transformation. *Cancer Sci,* 103, 670-6.

KULAK, M. V., CYR, A. R., WOODFIELD, G. W., BOGACHEK, M., SPANHEIMER, P. M., LI, T., PRICE, D. H., DOMANN, F. E. & WEIGEL, R. J. (2012) Transcriptional regulation of the GPX1 gene by TFAP2C and aberrant CpG methylation in human breast cancer. *Oncogene.*

KURIMOTO, K., YAMAJI, M., SEKI, Y. & SAITOU, M. (2008) Specification of the germ cell lineage in mice: a process orchestrated by the PR-domain proteins, Blimp1 and Prdm14. *Cell Cycle,* 7, 3514-8.

KUSANO, M., TOYOTA, M., SUZUKI, H., AKINO, K., AOKI, F., FUJITA, M., HOSOKAWA, M., SHINOMURA, Y., IMAI, K. & TOKINO, T. (2006) Genetic, epigenetic, and clinicopathologic features of gastric carcinomas with the CpG island methylator phenotype and an association with Epstein-Barr virus. *Cancer,* 106, 1467-79.

LANDOLIN, J. M., JOHNSON, D. S., TRINKLEIN, N. D., ALDRED, S. F., MEDINA, C., SHULHA, H., WENG, Z. & MYERS, R. M. (2010) Sequence features that drive human promoter function and tissue specificity. *Genome Res,* 20, 890-8.

LANGE, U. C., ADAMS, D. J., LEE, C., BARTON, S., SCHNEIDER, R., BRADLEY, A. & SURANI, M. A. (2008) Normal germ line establishment in mice carrying a deletion of the Ifitm/Fragilis gene family cluster. *Mol Cell Biol,* 28, 4688-96.

LEE, H. S., KIM, B. H., CHO, N. Y., YOO, E. J., CHOI, M., SHIN, S. H., JANG, J. J., SUH, K. S., KIM, Y. S. & KANG, G. H. (2009) Prognostic implications of and relationship between CpG island hypermethylation and repetitive DNA hypomethylation in hepatocellular carcinoma. *Clin Cancer Res,* 15, 812-20.

LEE, J. T., DAVIDOW, L. S. & WARSHAWSKY, D. (1999) Tsix, a gene antisense to Xist at the X-inactivation centre. *Nat Genet,* 21, 400-4.

LEE, M. G., VILLA, R., TROJER, P., NORMAN, J., YAN, K. P., REINBERG, D., DI CROCE, L. & SHIEKHATTAR, R. (2007) Demethylation of H3K27 regulates polycomb recruitment and H2A ubiquitination. *Science,* 318, 447-50.

LEE, S. H., APPLEBY, V., JEYAPALAN, J. N., PALMER, R. D., NICHOLSON, J. C., SOTTILE, V., GAO, E., COLEMAN, N. & SCOTTING, P. J. (2011) Variable methylation of the imprinted gene, SNRPN, supports a relationship between intracranial germ cell tumours and neural stem cells. *J Neurooncol,* 101, 419-28.

LEE, S. H., JEYAPALAN, J. N., APPLEBY, V., MOHAMED NOOR, D. A., SOTTILE, V. & SCOTTING, P. J. (2010) Dynamic methylation and expression of Oct4 in early neural stem cells. *J Anat,* 217, 203-13.

LEE, T. I., JENNER, R. G., BOYER, L. A., GUENTHER, M. G., LEVINE, .S. S., KUMAR, R. M., CHEVALIER, B., JOHNSTONE, S. E., COLE, M. F., ISONO, K., KOSEKI, H., FUCHIKAMI, T., ABE, K., MURRAY, H. L., ZUCKER, J. P., YUAN, B., BELL, G. W., HERBOLSHEIMER, E., HANNETT, N. M., SUN, K., ODOM, D. T., OTTE, A. P., VOLKERT, T. L., BARTEL, D. P., MELTON, D. A., GIFFORD, D. K., JAENISCH, R. & YOUNG, R. A. (2006) Control of developmental regulators by Polycomb in human embryonic stem cells. *Cell,* 125, 301-13.

LEONHARDT, H., PAGE, A. W., WEIER, H. U. & BESTOR, T. H. (1992) A targeting sequence directs DNA methyltransferase to sites of DNA replication in mammalian nuclei. *Cell,* 71, 865-73.

LI, E., BEARD, C. & JAENISCH, R. (1993) Role for DNA methylation in genomic imprinting. *Nature,* 366, 362-5.

LI, E., BESTOR, T. H. & JAENISCH, R. (1992) Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell,* 69, 915-26.

LI, H., BITLER, B. G., VATHIPADIEKAL, V., MARADEO, M. E., SLIFKER, M., CREASY, C. L., TUMMINO, P. J., CAIRNS, P., BIRRER, M. J. & ZHANG, R. (2012) ALDH1A1 is a novel EZH2 target gene in epithelial ovarian cancer identified by genome-wide approaches. *Cancer,* 5, 484-91. Epub 2011 Dec 5.

LI, J. Y., LEES-MURDOCK, D. J., XU, G. L. & WALSH, C. P. (2004) Timing of establishment of paternal methylation imprints in the mouse. *Genomics,* 84, 952-60.

LIAN, C. G., XU, Y., CEOL, C., WU, F., LARSON, A., DRESSER, K., XU, W., TAN, L., HU, Y., ZHAN, Q., LEE, C. W., HU, D., LIAN, B. Q., KLEFFEL, S., YANG, Y., NEISWENDER, J., KHORASANI, A. J., FANG, R., LEZCANO, C., DUNCAN, L. M., SCOLYER, R. A., THOMPSON, J. F., KAKAVAND, H., HOUVRAS, Y., ZON, L. I., MIHM, M. C., JR., KAISER, U. B., SCHATTON, T., WODA, B. A., MURPHY, G. F. & SHI, Y. G. (2012) Loss of 5-hydroxymethylcytosine is an epigenetic hallmark of melanoma. *Cell,* 150, 1135-46.

LIM, H. W., IWATANI, M., HATTORI, N., TANAKA, S., YAGI, S. & SHIOTA, K. (2010) Resistance to 5-aza-2'-deoxycytidine in genic regions compared to non-genic repetitive sequences. *J Reprod Dev,* 56, 86-93.

LIM, M. L., VASSILIEV, I., RICHINGS, N. M., FIRSOVA, A. B., ZHANG, C. & VERMA, P. J. (2011) A novel, efficient method to derive bovine and mouse embryonic stem cells with in vivo differentiation potential by treatment with 5-azacytidine. *Theriogenology,* 76, 133-42.

LIN, J., GILBERT, J., RUDEK, M. A., ZWIEBEL, J. A., GORE, S., JIEMJIT, A., ZHAO, M., BAKER, S. D., AMBINDER, R. F., HERMAN, J. G., DONEHOWER, R. C. & CARDUCCI, M. A. (2009) A phase I dose-finding study of 5-azacytidine in combination with sodium phenylbutyrate in patients with refractory solid tumors. *Clin Cancer Res,* 15, 6241-9.

LIN, L., WANG, Z., PRESCOTT, M. S., VAN DEKKEN, H., THOMAS, D. G., GIORDANO, T. J., CHANG, A. C., ORRINGER, M. B., GRUBER, S. B., MORAN, J. V., GLOVER, T. W. & BEER, D. G. (2006) Multiple forms of genetic instability within a 2-Mb chromosomal segment of 3q26.3-q27 are associated with development of esophageal adenocarcinoma. *Genes Chromosomes Cancer,* 45, 319-31.

LIND, G. E., SKOTHEIM, R. I., FRAGA, M. F., ABELER, V. M., ESTELLER, M. & LOTHE, R. A. (2006) Novel epigenetically deregulated genes in testicular cancer include homeobox genes and SCGB3A1 (HIN-1). *J Pathol,* 210, 441-9.

LIND, G. E., SKOTHEIM, R. I. & LOTHE, R. A. (2007) The epigenome of testicular germ cell tumors. *APMIS,* 115, 1147-60.

LIU, Y., OAKELEY, E. J., SUN, L. & JOST, J. P. (1998) Multiple domains are involved in the targeting of the mouse DNA methyltransferase to the DNA replication foci. *Nucleic Acids Res,* 26, 1038-45.

LOOIJENGA, L. H. (2009) Human testicular (non)seminomatous germ cell tumours: the clinical implications of recent pathobiological insights. *J Pathol,* 218, 146-62.

LOOIJENGA, L. H., DE MUNNIK, H. & OOSTERHUIS, J. W. (1999) A molecular model for the development of germ cell cancer. *Int J Cancer,* 83, 809-14.

LOOIJENGA, L. H., GILLIS, A. J., VAN GURP, R. J., VERKERK, A. J. & OOSTERHUIS, J. W. (1997) X inactivation in human testicular tumors. XIST expression and androgen receptor methylation status. *Am J Pathol,* 151, 581-90.

LOOIJENGA, L. H., HERSMUS, R., OOSTERHUIS, J. W., COOLS, M., DROP, S. L. & WOLFFENBUTTEL, K. P. (2007) Tumor risk in disorders of sex development (DSD). *Best Pract Res Clin Endocrinol Metab,* 21, 480-95.

LOOIJENGA, L. H., ROSENBERG, C., VAN GURP, R. J., GEELEN, E., VAN ECHTEN-ARENDS, J., DE JONG, B., MOSTERT, M. & WOLTER OOSTERHUIS, J. (2000) Comparative genomic hybridization of microdissected samples from different stages in the development of a seminoma and a non-seminoma. *J Pathol,* 191, 187-92.

LOOIJENGA, L. H., ZAFARANA, G., GRYGALEWICZ, B., SUMMERSGILL, B., DEBIEC-RYCHTER, M., VELTMAN, J., SCHOENMAKERS, E. F., RODRIGUEZ, S., JAFER, O., CLARK, J., VAN KESSEL, A. G., SHIPLEY, J., VAN GURP, R. J., GILLIS, A. J. & OOSTERHUIS, J. W. (2003) Role of gain of 12p in germ cell tumour development. *APMIS,* 111, 161-71; discussion 172-3.

LORIOT, A., DE PLAEN, E., BOON, T. & DE SMET, C. (2006) Transient down-regulation of DNMT1 methyltransferase leads to activation and stable hypomethylation of MAGE-A1 in melanoma cells. *J Biol Chem,* 281, 10118-26.

LUBBERT, M., SUCIU, S., BAILA, L., RUTER, B. H., PLATZBECKER, U., GIAGOUNIDIS, A., SELLESLAG, D., LABAR, B., GERMING, U., SALIH, H. R., BEELDENS, F., MUUS, P., PFLUGER, K. H., COENS, C., HAGEMEIJER, A., ECKART SCHAEFER, H., GANSER, A., AUL, C., DE WITTE, T. & WIJERMANS, P. W. (2008) Low-dose decitabine versus best supportive care in elderly patients with intermediate- or high-risk myelodysplastic syndrome (MDS) ineligible for intensive chemotherapy: final results of the randomized phase III study of the European Organisation for Research and Treatment of Cancer Leukemia Group and the German MDS Study Group. *J Clin Oncol,* 29, 1987-96.

LUCIFERO, D., MERTINEIT, C., CLARKE, H. J., BESTOR, T. H. & TRASLER, J. M. (2002) Methylation dynamics of imprinted genes in mouse germ cells. *Genomics,* 79, 530-8.

LUTKE HOLZIK, M. F., RAPLEY, E. A., HOEKSTRA, H. J., SLEIJFER, D. T., NOLTE, I. M. & SIJMONS, R. H. (2004) Genetic predisposition to testicular germ-cell tumours. *Lancet Oncol,* 5, 363-71.

LYNCH, J., KELLER, M., GUO, R. J., YANG, D. & TRABER, P. (2003) Cdx1 inhibits the proliferation of human colon cancer cells by reducing cyclin D1 gene expression. *Oncogene,* 22, 6395-407.

LYNCH, J., SUH, E. R., SILBERG, D. G., RULYAK, S., BLANCHARD, N. & TRABER, P. G. (2000) The caudal-related homeodomain protein Cdx1 inhibits proliferation of intestinal epithelial cells by down-regulation of D-type cyclins. *J Biol Chem,* 275, 4499-506.

LYON, M. F. (1999) X-chromosome inactivation. *Curr Biol,* 9, R235-7.

LYON, M. F. (2002) X-chromosome inactivation and human genetic disease. *Acta Paediatr Suppl,* 91, 107-12.

LYONS, R. M., COSGRIFF, T. M., MODI, S. S., GERSH, R. H., HAINSWORTH, J. D., COHN, A. L., MCINTYRE, H. J., FERNANDO, I. J., BACKSTROM, J. T. & BEACH, C. L. (2009) Hematologic response to three alternative dosing schedules of azacitidine in patients with myelodysplastic syndromes. *J Clin Oncol,* 27, 1850-6.

MAELFAIT, J. & BEYAERT, R. (2008) Non-apoptotic functions of caspase-8. *Biochem Pharmacol,* 76, 1365-73.

MAHAKALI ZAMA, A., HUDSON, F. P. R. & BEDELL, M. A. (2005) Analysis of hypomorphic KitISl mutants suggests different requirements for KITL in proliferation and migration of mouse primordial germ cells. *Biol Reprod,* 73, 639-47.

MAIER, S., NIMMRICH, I., KOENIG, T., EPPENBERGER-CASTORI, S., BOHLMANN, I., PARADISO, A., SPYRATOS, F., THOMSSEN, C., MUELLER, V., NAHRIG, J., SCHITTULLI, F., KATES, R., LESCHE, R., SCHWOPE, I., KLUTH, A., MARX, A., MARTENS, J. W., FOEKENS, J. A., SCHMITT, M. & HARBECK, N. (2007) DNA-methylation of the homeodomain transcription factor PITX2 reliably predicts risk of distant disease recurrence in tamoxifen-treated, node-negative breast cancer patients--Technical and clinical validation in a multi-centre setting in collaboration with the European Organisation for Research and Treatment of Cancer (EORTC) PathoBiology group. *Eur J Cancer,* 43, 1679-86.

MANTON, K. J., DOUGLAS, M. L., NETZEL-ARNETT, S., FITZPATRICK, D. R., NICOL, D. L., BOYD, A. W., CLEMENTS, J. A. & ANTALIS, T. M. (2005) Hypermethylation of the 5' CpG island of the gene encoding the serine protease Testisin promotes its loss in testicular tumorigenesis. *Br J Cancer,* 92, 760-9.

MARCUCCI, G., MAHARRY, K., WU, Y. Z., RADMACHER, M. D., MROZEK, K., MARGESON, D., HOLLAND, K. B., WHITMAN, S. P., BECKER, H., SCHWIND, S., METZELER, K. H., POWELL, B. L., CARTER, T. H., KOLITZ, J. E., WETZLER, M., CARROLL, A. J., BAER, M. R., CALIGIURI, M. A., LARSON, R. A. & BLOOMFIELD, C. D. (2010) IDH1 and IDH2 gene mutations identify novel molecular subsets within de novo cytogenetically normal acute myeloid leukemia: a Cancer and Leukemia Group B study. *J Clin Oncol,* 28, 2348-55.

MARGOT, J. B., AGUIRRE-ARTETA, A. M., DI GIACCO, B. V., PRADHAN, S., ROBERTS, R. J., CARDOSO, M. C. & LEONHARDT, H. (2000) Structure and function of the mouse DNA methyltransferase gene: Dnmt1 shows a tripartite structure. *J Mol Biol,* 297, 293-300.

MARGOT, J. B., CARDOSO, M. C. & LEONHARDT, H. (2001) Mammalian DNA methyltransferases show different subnuclear distributions. *J Cell Biochem,* 83, 373-9.

MARGOT, J. B., EHRENHOFER-MURRAY, A. E. & LEONHARDT, H. (2003) Interactions within the mammalian DNA methyltransferase family. *BMC Mol Biol,* 4, 7.

MARGUERON, R. & REINBERG, D. (2010) Chromatin structure and the inheritance of epigenetic information. *Nat Rev Genet,* **11,** 285-96.

MARSIT, C. J., HOUSEMAN, E. A., CHRISTENSEN, B. C., EDDY, K., BUENO, R., SUGARBAKER, D. J., NELSON, H. H., KARAGAS, M. R. & KELSEY, K. T. (2006) Examination of a CpG island methylator phenotype and implications of methylation profiles in solid tumors. *Cancer Res,* 66, 10621-9.

MARTIN-PEREZ, D., SANCHEZ, E., MAESTRE, L., SUELA, J., VARGIU, P., DI LISIO, L., MARTINEZ, N., ALVES, J., PIRIS, M. A. & SANCHEZ-BEATO, M. (2010) Deregulated expression of the polycomb-group protein SUZ12 target genes characterizes mantle cell lymphoma. *Am J Pathol,* 177, 930-42.

MARTIN-SUBERO, J. I., AMMERPOHL, O., BIBIKOVA, M., WICKHAM-GARCIA, E., AGIRRE, X., ALVAREZ, S., BRUGGEMANN, M., BUG, S., CALASANZ, M. J., DECKERT, M., DREYLING, M., DU, M. Q., DURIG, J., DYER, M. J., FAN, J. B., GESK, S., HANSMANN, M. L., HARDER, L., HARTMANN, S., KLAPPER, W., KUPPERS, R., MONTESINOS-RONGEN, M., NAGEL, I., POTT, C., RICHTER, J., ROMAN-GOMEZ, J., SEIFERT, M., STEIN, H., SUELA, J., TRUMPER, L., VATER, I., PROSPER, F., HAFERLACH, C., CRUZ CIGUDOSA, J. & SIEBERT, R. (2009) A comprehensive microarray-based DNA methylation study of 367 hematological neoplasms. *PLoS One,* 4, e6986.

MARTIN, C. & ZHANG, Y. (2005) The diverse functions of histone lysine methylation. *Nat Rev Mol Cell Biol,* 6, 838-49.

MARTINEZ, J. G., PEREZ-ESCUREDO, J., CASTRO-SANTOS, P., MARCOS, C. A., PENDAS, J. L., FRAGA, M. F. & HERMSEN, M. A. (2012) Hypomethylation of LINE-1, and not centromeric SAT-alpha, is associated with centromeric instability in head and neck squamous cell carcinoma. *Cell Oncol (Dordr),* 35, 259-67.

MARTINEZ, R., MARTIN-SUBERO, J. I., ROHDE, V., KIRSCH, M., ALAMINOS, M., FERNANDEZ, A. F., ROPERO, S., SCHACKERT, G. & ESTELLER, M. (2009) A microarray-based DNA methylation study of glioblastoma multiforme. *Epigenetics,* 4, 255-64.

MASQUE-SOLER, N., SZCZEPANOWSKI, M., LEUSCHNER, I., VOKUHL, C., HAAG, J., CALAMINUS, G. & KLAPPER, W. (2012) Absence of BRAF mutation in pediatric and adolescent germ cell tumors indicate biological differences to adult tumors. *Pediatr Blood Cancer,* 59, 732-5.

MASUMOTO, J., DOWDS, T. A., SCHANER, P., CHEN, F. F., OGURA, Y., LI, M., ZHU, L., KATSUYAMA, T., SAGARA, J., TANIGUCHI, S., GUMUCIO, D. L., NUNEZ, G. & INOHARA, N. (2003) ASC is an activating adaptor for NF-kappa B and caspase-8-dependent apoptosis. *Biochem Biophys Res Commun,* 303, 69-73.

MASUMOTO, J., TANIGUCHI, S., AYUKAWA, K., SARVOTHAM, H., KISHINO, T., NIIKAWA, N., HIDAKA, E., KATSUYAMA, T., HIGUCHI, T. & SAGARA, J. (1999) ASC, a novel 22-kDa protein, aggregates during apoptosis of human promyelocytic leukemia HL-60 cells. *J Biol Chem,* 274, 33835-8.

MCCABE, M. T., BRANDES, J. C. & VERTINO, P. M. (2009) Cancer DNA methylation: molecular mechanisms and clinical implications. *Clin Cancer Res,* 15, 3927-37.

MCCABE, M. T., DAVIS, J. N. & DAY, M. L. (2005) Regulation of DNA methyltransferase 1 by the pRb/E2F1 pathway. *Cancer Res,* 65, 3624-32.

MCRONALD, F. E., MORRIS, M. R., GENTLE, D., WINCHESTER, L., BABAN, D., RAGOUSSIS, J., CLARKE, N. W., BROWN, M. D., KISHIDA, T., YAO, M., LATIF, F. & MAHER, E. R. (2009) CpG methylation profiling in VHL related and VHL unrelated renal cell carcinoma. *Mol Cancer,* 8, 31.

MERAJVER, S. D., PHAM, T. M., CADUFF, R. F., CHEN, M., POY, E. L., COONEY, K. A., WEBER, B. L., COLLINS, F. S., JOHNSTON, C. & FRANK, T. S. (1995) Somatic mutations in the BRCA1 gene in sporadic ovarian tumours. *Nat Genet,* 9, 439-43.

METZGER, E., WISSMANN, M., YIN, N., MULLER, J. M., SCHNEIDER, R., PETERS, A. H., GUNTHER, T., BUETTNER, R. & SCHULE, R. (2005) LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription. *Nature,* 437, 436-9.

MIKESKA, T., BOCK, C., DO, H. & DOBROVIC, A. (2012) DNA methylation biomarkers in cancer: progress towards clinical implementation. *Expert Rev Mol Diagn,* 12, 473-87.

MILANI, L., LUNDMARK, A., KIIALAINEN, A., NORDLUND, J., FLAEGSTAD, T., FORESTIER, E., HEYMAN, M., JONMUNDSSON, G., KANERVA, J., SCHMIEGELOW, K., SODERHALL, S., GUSTAFSSON, M. G., LONNERHOLM, G. & SYVANEN, A. C. (2010) DNA methylation for subtype classification and prediction of treatment outcome in patients with childhood acute lymphoblastic leukemia. *Blood,* 115, 1214-25.

MILDE, T., OEHME, I., KORSHUNOV, A., KOPP-SCHNEIDER, A., REMKE, M., NORTHCOTT, P., DEUBZER, H. E., LODRINI, M., TAYLOR, M. D., VON DEIMLING, A., PFISTER, S. & WITT, O. (2010) HDAC5 and HDAC9 in medulloblastoma: novel markers for risk stratification and role in tumor cell growth. *Clin Cancer Res,* 16, 3240-52.

MILIA-ARGEITI, E., HUET, E., LABROPOULOU, V. T., MOURAH, S., FENICHEL, P., KARAMANOS, N. K., MENASHI, S. & THEOCHARIS, A. D. (2012) Imbalance of MMP-2 and MMP-9 expression versus TIMP-1 and TIMP-2 reflects increased invasiveness of human testicular germ cell tumours. *Int J Androl,* 35, 835-44.

MIMS, A., WALKER, A. R., HUANG, X., SUN, J., WANG, H., SANTHANAM, R., DORRANCE, A. M., WALKER, C., HOELLERBAUER, P., TARIGHAT, S. S., CHAN, K. K., KLISOVIC, R. B., PERROTTI, D., CALIGIURI, M. A., BYRD, J. C., CHEN, C. S., JAMES LEE, L., JACOB, S., MROZEK, K., BLOOMFIELD, C. D., BLUM, W., GARZON, R., SCHWIND, S. & MARCUCCI, G. (2012) Increased anti-leukemic activity of decitabine via AR-42-induced upregulation of miR-29b: a novel epigenetic-targeting approach in acute myeloid leukemia. *Leukemia.*

MIZUNO, Y. G., A., GOTOH, A., KAMIDONO, S. & KITAZAWA, S. (1993) [Establishment and characterization of a new human testicular germ cell tumor cell line (TCam-2)]. *Nihon Hinyokika Gakkai Zasshi.*

MOHANDAS, T., SPARKES, R. S. & SHAPIRO, L. J. (1981) Reactivation of an inactive human X chromosome: evidence for X inactivation by DNA methylation. *Science,* 211, 393-6.

MOLYNEAUX, K. A., STALLOCK, J., SCHAIBLE, K. & WYLIE, C. (2001) Time-lapse analysis of living mouse germ cell migration. *Dev Biol,* 240, 488-98.

MORENO, D. A., SCRIDELI, C. A., CORTEZ, M. A. A., DE PAULA QUEIROZ, R., VALERA, E. T., DA SILVA SILVEIRA, V., YUNES, J. A., BRANDALISE, S. R. & TONE, L. G. (2010) research paper: Differential expression of HDAC3, HDAC7 and HDAC9 is associated with prognosis and survival in childhood acute lymphoblastic leukaemia. *British Journal of Haematology,* 150, 665-673.

MOSTERT, M., ROSENBERG, C., STOOP, H., SCHUYER, M., TIMMER, A., OOSTERHUIS, W. & LOOIJENGA, L. (2000) Comparative genomic and in situ hybridization of germ cell tumors of the infantile testis. *Lab Invest,* 80, 1055-64.

MULERO-NAVARRO, S., CARVAJAL-GONZALEZ, J. M., HERRANZ, M., BALLESTAR, E., FRAGA, M. F., ROPERO, S., ESTELLER, M. & FERNANDEZ-SALGUERO, P. M. (2006) The dioxin receptor is silenced by promoter hypermethylation in human acute lymphoblastic leukemia through inhibition of Sp1 binding. *Carcinogenesis,* 27, 1099-104.

MUND, C., HACKANSON, B., STRESEMANN, C., LUBBERT, M. & LYKO, F. (2005) Characterization of DNA demethylation effects induced by 5-Aza-2'-deoxycytidine in patients with myelodysplastic syndrome. *Cancer Res,* 65, 7086-90.

MURRAY, M. J. & NICHOLSON, J. C. (2009) Germ cell tumours in children and adolescents. *Paediatrics and Child Health,* 20, 109-116.

MURRAY, M. J., SAINI, H. K., VAN DONGEN, S., PALMER, R. D., MURALIDHAR, B., PETT, M. R., PIIPARI, M., THORNTON, C. M., NICHOLSON, J. C., ENRIGHT, A. J. & COLEMAN, N. (2010) The two most common histological subtypes of malignant germ cell tumour are distinguished by global microRNA profiles, associated with differential transcription factor expression. *Mol Cancer,* 9, 290.

NAKAYAMA, M., BENNETT, C. J., HICKS, J. L., EPSTEIN, J. I., PLATZ, E. A., NELSON, W. G. & DE MARZO, A. M. (2003) Hypermethylation of the human glutathione S-transferase-pi gene (GSTP1) CpG island is present in a subset of proliferative inflammatory atrophy lesions but not in normal or hyperplastic epithelium of the prostate: a detailed study using laser-capture microdissection. *Am J Pathol,* 163, 923-33.

NATHANSON, K. L., KANETSKY, P. A., HAWES, R., VAUGHN, D. J., LETRERO, R., TUCKER, K., FRIEDLANDER, M., PHILLIPS, K. A., HOGG, D., JEWETT, M. A., LOHYNSKA, R., DAUGAARD, G., RICHARD, S., CHOMPRET, A., BONAITI-PELLIE, C., HEIDENREICH, A., OLAH, E., GECZI, L., BODROGI, I., ORMISTON, W. J., DALY, P. A., OOSTERHUIS, J. W., GILLIS, A. J., LOOIJENGA, L. H., GUILFORD, P., FOSSA, S. D., HEIMDAL, K., TJULANDIN, S. A., LIUBCHENKO, L., STOLL, H., WEBER, W., RUDD, M., HUDDART, R., CROCKFORD, G. P., FORMAN, D., OLIVER, D. T., EINHORN, L., WEBER, B. L., KRAMER, J., MCMASTER, M., GREENE, M. H., PIKE, M., CORTESSIS, V., CHEN, C., SCHWARTZ, S. M., BISHOP, D. T., EASTON, D. F., STRATTON, M. R. & RAPLEY, E. A. (2005) The Y deletion gr/gr and susceptibility to testicular germ cell tumor. *Am J Hum Genet,* 77, 1034-43.

NETTERSHEIM, D., GILLIS, A. J., LOOIJENGA, L. H. & SCHORLE, H. (2011) TGF-beta1, EGF and FGF4 synergistically induce differentiation of the seminoma cell line TCam-2 into a cell type resembling mixed non-seminoma. *Int J Androl,* 34, e189-203.

NETTO, G. J., NAKAI, Y., NAKAYAMA, M., JADALLAH, S., TOUBAJI, A., NONOMURA, N., ALBADINE, R., HICKS, J. L., EPSTEIN, J. I., YEGNASUBRAMANIAN, S., NELSON, W. G. & DE MARZO, A. M. (2008) Global DNA hypomethylation in intratubular germ cell neoplasia and seminoma, but not in nonseminomatous male germ cell tumors. *Mod Pathol,* 21, 1337-44.

NIMMRICH, I., SIEUWERTS, A. M., MEIJER-VAN GELDER, M. E., SCHWOPE, I., BOLT-DE VRIES, J., HARBECK, N., KOENIG, T., HARTMANN, O., KLUTH, A., DIETRICH, D., MAGDOLEN, V., PORTENGEN, H., LOOK, M. P., KLIJN, J. G., LESCHE, R., SCHMITT, M., MAIER, S., FOEKENS, J. A. & MARTENS, J. W. (2008) DNA hypermethylation of PITX2 is a marker of poor prognosis in untreated lymph node-negative hormone receptor-positive breast cancer patients. *Breast Cancer Res Treat,* 111, 429-37.

NISHIGAKI, M., AOYAGI, K., DANJOH, I., FUKAYA, M., YANAGIHARA, K., SAKAMOTO, H., YOSHIDA, T. & SASAKI, H. (2005) Discovery of aberrant expression of R-RAS by cancer-linked DNA hypomethylation in gastric cancer using microarrays. *Cancer Res,* 65, 2115-24.

NOUSHMEHR, H., WEISENBERGER, D. J., DIEFES, K., PHILLIPS, H. S., PUJARA, K., BERMAN, B. P., PAN, F., PELLOSKI, C. E., SULMAN, E. P., BHAT, K. P., VERHAAK, R. G., HOADLEY, K. A., HAYES, D. N., PEROU, C. M., SCHMIDT, H. K., DING, L., WILSON, R. K., VAN DEN BERG, D., SHEN, H., BENGTSSON, H., NEUVIAL, P., COPE, L. M., BUCKLEY, J., HERMAN, J. G., BAYLIN, S. B., LAIRD, P. W. & ALDAPE, K. (2010) Identification of a CpG island methylator phenotype that defines a distinct subgroup of glioma. *Cancer Cell,* 17, 510-22.

O'NEILL, M. J. (2005) The influence of non-coding RNAs on allele-specific gene expression in mammals. *Hum Mol Genet,* 14 Spec No 1, R113-20.

OGINO, S., KAWASAKI, T., NOSHO, K., OHNISHI, M., SUEMOTO, Y., KIRKNER, G. J. & FUCHS, C. S. (2008) LINE-1 hypomethylation is inversely associated with microsatellite instability and CpG island methylator phenotype in colorectal cancer. *Int J Cancer,* 122, 2767-73.

OGOSHI, K., HASHIMOTO, S., NAKATANI, Y., QU, W., OSHIMA, K., TOKUNAGA, K., SUGANO, S., HATTORI, M., MORISHITA, S. & MATSUSHIMA, K. (2011) Genome-wide profiling of DNA methylation in human cancer cells. *Genomics,* 98, 280-7.

OHINATA, Y., PAYER, B., O'CARROLL, D., ANCELIN, K., ONO, Y., SANO, M., BARTON, S. C., OBUKHANYCH, T., NUSSENZWEIG, M., TARAKHOVSKY, A., SAITOU, M. & SURANI, M. A. (2005) Blimp1 is a critical determinant of the germ cell lineage in mice. *Nature,* 436, 207-13.

OHTANI-FUJITA, N., DRYJA, T. P., RAPAPORT, J. M., FUJITA, T., MATSUMURA, S., OZASA, K., WATANABE, Y., HAYASHI, K., MAEDA, K., KINOSHITA, S., MATSUMURA, T., OHNISHI, Y., HOTTA, Y., TAKAHASHI, R., KATO, M. V., ISHIZAKI, K., SASAKI, M. S., HORSTHEMKE, B., MINODA, K. & SAKAI, T. (1997) Hypermethylation in the retinoblastoma gene is associated with unilateral, sporadic retinoblastoma. *Cancer Genet Cytogenet,* 98, 43-9.

OKAMOTO, I., OTTE, A. P., ALLIS, C. D., REINBERG, D. & HEARD, E. (2004) Epigenetic dynamics of imprinted X inactivation during early mouse development. *Science,* 303, 644-9.

OKANO, M., BELL, D. W., HABER, D. A. & LI, E. (1999) DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell,* 99, 247-57.

OKANO, M., XIE, S. & LI, E. (1998) Dnmt2 is not required for de novo and maintenance methylation of viral DNA in embryonic stem cells. *Nucleic Acids Res,* 26, 2536-40.

OKPANYI, V., SCHNEIDER, D. T., ZAHN, S., SIEVERS, S., CALAMINUS, G., NICHOLSON, J. C., PALMER, R. D., LEUSCHNER, I., BORKHARDT, A. & SCHONBERGER, S. (2011) Analysis of the adenomatous polyposis coli (APC) gene in childhood and adolescent germ cell tumors. *Pediatr Blood Cancer,* 56, 384-91.

OLASZ, J., MANDOKY, L., GECZI, L., BODROGI, I., CSUKA, O. & BAK, M. (2005) Influence of hMLH1 methylation, mismatch repair deficiency and microsatellite instability on chemoresistance of testicular germ-cell tumors. *Anticancer Res,* 25, 4319-24.

OOI, S. K., QIU, C., BERNSTEIN, E., LI, K., JIA, D., YANG, Z., ERDJUMENT-BROMAGE, H., TEMPST, P., LIN, S. P., ALLIS, C. D., CHENG, X. & BESTOR, T. H. (2007) DNMT3L connects unmethylated lysine 4 of histone H3 to de novo methylation of DNA. *Nature,* 448, 714-7.

OOSTERHUIS, J. W., KERSEMAEKERS, A. M., JACOBSEN, G. K., TIMMER, A., STEYERBERG, E. W., MOLIER, M., VAN WEEREN, P. C., STOOP, H. & LOOIJENGA, L. H. (2003) Morphology of testicular parenchyma adjacent to germ cell tumours. An interim report. *APMIS,* 111, 32-40; discussion 41-2.

OOSTERHUIS, J. W. & LOOIJENGA, L. H. (2005) Testicular germ-cell tumours in a broader perspective. *Nat Rev Cancer,* 5, 210-22.

OPAVSKY, R., WANG, S. H., TRIKHA, P., RAVAL, A., HUANG, Y., WU, Y. Z., RODRIGUEZ, B., KELLER, B., LIYANARACHCHI, S., WEI, G., DAVULURI, R. V., WEINSTEIN, M., FELSHER, D., OSTROWSKI, M., LEONE, G. & PLASS, C. (2007) CpG island methylation in a mouse model of lymphoma is driven by the genetic configuration of tumor cells. *PLoS Genet,* 3, 1757-69.

OTTESEN, A. M., SKAKKEBAEK, N. E., LUNDSTEEN, C., LEFFERS, H., LARSEN, J. & RAJPERT-DE MEYTS, E. (2003) High-resolution comparative genomic hybridization detects extra chromosome arm 12p material in most cases of carcinoma in situ adjacent to overt germ cell tumors, but not before the invasive tumor development. *Genes Chromosomes Cancer,* 38, 117-25.

PAL, A., SRIVASTAVA, T., SHARMA, M. K., MEHNDIRATTA, M., DAS, P., SINHA, S. & CHATTOPADHYAY, P. (2009) Aberrant methylation and associated transcriptional mobilization of Alu elements contributes to genomic instability in hypoxia. *J Cell Mol Med,* 14, 2646-54.

PALMER, R. D., BARBOSA-MORAIS, N. L., GOODING, E. L., MURALIDHAR, B., THORNTON, C. M., PETT, M. R., ROBERTS, I., SCHNEIDER, D. T., THORNE, N., TAVARE, S., NICHOLSON, J. C. & COLEMAN, N. (2008) Pediatric malignant germ cell tumors show characteristic transcriptome profiles. *Cancer Res,* 68, 4239-47.

PALMISANO, W. A., DIVINE, K. K., SACCOMANNO, G., GILLILAND, F. D., BAYLIN, S. B., HERMAN, J. G. & BELINSKY, S. A. (2000) Predicting lung cancer by detecting aberrant promoter methylation in sputum. *Cancer Res,* 60, 5954-8.

PARK, S. Y., LEE, J. H., HA, M., NAM, J. W. & KIM, V. N. (2009a) miR-29 miRNAs activate p53 by targeting p85 alpha and CDC42. *Nat Struct Mol Biol,* 16, 23-9.

PARK, S. Y., YOO, E. J., CHO, N. Y., KIM, N. & KANG, G. H. (2009b) Comparison of CpG island hypermethylation and repetitive DNA hypomethylation in premalignant stages of gastric cancer, stratified for Helicobacter pylori infection. *J Pathol,* 219, 410-6.

PASMANT, E., MASLIAH-PLANCHON, J., LEVY, P., LAURENDEAU, I., ORTONNE, N., PARFAIT, B., VALEYRIE-ALLANORE, L., LEROY, K., WOLKENSTEIN, P., VIDAUD, M., VIDAUD, D. & BIECHE, I. (2011) Identification of genes potentially involved in the increased risk of malignancy in NF1-microdeleted patients. *Mol Med,* 17, 79-87.

PEINADO, H., BALLESTAR, E., ESTELLER, M. & CANO, A. (2004) Snail mediates E-cadherin repression by the recruitment of the Sin3A/histone deacetylase 1 (HDAC1)/HDAC2 complex. *Mol Cell Biol,* 24, 306-19.

PELTOMAKI, P. (1991) DNA methylation changes in human testicular cancer. *Biochim Biophys Acta,* 1096, 187-96.

PENNA, E., ORSO, F., CIMINO, D., TENAGLIA, E., LEMBO, A., QUAGLINO, E., POLISENO, L., HAIMOVIC, A., OSELLA-ABATE, S., DE PITTA, C., PINATEL, E., STADLER, M. B., PROVERO, P., BERNENGO, M. G., OSMAN, I. & TAVERNA, D. (2011) microRNA-214 contributes to melanoma tumour progression through suppression of TFAP2C. *EMBO J,* 30, 1990-2007.

PENNY, G. D., KAY, G. F., SHEARDOWN, S. A., RASTAN, S. & BROCKDORFF, N. (1996) Requirement for Xist in X chromosome inactivation. *Nature,* 379, 131-7.

PERA, M. F., BLASCO LAFITA, M. J. & MILLS, J. (1987) Cultured stem-cells from human testicular teratomas: the nature of human embryonal carcinoma, and its comparison with two types of yolk-sac carcinoma. *Int J Cancer,* 40, 334-43.

PIERCE, T., WORMAN, H. J. & HOLY, J. (1999) Neuronal differentiation of NT2/D1 teratocarcinoma cells is accompanied by a loss of lamin A/C expression and an increase in lamin B1 expression. *Exp Neurol,* 157, 241-50.

PILOZZI, E., ONELLI, M. R., ZIPARO, V., MERCANTINI, P. & RUCO, L. (2004) CDX1 expression is reduced in colorectal carcinoma and is associated with promoter hypermethylation. *J Pathol,* 204, 289-95.

POGRIBNY, I. P., FILKOWSKI, J. N., TRYNDYAK, V. P., GOLUBOV, A., SHPYLEVA, S. I. & KOVALCHUK, O. (2010) Alterations of microRNAs and their targets are associated with acquired resistance of MCF-7 breast cancer cells to cisplatin. *Int J Cancer,* 127, 1785-94.

POHLMANN, P., DILEONE, L. P., CANCELLA, A. I., CALDAS, A. P., DAL LAGO, L., CAMPOS, O., JR., MONEGO, E., RIVOIRE, W. & SCHWARTSMANN, G. (2002) Phase II trial of cisplatin plus decitabine, a new DNA hypomethylating agent, in patients with advanced squamous cell carcinoma of the cervix. *Am J Clin Oncol,* 25, 496-501.

POPLINSKI, A., TUTTELMANN, F., KANBER, D., HORSTHEMKE, B. & GROMOLL, J. (2010) Idiopathic male infertility is strongly associated with aberrant methylation of MEST and IGF2/H19 ICR1. *Int J Androl,* 33, 642-9.

POPP, C., DEAN, W., FENG, S., COKUS, S. J., ANDREWS, S., PELLEGRINI, M., JACOBSEN, S. E. & REIK, W. (2010) Genome-wide erasure of DNA methylation in mouse primordial germ cells is affected by AID deficiency. *Nature,* 463, 1101-5.

PORTELA, A. & ESTELLER, M. (2010) Epigenetic modifications and human disease. *Nat Biotechnol,* 28, 1057-68.

PRADHAN, S. & KIM, G. D. (2002) The retinoblastoma gene product interacts with maintenance human DNA (cytosine-5) methyltransferase and modulates its activity. *EMBO J,* 21, 779-88.

PRAK, E. T. & KAZAZIAN, H. H., JR. (2000) Mobile elements and the human genome. *Nat Rev Genet,* 1, 134-44.

PROKHORTCHOUK, A., HENDRICH, B., JORGENSEN, H., RUZOV, A., WILM, M., GEORGIEV, G., BIRD, A. & PROKHORTCHOUK, E. (2001) The p120 catenin partner Kaiso is a DNA methylation-dependent transcriptional repressor. *Genes Dev,* 15, 1613-8.

QIN, T., JELINEK, J., SI, J., SHU, J. & ISSA, J. P. (2009) Mechanisms of resistance to 5-aza-2'-deoxycytidine in human cancer cell lines. *Blood,* 113, 659-67.

QIN, T., YOUSSEF, E. M., JELINEK, J., CHEN, R., YANG, A. S., GARCIA-MANERO, G. & ISSA, J. P. (2007) Effect of cytarabine and decitabine in combination in human leukemic cell lines. *Clin Cancer Res,* 13, 4225-32.

RAHNAMA, F., SHAFIEI, F., GLUCKMAN, P. D., MITCHELL, M. D. & LOBIE, P. E. (2006) Epigenetic regulation of human trophoblastic cell migration and invasion. *Endocrinology,* 147, 5275-83.

RAKYAN, V. K., HILDMANN, T., NOVIK, K. L., LEWIN, J., TOST, J., COX, A. V., ANDREWS, T. D., HOWE, K. L., OTTO, T., OLEK, A., FISCHER, J., GUT, I. G., BERLIN, K. & BECK, S. (2004) DNA methylation profiling of the human major histocompatibility complex: a pilot study for the human epigenome project. *PLoS Biol,* 2, e405.

RAMACHANDRAN, K., GORDIAN, E. & SINGAL, R. (2011) 5-azacytidine reverses drug resistance in bladder cancer cells. *Anticancer Res,* 31, 3757-66.

RAMSAHOYE, B. H., BINISZKIEWICZ, D., LYKO, F., CLARK, V., BIRD, A. P. & JAENISCH, R. (2000) Non-CpG methylation is prevalent in embryonic stem cells and may be mediated by DNA methyltransferase 3a. *Proc Natl Acad Sci U S A,* 97, 5237-42.

RAPLEY, E. A., BARFOOT, R., BONAITI-PELLIE, C., CHOMPRET, A., FOULKES, W., PERUSINGHE, N., REEVE, A., ROYER-POKORA, B., SCHUMACHER, V., SHELLING, A., SKEEN, J., DE TOURREIL, S., WEIRICH, A., PRITCHARD-JONES, K., STRATTON, M. R. & RAHMAN, N. (2000) Evidence for susceptibility genes to familial Wilms tumour in addition to WT1, FWT1 and FWT2. *Br J Cancer,* 83, 177-83.

RAPLEY, E. A., CROCKFORD, G. P., EASTON, D. F., STRATTON, M. R. & BISHOP, D. T. (2003) Localisation of susceptibility genes for familial testicular germ cell tumour. *APMIS,* 111, 128-33; discussion 33-5.

RAUCH, T. A., WU, X., ZHONG, X., RIGGS, A. D. & PFEIFER, G. P. (2009) A human B cell methylome at 100-base pair resolution. *Proc Natl Acad Sci U S A,* 106, 671-8.

REA, S., EISENHABER, F., O'CARROLL, D., STRAHL, B. D., SUN, Z. W., SCHMID, M., OPRAVIL, S., MECHTLER, K., PONTING, C. P., ALLIS, C. D. & JENUWEIN, T. (2000) Regulation of chromatin structure by site-specific histone H3 methyltransferases. *Nature,* 406, 593-9.

REIK, W., BROWN, K. W., SCHNEID, H., LE BOUC, Y., BICKMORE, W. & MAHER, E. R. (1995) Imprinting mutations in the Beckwith-Wiedemann syndrome suggested by altered imprinting pattern in the IGF2-H19 domain. *Hum Mol Genet,* 4, 2379-85.

REUTER, V. E. (2005) Origins and molecular biology of testicular germ cell tumors. *Mod Pathol,* 18 Suppl 2, S51-60.

RICHARDSON, B. E. & LEHMANN, R. (2010) Mechanisms guiding primordial germ cell migration: strategies from different organisms. *Nat Rev Mol Cell Biol,* 11, 37-49.

RICHTER, J., AMMERPOHL, O., MARTIN-SUBERO, J. I., MONTESINOS-RONGEN, M., BIBIKOVA, M., WICKHAM-GARCIA, E., WIESTLER, O. D., DECKERT, M. & SIEBERT, R. (2009) Array-based DNA methylation profiling of primary lymphomas of the central nervous system. *BMC Cancer,* 9, 455.

RICKERT, C. H. (1999) Neuropathology and prognosis of foetal brain tumours. *Acta Neuropathol,* 98, 567-76.

RIUS, M. & LYKO, F. (2012) Epigenetic cancer therapy: rationales, targets and drugs. *Oncogene,* 31, 4257-65.

RODRIGUEZ, S., JAFER, O., GOKER, H., SUMMERSGILL, B. M., ZAFARANA, G., GILLIS, A. J., VAN GURP, R. J., OOSTERHUIS, J. W., LU, Y. J., HUDDART, R., COOPER, C. S., CLARK, J., LOOIJENGA, L. H. & SHIPLEY, J. M. (2003) Expression profile of genes from 12p in testicular germ cell tumors of adolescents and adults associated with i(12p) and amplification at 12p11.2-p12.1. *Oncogene,* 22, 1880-91.

ROELOFS, H., MOSTERT, M. C., POMPE, K., ZAFARANA, G., VAN OORSCHOT, M., VAN GURP, R. J., GILLIS, A. J., STOOP, H., BEVERLOO, B., OOSTERHUIS, J. W., BOKEMEYER, C. & LOOIJENGA, L. H. (2000) Restricted 12p amplification and RAS mutation in human germ cell tumors of the adult testis. *Am J Pathol,* 157, 1155-66.

ROLL, J. D., RIVENBARK, A. G., JONES, W. D. & COLEMAN, W. B. (2008) DNMT3b overexpression contributes to a hypermethylator phenotype in human breast cancer cell lines. *Mol Cancer,* 7, 15.

ROMAN-GOMEZ, J., JIMENEZ-VELASCO, A., AGIRRE, X., CASTILLEJO, J. A., NAVARRO, G., SAN JOSE-ENERIZ, E., GARATE, L., CORDEU, L., CERVANTES, F., PROSPER, F., HEINIGER, A. & TORRES, A. (2007) Epigenetic regulation of human cancer/testis antigen gene, HAGE, in chronic myeloid leukemia. *Haematologica,* 92, 153-62.

ROMAN-GOMEZ, J., JIMENEZ-VELASCO, A., AGIRRE, X., CASTILLEJO, J. A., NAVARRO, G., SAN JOSE-ENERIZ, E., GARATE, L., CORDEU, L., CERVANTES, F., PROSPER, F., HEINIGER, A. & TORRES, A. (2008) Repetitive DNA hypomethylation in the advanced phase of chronic myeloid leukemia. *Leuk Res,* 32, 487-90.

ROMAN-GOMEZ, J., JIMENEZ-VELASCO, A., AGIRRE, X., CERVANTES, F., SANCHEZ, J., GARATE, L., BARRIOS, M., CASTILLEJO, J. A., NAVARRO, G., COLOMER, D., PROSPER, F., HEINIGER, A. & TORRES, A. (2005) Promoter hypomethylation of the LINE-1 retrotransposable elements activates sense/antisense transcription and marks the progression of chronic myeloid leukemia. *Oncogene,* 24, 7213-23.

ROSENBERG, C., VAN GURP, R. J., GEELEN, E., OOSTERHUIS, J. W. & LOOIJENGA, L. H. (2000) Overrepresentation of the short arm of chromosome 12 is related to invasive growth of human testicular seminomas and nonseminomas. *Oncogene,* 19, 5858-62.

ROSS, J. H., RYBICKI, L. & KAY, R. (2002) Clinical behavior and a contemporary management algorithm for prepubertal testis tumors: a summary of the Prepubertal Testis Tumor Registry. *J Urol,* 168, 1675-8; discussion 1678-9.

ROZENBERG, J. M., SHLYAKHTENKO, A., GLASS, K., RISHI, V., MYAKISHEV, M. V., FITZGERALD, P. C. & VINSON, C. (2008) All and only CpG containing sequences are enriched in promoters abundantly bound by RNA polymerase II in multiple tissues. *BMC Genomics,* 9.

RUNYAN, C., GU, Y., SHOEMAKER, A., LOOIJENGA, L. & WYLIE, C. (2008) The distribution and behavior of extragonadal primordial germ cells in Bax mutant mice suggest a novel origin for sacrococcygeal germ cell tumors. *Int J Dev Biol,* 52, 333-44.

RUNYAN, C., SCHAIBLE, K., MOLYNEAUX, K., WANG, Z., LEVIN, L. & WYLIE, C. (2006) Steel factor controls midline cell death of primordial germ cells and is essential for their normal proliferation and migration. *Development,* 133, 4861-9.

SAAVEDRA-CASTILLO, E., CORTES-GUTIERREZ, E. I., DAVILA-RODRIGUEZ, M. I., REYES-MARTINEZ, M. E. & OLIVEROS-RODRIGUEZ, A. (2005) 47,XXY female with testicular feminization and positive SRY: a case report. *J Reprod Med,* 50, 138-40.

SABA, H. I. (2007) Decitabine in the treatment of myelodysplastic syndromes. *Ther Clin Risk Manag,* 3, 807-17.

SABRY, M., TSIROGIANNI, M., BAKHSH, I. A., NORTH, J., SIVAKUMARAN, J., GIANNOPOULOS, K., ANDERSON, R., MACKINNON, S. & LOWDELL, M. W. (2011) Leukemic priming of resting NK cells is killer Ig-like receptor independent but requires CD15-mediated CD2 ligation and natural cytotoxicity receptors. *J Immunol,* 187, 6227-34.

SADO, T., FENNER, M. H., TAN, S. S., TAM, P., SHIODA, T. & LI, E. (2000) X inactivation in the mouse embryo deficient for Dnmt1: distinct effect of hypomethylation on imprinted and random X inactivation. *Dev Biol,* 225, 294-303.

SADO, T., WANG, Z., SASAKI, H. & LI, E. (2001) Regulation of imprinted X-chromosome inactivation in mice by Tsix. *Development,* 128, 1275-86.

SAGA, Y. (2008) Mouse germ cell development during embryogenesis. *Curr Opin Genet Dev,* 18, 337-41.

SAIED, M. H., MARZEC, J., KHALID, S., SMITH, P., DOWN, T. A., RAKYAN, V. K., MOLLOY, G., RAGHAVAN, M., DEBERNARDI, S. & YOUNG, B. D. (2012) Genome wide analysis of acute myeloid leukemia reveal leukemia specific methylome and subtype specific hypomethylation of repeats. *PLoS One,* 7, e33213.

SAITOU, M., BARTON, S. C. & SURANI, M. A. (2002) A molecular programme for the specification of germ cell fate in mice. *Nature,* 418, 293-300.

SAITOU, M., KAGIWADA, S. & KURIMOTO, K. (2012) Epigenetic reprogramming in mouse pre-implantation development and primordial germ cells. *Development,* 139, 15-31.

SAKUMA, Y., SAKURAI, S., OGUNI, S., HIRONAKA, M. & SAITO, K. (2003) Alterations of the c-kit gene in testicular germ cell tumors. *Cancer Sci,* 94, 486-91.

SAKUMA, Y., SAKURAI, S., OGUNI, S., SATOH, M., HIRONAKA, M. & SAITO, K. (2004) c-kit gene mutations in intracranial germinomas. *Cancer Sci,* 95, 716-20.

SALONEN, J., RAJPERT-DE MEYTS, E., MANNISTO, S., NIELSEN, J. E., GRAEM, N., TOPPARI, J. & HEIKINHEIMO, M. (2010) Differential developmental expression of transcription factors GATA-4 and GATA-6, their cofactor FOG-2 and downstream target genes in testicular carcinoma in situ and germ cell tumors. *Eur J Endocrinol,* 162, 625-31.

SANDBERG, A. A., MELONI, A. M. & SUIJKERBUIJK, R. F. (1996) Reviews of chromosome studies in urological tumors. III. Cytogenetics and genes in testicular tumors. *J Urol,* 155, 1531-56.

SARRAF, S. A. & STANCHEVA, I. (2004) Methyl-CpG binding protein MBD1 couples histone H3 methylation at lysine 9 by SETDB1 to DNA replication and chromatin assembly. *Mol Cell,* 15, 595-605.

SASAKI, H. & MATSUI, Y. (2008) Epigenetic events in mammalian germ-cell development: reprogramming and beyond. *Nat Rev Genet,* 9, 129-40.

SASSAMAN, D. M., DOMBROSKI, B. A., MORAN, J. V., KIMBERLAND, M. L., NAAS, T. P., DEBERARDINIS, R. J., GABRIEL, A., SWERGOLD, G. D. & KAZAZIAN, H. H., JR. (1997) Many human L1 elements are capable of retrotransposition. *Nat Genet,* 16, 37-43.

SAWAN, C., VAISSIERE, T., MURR, R. & HERCEG, Z. (2008) Epigenetic drivers and genetic passengers on the road to cancer. *Mutat Res,* 642, 1-13.

SCHMID, M., GUTTENBACH, M., ENDERS, H. & TERRUHN, V. (1992) A 47,XXY female with unusual genitalia. *Hum Genet,* 90, 346-9.

SCHMIDT, B., LIEBENBERG, V., DIETRICH, D., SCHLEGEL, T., KNEIP, C., SEEGEBARTH, A., FLEMMING, N., SEEMANN, S., DISTLER, J., LEWIN, J., TETZNER, R., WEICKMANN, S., WILLE, U., LILOGLOU, T., RAJI, O., WALSHAW, M., FLEISCHHACKER, M., WITT, C. & FIELD, J. K. (2010) SHOX2 DNA methylation is a biomarker for the diagnosis of lung cancer based on bronchial aspirates. *BMC Cancer,* 10, 600.

SCHMOLL, H. J. (2002) Extragonadal germ cell tumors. *Ann Oncol,* 13 Suppl 4, 265-72.

SCHNEIDER, D. T., CALAMINUS, G., KOCH, S., TESKE, C., SCHMIDT, P., HAAS, R. J., HARMS, D. & GOBEL, U. (2004) Epidemiologic analysis of 1,442 children and adolescents registered in the German germ cell tumor protocols. *Pediatr Blood Cancer,* 42, 169-75.

SCHNEIDER, D. T., SCHUSTER, A. E., FRITSCH, M. K., HU, J., OLSON, T., LAUER, S., GOBEL, U. & PERLMAN, E. J. (2001) Multipoint imprinting analysis indicates a common precursor cell for gonadal and nongonadal pediatric germ cell tumors. *Cancer Res,* 61, 7268-76.

SCHULZ, W. A., STEINHOFF, C. & FLORL, A. R. (2006) Methylation of endogenous human retroelements in health and disease. *Curr Top Microbiol Immunol,* 310, 211-50.

SCHWARTSMANN, G., SCHUNEMANN, H., GORINI, C. N., FILHO, A. F., GARBINO, C., SABINI, G., MUSE, I., DILEONE, L. & MANS, D. R. (2000) A phase I trial of cisplatin plus decitabine, a new DNA-hypomethylating agent, in patients with advanced solid tumors and a follow-up early phase II evaluation in patients with inoperable non-small cell lung cancer. *Invest New Drugs,* 18, 83-91.

SCHWARTZENTRUBER, J., KORSHUNOV, A., LIU, X. Y., JONES, D. T., PFAFF, E., JACOB, K., STURM, D., FONTEBASSO, A. M., QUANG, D. A., TONJES, M., HOVESTADT, V., ALBRECHT, S., KOOL, M., NANTEL, A., KONERMANN, C., LINDROTH, A., JAGER, N., RAUSCH, T., RYZHOVA, M., KORBEL, J. O., HIELSCHER, T., HAUSER, P., GARAMI, M., KLEKNER, A., BOGNAR, L., EBINGER, M., SCHUHMANN, M. U., SCHEURLEN, W., PEKRUN, A., FRUHWALD, M. C., ROGGENDORF, W., KRAMM, C., DURKEN, M., ATKINSON, J., LEPAGE, P., MONTPETIT, A., ZAKRZEWSKA, M., ZAKRZEWSKI, K., LIBERSKI, P. P., DONG, Z., SIEGEL, P., KULOZIK, A. E., ZAPATKA, M., GUHA, A., MALKIN, D., FELSBERG, J., REIFENBERGER, G., VON DEIMLING, A., ICHIMURA, K., COLLINS, V. P., WITT, H., MILDE, T., WITT, O., ZHANG, C., CASTELO-BRANCO, P., LICHTER, P., FAURY, D., TABORI, U., PLASS, C., MAJEWSKI, J., PFISTER, S. M. & JABADO, N. (2012) Driver mutations in histone H3.3 and chromatin remodelling genes in paediatric glioblastoma. *Nature,* 482, 226-31.

SEGURA-PACHECO, B., TREJO-BECERRIL, C., PEREZ-CARDENAS, E., TAJA-CHAYEB, L., MARISCAL, I., CHAVEZ, A., ACUNA, C., SALAZAR, A. M., LIZANO, M. & DUENAS-GONZALEZ, A. (2003) Reactivation of tumor suppressor genes by the cardiovascular drugs hydralazine and procainamide and their potential use in cancer therapy. *Clin Cancer Res,* 9, 1596-603.

SEKI, Y., HAYASHI, K., ITOH, K., MIZUGAKI, M., SAITOU, M. & MATSUI, Y. (2005) Extensive and orderly reprogramming of genome-wide chromatin modifications associated with specification and early development of germ cells in mice. *Dev Biol,* 278, 440-58.

SEKI, Y., YAMAJI, M., YABUTA, Y., SANO, M., SHIGETA, M., MATSUI, Y., SAGA, Y., TACHIBANA, M., SHINKAI, Y. & SAITOU, M. (2007) Cellular dynamics associated with the genome-wide epigenetic reprogramming in migrating primordial germ cells in mice. *Development,* 134, 2627-38.

SHAHBAZIAN, M. D. & GRUNSTEIN, M. (2007) Functions of site-specific histone acetylation and deacetylation. *Annu Rev Biochem,* 76, 75-100.

SHAHRZAD, S., BERTRAND, K., MINHAS, K. & COOMBER, B. L. (2007) Induction of DNA hypomethylation by tumor hypoxia. *Epigenetics,* 2, 119-25.

SHARP, A. J., STATHAKI, E., MIGLIAVACCA, E., BRAHMACHARY, M., MONTGOMERY, S. B., DUPRE, Y. & ANTONARAKIS, S. E. (2011) DNA methylation profiles of human active and inactive X chromosomes. *Genome Res,* 21, 1592-600.

SHARPE, R. M. (1993) Declining sperm counts in men--is there an endocrine cause? *J Endocrinol,* 136, 357-60.

SHARPE, R. M. (2003) The 'oestrogen hypothesis'- where do we stand now? *Int J Androl,* 26, 2-15.

SHARPE, R. M. & SKAKKEBAEK, N. E. (1993) Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? *Lancet,* 341, 1392-5.

SHEMER, R., BIRGER, Y., RIGGS, A. D. & RAZIN, A. (1997) Structure of the imprinted mouse Snrpn gene and establishment of its parental-specific methylation pattern. *Proc Natl Acad Sci U S A,* 94, 10267-72.

SHEN, J., WANG, S., ZHANG, Y. J., WU, H. C., KIBRIYA, M. G., JASMINE, F., AHSAN, H., WU, D. P., SIEGEL, A. B., REMOTTI, H. & SANTELLA, R. M. (2013) Exploring genome-wide DNA methylation profiles altered in hepatocellular carcinoma using Infinium HumanMethylation 450 BeadChips. *Epigenetics,* 8, 34-43.

SHI, Y., LAN, F., MATSON, C., MULLIGAN, P., WHETSTINE, J. R., COLE, P. A. & CASERO, R. A. (2004) Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell,* 119, 941-53.

SHIM, Y. H., PARK, H. J., CHOI, M. S., KIM, J. S., KIM, H., KIM, J. J., JANG, J. J. & YU, E. (2003) Hypermethylation of the p16 gene and lack of p16 expression in hepatoblastoma. *Mod Pathol,* 16, 430-6.

SHINJO, K., OKAMOTO, Y., AN, B., YOKOYAMA, T., TAKEUCHI, I., FUJII, M., OSADA, H., USAMI, N., HASEGAWA, Y., ITO, H., HIDA, T., FUJIMOTO, N., KISHIMOTO, T., SEKIDO, Y. & KONDO, Y. (2012) Integrated analysis of genetic and epigenetic alterations reveals CpG island methylator phenotype associated with distinct clinical characters of lung adenocarcinoma. *Carcinogenesis,* 33, 1277-85.

SILTANEN, S., ANTTONEN, M., HEIKKILA, P., NARITA, N., LAITINEN, M., RITVOS, O., WILSON, D. B. & HEIKINHEIMO, M. (1999) Transcription factor GATA-4 is expressed in pediatric yolk sac tumors. *Am J Pathol,* 155, 1823-9.

SILVER, D. P., RICHARDSON, A. L., EKLUND, A. C., WANG, Z. C., SZALLASI, Z., LI, Q., JUUL, N., LEONG, C. O., CALOGRIAS, D., BURAIMOH, A., FATIMA, A., GELMAN, R. S., RYAN, P. D., TUNG, N. M., DE NICOLO, A., GANESAN, S., MIRON, A., COLIN, C., SGROI, D. C., ELLISEN, L. W., WINER, E. P. & GARBER, J. E. (2010) Efficacy of neoadjuvant Cisplatin in triple-negative breast cancer. *J Clin Oncol,* 28, 1145-53.

SIRAJ, A. K., HUSSAIN, A. R., AL-RASHEED, M., AHMED, M., BAVI, P., ALSOBHI, S. A., AL-NUAIM, A., UDDIN, S. & AL-KURAYA, K. (2011) Demethylation of TMS1 gene sensitizes thyroid cancer cells to TRAIL-induced apoptosis. *J Clin Endocrinol Metab,* 96, E215-24.

SKAKKEBAEK, N. E. (1978) Carcinoma in situ of the testis: frequency and relationship to invasive germ cell tumours in infertile men. *Histopathology,* 2, 157-70.

SKOTHEIM, R. I., MONNI, O., MOUSSES, S., FOSSA, S. D., KALLIONIEMI, O. P., LOTHE, R. A. & KALLIONIEMI, A. (2002) New insights into testicular germ cell tumorigenesis from gene expression profiling. *Cancer Res,* 62, 2359-64.

SMALLWOOD, S. A., TOMIZAWA, S., KRUEGER, F., RUF, N., CARLI, N., SEGONDS-PICHON, A., SATO, S., HATA, K., ANDREWS, S. R. & KELSEY, G. (2011) Dynamic CpG island methylation landscape in oocytes and preimplantation embryos. *Nat Genet,* 43, 811-4.

SMIRAGLIA, D. J. & PLASS, C. (2002) The study of aberrant methylation in cancer via restriction landmark genomic scanning. *Oncogene,* 21, 5414-26.

SMIRAGLIA, D. J., RUSH, L. J., FRUHWALD, M. C., DAI, Z., HELD, W. A., COSTELLO, J. F., LANG, J. C., ENG, C., LI, B., WRIGHT, F. A., CALIGIURI, M. A. & PLASS, C. (2001) Excessive CpG

island hypermethylation in cancer cell lines versus primary human malignancies. *Hum Mol Genet,* 10, 1413-9.

SMIRAGLIA, D. J., SZYMANSKA, J., KRAGGERUD, S. M., LOTHE, R. A., PELTOMAKI, P. & PLASS, C. (2002) Distinct epigenetic phenotypes in seminomatous and nonseminomatous testicular germ cell tumors. *Oncogene,* 21, 3909-16.

SMITH-SORENSEN, B., LIND, G. E., SKOTHEIM, R. I., FOSSA, S. D., FODSTAD, O., STENWIG, A. E., JAKOBSEN, K. S. & LOTHE, R. A. (2002) Frequent promoter hypermethylation of the O6-Methylguanine-DNA Methyltransferase (MGMT) gene in testicular cancer. *Oncogene,* 21, 8878-84.

SOHN, B. H., PARK, I. Y., LEE, J. J., YANG, S. J., JANG, Y. J., PARK, K. C., KIM, D. J., LEE, D. C., SOHN, H. A., KIM, T. W., YOO, H. S., CHOI, J. Y., BAE, Y. S. & YEOM, Y. I. (2010) Functional switching of TGF-beta1 signaling in liver cancer via epigenetic modulation of a single CpG site in TTP promoter. *Gastroenterology,* 138, 1898-908.

SONNE, S. B., ALMSTRUP, K., DALGAARD, M., JUNCKER, A. S., EDSGARD, D., RUBAN, L., HARRISON, N. J., SCHWAGER, C., ABDOLLAHI, A., HUBER, P. E., BRUNAK, S., GJERDRUM, L. M., MOORE, H. D., ANDREWS, P. W., SKAKKEBAEK, N. E., RAJPERT-DE MEYTS, E. & LEFFERS, H. (2009) Analysis of gene expression profiles of microdissected cell populations indicates that testicular carcinoma in situ is an arrested gonocyte. *Cancer Res,* 69, 5241-50.

SPEEK, M. (2001) Antisense promoter of human L1 retrotransposon drives transcription of adjacent cellular genes. *Mol Cell Biol,* 21, 1973-85.

SPERGER, J. M., CHEN, X., DRAPER, J. S., ANTOSIEWICZ, J. E., CHON, C. H., JONES, S. B., BROOKS, J. D., ANDREWS, P. W., BROWN, P. O. & THOMSON, J. A. (2003) Gene expression patterns in human embryonic stem cells and human pluripotent germ cell tumors. *Proc Natl Acad Sci U S A,* 100, 13350-5.

STAVROPOULOS, N., LU, N. & LEE, J. T. (2001) A functional role for Tsix transcription in blocking Xist RNA accumulation but not in X-chromosome choice. *Proc Natl Acad Sci U S A,* 98, 10232-7.

STEGMANN, A. P., HONDERS, M. W., WILLEMZE, R. & LANDEGENT, J. E. (1995) De novo induced mutations in the deoxycytidine kinase (dck) gene in rat leukemic clonal cell lines confer resistance to cytarabine (AraC) and 5-aza-2'-deoxycytidine (DAC). *Leukemia,* 9, 1032-8.

STRESEMANN, C. & LYKO, F. (2008) Modes of action of the DNA methyltransferase inhibitors azacytidine and decitabine. *Int J Cancer,* 123, 8-13.

SUGIMOTO, K., NAKAHARA, I. & NISHIKAWA, M. (2002) Bilateral metachronous germinoma of the basal ganglia occurring long after total removal of a mature pineal teratoma: case report. *Neurosurgery,* 50, 613-6; discussion 616-7.

SUH, E. R., HA, C. S., RANKIN, E. B., TOYOTA, M. & TRABER, P. G. (2002) DNA methylation down-regulates CDX1 gene expression in colorectal cancer cell lines. *J Biol Chem,* 277, 35795-800.

SUMMERSGILL, B., OSIN, P., LU, Y. J., HUDDART, R. & SHIPLEY, J. (2001) Chromosomal imbalances associated with carcinoma in situ and associated testicular germ cell tumours of adolescents and adults. *Br J Cancer,* 85, 213-20.

SUN, W., LIU, Y., GLAZER, C. A., SHAO, C., BHAN, S., DEMOKAN, S., ZHAO, M., RUDEK, M. A., HA, P. K. & CALIFANO, J. A. (2010) TKTL1 is activated by promoter hypomethylation and contributes to head and neck squamous cell carcinoma carcinogenesis through increased aerobic glycolysis and HIF1alpha stabilization. *Clin Cancer Res,* 16, 857-66.

SUNAMI, E., SHINOZAKI, M., HIGANO, C. S., WOLLMAN, R., DORFF, T. B., TUCKER, S. J., MARTINEZ, S. R., MIZUNO, R., SINGER, F. R. & HOON, D. S. (2009) Multimarker

circulating DNA assay for assessing blood of prostate cancer patients. *Clin Chem,* 55, 559-67.

SURANI, M. A., ANCELIN, K., HAJKOVA, P., LANGE, U. C., PAYER, B., WESTERN, P. & SAITOU, M. (2004) Mechanism of mouse germ cell specification: a genetic program regulating epigenetic reprogramming. *Cold Spring Harb Symp Quant Biol,* 69, 1-9.

SUZUKI, R. & SHIMODAIRA, H. (2006) Pvclust: an R package for assessing the uncertainty in hierarchical clustering. *Bioinformatics,* 22, 1540-2.

SUZUKI, T., FUJII, M. & AYUSAWA, D. (2002) Demethylation of classical satellite 2 and 3 DNA with chromosomal instability in senescent human fibroblasts. *Exp Gerontol,* 37, 1005-14.

TACCAGNI, G. L., PARAFIORITI, A., DELL'ANTONIO, G. & CRESPI, G. (1989) Mixed germ cell tumour of the mediastinum (seminoma, embryonal carcinoma, choriocarcinoma and teratoma). Light and electron microscopic cytology and histological investigation. *Pathol Res Pract,* 185, 506-10; discussion 511-3.

TACHIBANA, M., UEDA, J., FUKUDA, M., TAKEDA, N., OHTA, T., IWANARI, H., SAKIHAMA, T., KODAMA, T., HAMAKUBO, T. & SHINKAI, Y. (2005) Histone methyltransferases G9a and GLP form heteromeric complexes and are both crucial for methylation of euchromatin at H3-K9. *Genes Dev,* 19, 815-26.

TAHILIANI, M., KOH, K. P., SHEN, Y., PASTOR, W. A., BANDUKWALA, H., BRUDNO, Y., AGARWAL, S., IYER, L. M., LIU, D. R., ARAVIND, L. & RAO, A. (2009) Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science,* 324, 930-5.

TAKAHASHI, K. & YAMANAKA, S. (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell,* 126, 663-76.

TAKAI, D. & JONES, P. A. (2002) Comprehensive analysis of CpG islands in human chromosomes 21 and 22. *Proc Natl Acad Sci U S A,* 99, 3740-5.

TAKEUCHI, S., TAKASATO, Y., MASAOKA, H., HAYAKAWA, T., OTANI, N., YOSHINO, Y. & YATSUSHIGE, H. (2009) Malignant transformation of an intracranial germinoma into a choriocarcinoma. *BMJ Case Rep,* 2009.

TAKEUCHI, T., TANIGAWA, Y., MINAMIDE, R., IKENISHI, K. & KOMIYA, T. (2010) Analysis of SDF-1/CXCR4 signaling in primordial germ cell migration and survival or differentiation in Xenopus laevis. *Mech Dev,* 127, 146-58.

TAMARU, H. & SELKER, E. U. (2001) A histone H3 methyltransferase controls DNA methylation in Neurospora crassa. *Nature,* 414, 277-83.

TAN, C. & SCOTTING, P. J. (2012) Stem cell research points the way to the cell of origin for intracranial germ cell tumours. *J Pathol,* 229, 4-11.

TANAKA, S. S., YAMAGUCHI, Y. L., TSOI, B., LICKERT, H. & TAM, P. P. (2005) IFITM/Mil/fragilis family proteins IFITM1 and IFITM3 play distinct roles in mouse primordial germ cell homing and repulsion. *Dev Cell,* 9, 745-56.

TEILUM, G. (1958) Classification of testicular and ovarian androblastoma and Sertoli cell tumors; a survey of comparative studies with consideration of histogenesis, endocrinology, and embryological theories. *Cancer,* 11, 769-82.

TEILUM, G. (1965) Classification of endodermal sinus tumour (mesoblatoma vitellinum) and so-called "embryonal carcinoma" of the ovary. *Acta Pathol Microbiol Scand,* 64, 407-29.

TIAN, Y., HOU, Y., ZHOU, X., CHENG, H. & ZHOU, R. (2011) Tumor suppressor RASSF1A promoter: p53 binding and methylation. *PLoS One,* 6, e17017.

TOMLINS, S. A., LAXMAN, B., DHANASEKARAN, S. M., HELGESON, B. E., CAO, X., MORRIS, D. S., MENON, A., JING, X., CAO, Q., HAN, B., YU, J., WANG, L., MONTIE, J. E., RUBIN, M. A., PIENTA, K. J., ROULSTON, D., SHAH, R. B., VARAMBALLY, S., MEHRA, R. & CHINNAIYAN,

A. M. (2007) Distinct classes of chromosomal rearrangements create oncogenic ETS gene fusions in prostate cancer. *Nature,* 448, 595-9.

TONG, W. G., WIERDA, W. G., LIN, E., KUANG, S. Q., BEKELE, B. N., ESTROV, Z., WEI, Y., YANG, H., KEATING, M. J. & GARCIA-MANERO, G. (2010) Genome-wide DNA methylation profiling of chronic lymphocytic leukemia allows identification of epigenetically repressed molecular pathways with clinical impact. *Epigenetics,* 5, 499-508.

TOYOTA, M., AHUJA, N., OHE-TOYOTA, M., HERMAN, J. G., BAYLIN, S. B. & ISSA, J. P. (1999) CpG island methylator phenotype in colorectal cancer. *Proc Natl Acad Sci U S A,* 96, 8681-6.

TRAVIS, L. B., BEARD, C., ALLAN, J. M., DAHL, A. A., FELDMAN, D. R., OLDENBURG, J., DAUGAARD, G., KELLY, J. L., DOLAN, M. E., HANNIGAN, R., CONSTINE, L. S., OEFFINGER, K. C., OKUNIEFF, P., ARMSTRONG, G., WILJER, D., MILLER, R. C., GIETEMA, J. A., VAN LEEUWEN, F. E., WILLIAMS, J. P., NICHOLS, C. R., EINHORN, L. H. & FOSSA, S. D. (2010) Testicular cancer survivorship: research strategies and recommendations. *J Natl Cancer Inst,* 102, 1114-30.

TURCAN, S., ROHLE, D., GOENKA, A., WALSH, L. A., FANG, F., YILMAZ, E., CAMPOS, C., FABIUS, A. W., LU, C., WARD, P. S., THOMPSON, C. B., KAUFMAN, A., GURYANOVA, O., LEVINE, R., HEGUY, A., VIALE, A., MORRIS, L. G., HUSE, J. T., MELLINGHOFF, I. K. & CHAN, T. A. (2012) IDH1 mutation is sufficient to establish the glioma hypermethylator phenotype. *Nature,* 483, 479-83.

USHIDA, H., KAWAKAMI, T., MINAMI, K., CHANO, T., OKABE, H., OKADA, Y. & OKAMOTO, K. (2011) Methylation profile of DNA repetitive elements in human testicular germ cell tumor. *Mol Carcinog.*

UTSUKI, S., OKA, H., SAGIUCHI, T., SHIMIZU, S., SUZUKI, S. & FUJII, K. (2007) Malignant transformation of intracranial mature teratoma to yolk sac tumor after late relapse. Case report. *J Neurosurg,* 106, 1067-1069.

VAN DE GEIJN, G. J., HERSMUS, R. & LOOIJENGA, L. H. (2009) Recent developments in testicular germ cell tumor research. *Birth Defects Res C Embryo Today,* 87, 96-113.

VAN DER AUWERA, I., YU, W., SUO, L., VAN NESTE, L., VAN DAM, P., VAN MARCK, E. A., PAUWELS, P., VERMEULEN, P. B., DIRIX, L. Y. & VAN LAERE, S. J. (2010) Array-based DNA methylation profiling for breast cancer subtype discrimination. *PLoS One,* 5, e12616.

VAN NESTE, L., HERMAN, J. G., OTTO, G., BIGLEY, J. W., EPSTEIN, J. I. & VAN CRIEKINGE, W. (2012) The epigenetic promise for prostate cancer diagnosis. *Prostate,* 72, 1248-61.

VELTMAN, I. M., SCHEPENS, M. T., LOOIJENGA, L. H., STRONG, L. C. & VAN KESSEL, A. G. (2003) Germ cell tumours in neonates and infants: a distinct subgroup? *APMIS,* 111, 152-60; discussion 160.

VERTINO, P. M., ISSA, J. P., PEREIRA-SMITH, O. M. & BAYLIN, S. B. (1994) Stabilization of DNA methyltransferase levels and CpG island hypermethylation precede SV40-induced immortalization of human fibroblasts. *Cell Growth Differ,* 5, 1395-402.

VESTERGAARD, J., LIND-THOMSEN, A., PEDERSEN, M. W., JARMER, H. O., BAK, M., HASHOLT, L., TOMMERUP, N., TUMER, Z. & LARSEN, L. A. (2008) GLI1 is involved in cell cycle regulation and proliferation of NT2 embryonal carcinoma stem cells. *DNA Cell Biol,* 27, 251-6.

VON DER MAASE, H., RORTH, M., WALBOM-JORGENSEN, S., SORENSEN, B. L., CHRISTOPHERSEN, I. S., HALD, T., JACOBSEN, G. K., BERTHELSEN, J. G. & SKAKKEBAEK, N. E. (1986) Carcinoma in situ of contralateral testis in patients with testicular germ cell cancer: study of 27 cases in 500 patients. *Br Med J (Clin Res Ed),* 293, 1398-401.

VON EYBEN, F. E., LIU, F. J., AMATO, R. J. & FRITSCHE, H. A. (2000) Lactate dehydrogenase isoenzyme 1 is the most important LD isoenzyme in patients with testicular germ cell tumor. *Acta Oncol,* 39, 509-17.

WANDERAS, E. H., FOSSA, S. D. & TRETLI, S. (1997) Risk of a second germ cell cancer after treatment of a primary germ cell cancer in 2201 Norwegian male patients. *Eur J Cancer,* 33, 244-52.

WANG, P., DONG, Q., ZHANG, C., KUAN, P. F., LIU, Y., JECK, W. R., ANDERSEN, J. B., JIANG, W., SAVICH, G. L., TAN, T. X., AUMAN, J. T., HOSKINS, J. M., MISHER, A. D., MOSER, C. D., YOURSTONE, S. M., KIM, J. W., CIBULSKIS, K., GETZ, G., HUNT, H. V., THORGEIRSSON, S. S., ROBERTS, L. R., YE, D., GUAN, K. L., XIONG, Y., QIN, L. X. & CHIANG, D. Y. (2012) Mutations in isocitrate dehydrogenase 1 and 2 occur frequently in intrahepatic cholangiocarcinomas and share hypermethylation targets with glioblastomas. *Oncogene.*

WANG, Z., ZANG, C., ROSENFELD, J. A., SCHONES, D. E., BARSKI, A., CUDDAPAH, S., CUI, K., ROH, T. Y., PENG, W., ZHANG, M. Q. & ZHAO, K. (2008) Combinatorial patterns of histone acetylations and methylations in the human genome. *Nat Genet,* 40, 897-903.

WEBER, B., KIMHI, S., HOWARD, G., EDEN, A. & LYKO, F. (2010a) Demethylation of a LINE-1 antisense promoter in the cMet locus impairs Met signalling through induction of illegitimate transcription. *Oncogene,* 29, 5775-84.

WEBER, S., ECKERT, D., NETTERSHEIM, D., GILLIS, A. J., SCHAFER, S., KUCKENBERG, P., EHLERMANN, J., WERLING, U., BIERMANN, K., LOOIJENGA, L. H. & SCHORLE, H. (2010b) Critical function of AP-2 gamma/TCFAP2C in mouse embryonic germ cell maintenance. *Biol Reprod,* 82, 214-23.

WEISENBERGER, D. J., SIEGMUND, K. D., CAMPAN, M., YOUNG, J., LONG, T. I., FAASSE, M. A., KANG, G. H., WIDSCHWENDTER, M., WEENER, D., BUCHANAN, D., KOH, H., SIMMS, L., BARKER, M., LEGGETT, B., LEVINE, J., KIM, M., FRENCH, A. J., THIBODEAU, S. N., JASS, J., HAILE, R. & LAIRD, P. W. (2006) CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. *Nat Genet,* 38, 787-93.

WEISS, G., COTTRELL, S., DISTLER, J., SCHATZ, P., KRISTIANSEN, G., ITTMANN, M., HAEFLIGER, C., LESCHE, R., HARTMANN, A., CORMAN, J. & WHEELER, T. (2009) DNA methylation of the PITX2 gene promoter region is a strong independent prognostic marker of biochemical recurrence in patients with prostate cancer after radical prostatectomy. *J Urol,* 181, 1678-85.

WERMANN, H., STOOP, H., GILLIS, A. J., HONECKER, F., VAN GURP, R. J., AMMERPOHL, O., RICHTER, J., OOSTERHUIS, J. W., BOKEMEYER, C. & LOOIJENGA, L. H. (2010) Global DNA methylation in fetal human germ cells and germ cell tumours: association with differentiation and cisplatin resistance. *J Pathol,* 221, 433-42.

WILTING, R. H. & DANNENBERG, J. H. (2012) Epigenetic mechanisms in tumorigenesis, tumor cell heterogeneity and drug resistance. *Drug Resist Updat,* 15, 21-38.

WOLFF, E. M., BYUN, H. M., HAN, H. F., SHARMA, S., NICHOLS, P. W., SIEGMUND, K. D., YANG, A. S., JONES, P. A. & LIANG, G. (2010) Hypomethylation of a LINE-1 promoter activates an alternate transcript of the MET oncogene in bladders with cancer. *PLoS Genet,* 6, e1000917.

WONG, J. M., CHI, S. N., MARCUS, K. J., LEVINE, B. S., ULLRICH, N. J., MACDONALD, S., LECHPAMMER, M. & GOUMNEROVA, L. C. (2010) Germinoma with malignant transformation to nongerminomatous germ cell tumor. *J Neurosurg Pediatr,* 6, 295-8.

WONG, N. A., BRITTON, M. P., CHOI, G. S., STANTON, T. K., BICKNELL, D. C., WILDING, J. L. & BODMER, W. F. (2004) Loss of CDX1 expression in colorectal carcinoma: promoter

methylation, mutation, and loss of heterozygosity analyses of 37 cell lines. *Proc Natl Acad Sci U S A,* 101, 574-9.

WOSSIDLO, M., NAKAMURA, T., LEPIKHOV, K., MARQUES, C. J., ZAKHARTCHENKO, V., BOIANI, M., ARAND, J., NAKANO, T., REIK, W. & WALTER, J. (2011) 5-Hydroxymethylcytosine in the mammalian zygote is linked with epigenetic reprogramming. *Nat Commun,* 2, 241.

WU, X., RAUCH, T. A., ZHONG, X., BENNETT, W. P., LATIF, F., KREX, D. & PFEIFER, G. P. (2010a) CpG island hypermethylation in human astrocytomas. *Cancer Res,* 70, 2718-27.

WU, Y., ALVAREZ, M., SLAMON, D. J., KOEFFLER, P. & VADGAMA, J. V. (2010b) Caspase 8 and maspin are downregulated in breast cancer cells due to CpG site promoter methylation. *BMC Cancer,* 10, 32.

XIONG, Y., FANG, J. H., YUN, J. P., YANG, J., ZHANG, Y., JIA, W. H. & ZHUANG, S. M. (2010) Effects of microRNA-29 on apoptosis, tumorigenicity, and prognosis of hepatocellular carcinoma. *Hepatology,* 51, 836-45.

XU, G. L., BESTOR, T. H., BOURC'HIS, D., HSIEH, C. L., TOMMERUP, N., BUGGE, M., HULTEN, M., QU, X., RUSSO, J. J. & VIEGAS-PEQUIGNOT, E. (1999) Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene. *Nature,* 402, 187-91.

YAMAJI, M., SEKI, Y., KURIMOTO, K., YABUTA, Y., YUASA, M., SHIGETA, M., YAMANAKA, K., OHINATA, Y. & SAITOU, M. (2008) Critical function of Prdm14 for the establishment of the germ cell lineage in mice. *Nat Genet,* 40, 1016-22.

YAN, H., PARSONS, D. W., JIN, G., MCLENDON, R., RASHEED, B. A., YUAN, W., KOS, I., BATINIC-HABERLE, I., JONES, S., RIGGINS, G. J., FRIEDMAN, H., FRIEDMAN, A., REARDON, D., HERNDON, J., KINZLER, K. W., VELCULESCU, V. E., VOGELSTEIN, B. & BIGNER, D. D. (2009) IDH1 and IDH2 mutations in gliomas. *N Engl J Med,* 360, 765-73.

YANG, A. S., ESTECIO, M. R., DOSHI, K., KONDO, Y., TAJARA, E. H. & ISSA, J. P. (2004) A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. *Nucleic Acids Res,* 32, e38.

YANG, H., LIU, Y., BAI, F., ZHANG, J. Y., MA, S. H., LIU, J., XU, Z. D., ZHU, H. G., LING, Z. Q., YE, D., GUAN, K. L. & XIONG, Y. (2012) Tumor development is associated with decrease of TET gene expression and 5-methylcytosine hydroxylation. *Oncogene.*

YAO, Z. X., HAN, Z., XU, J., GREESON, J., LECANU, L. & PAPADOPOULOS, V. (2007) 22R-Hydroxycholesterol induces differentiation of human NT2 precursor (Ntera2/D1 teratocarcinoma) cells. *Neuroscience,* 148, 441-53.

YAQINUDDIN, A., QURESHI, S. A., QAZI, R. & ABBAS, F. (2008) Down-regulation of DNMT3b in PC3 cells effects locus-specific DNA methylation, and represses cellular growth and migration. *Cancer Cell Int,* 8, 13.

YEGNASUBRAMANIAN, S., HAFFNER, M. C., ZHANG, Y., GUREL, B., CORNISH, T. C., WU, Z., IRIZARRY, R. A., MORGAN, J., HICKS, J., DEWEESE, T. L., ISAACS, W. B., BOVA, G. S., DE MARZO, A. M. & NELSON, W. G. (2008) DNA hypomethylation arises later in prostate cancer progression than CpG island hypermethylation and contributes to metastatic tumor heterogeneity. *Cancer Res,* 68, 8954-67.

YEGNASUBRAMANIAN, S., KOWALSKI, J., GONZALGO, M. L., ZAHURAK, M., PIANTADOSI, S., WALSH, P. C., BOVA, G. S., DE MARZO, A. M., ISAACS, W. B. & NELSON, W. G. (2004) Hypermethylation of CpG islands in primary and metastatic human prostate cancer. *Cancer Res,* 64, 1975-86.

YOO, C. B., JEONG, S., EGGER, G., LIANG, G., PHIASIVONGSA, P., TANG, C., REDKAR, S. & JONES, P. A. (2007) Delivery of 5-aza-2'-deoxycytidine to cells using oligodeoxynucleotides. *Cancer Res,* 67, 6400-8.

YOU, A., TONG, J. K., GROZINGER, C. M. & SCHREIBER, S. L. (2001) CoREST is an integral component of the CoREST- human histone deacetylase complex. *Proc Natl Acad Sci U S A,* 98, 1454-8.

YUILLE, M. R., CONDIE, A., STONE, E. M., WILSHER, J., BRADSHAW, P. S., BROOKS, L. & CATOVSKY, D. (2001) TCL1 is activated by chromosomal rearrangement or by hypomethylation. *Genes Chromosomes Cancer,* 30, 336-41.

ZHAN, S., SHAPIRO, D. N. & HELMAN, L. J. (1995) Loss of imprinting of IGF2 in Ewing's sarcoma. *Oncogene,* 11, 2503-7.

ZHANG, C., KAWAKAMI, T., OKADA, Y. & OKAMOTO, K. (2005) Distinctive epigenetic phenotype of cancer testis antigen genes among seminomatous and nonseminomatous testicular germ-cell tumors. *Genes Chromosomes Cancer,* 43, 104-12.

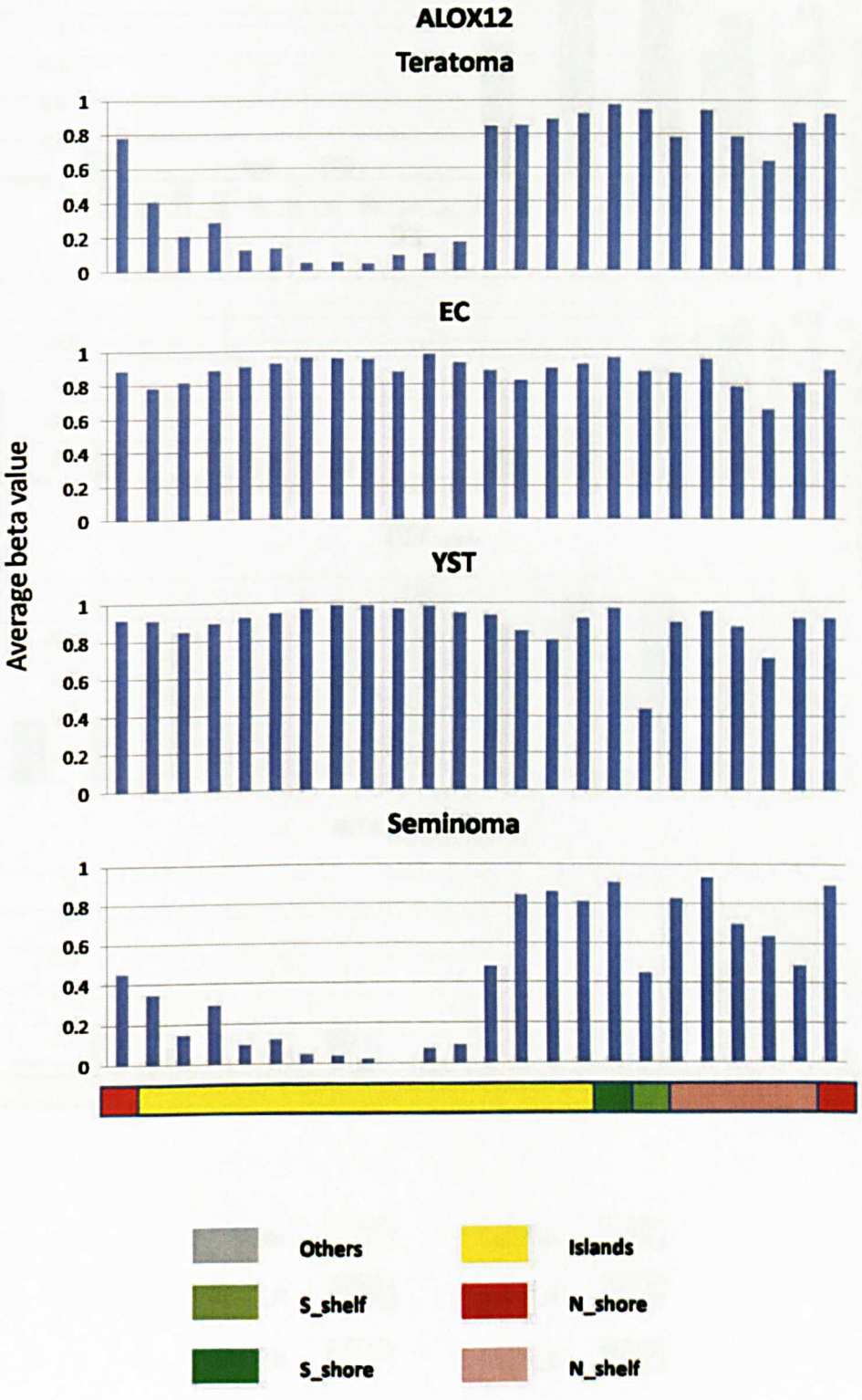
ZHANG, J., BENAVENTE, C. A., MCEVOY, J., FLORES-OTERO, J., DING, L., CHEN, X., ULYANOV, A., WU, G., WILSON, M., WANG, J., BRENNAN, R., RUSCH, M., MANNING, A. L., MA, J., EASTON, J., SHURTLEFF, S., MULLIGHAN, C., POUNDS, S., MUKATIRA, S., GUPTA, P., NEALE, G., ZHAO, D., LU, C., FULTON, R. S., FULTON, L. L., HONG, X., DOOLING, D. J., OCHOA, K., NAEVE, C., DYSON, N. J., MARDIS, E. R., BAHRAMI, A., ELLISON, D., WILSON, R. K., DOWNING, J. R. & DYER, M. A. (2012) A novel retinoblastoma therapy from genomic and epigenetic analyses. *Nature,* 481, 329-34.

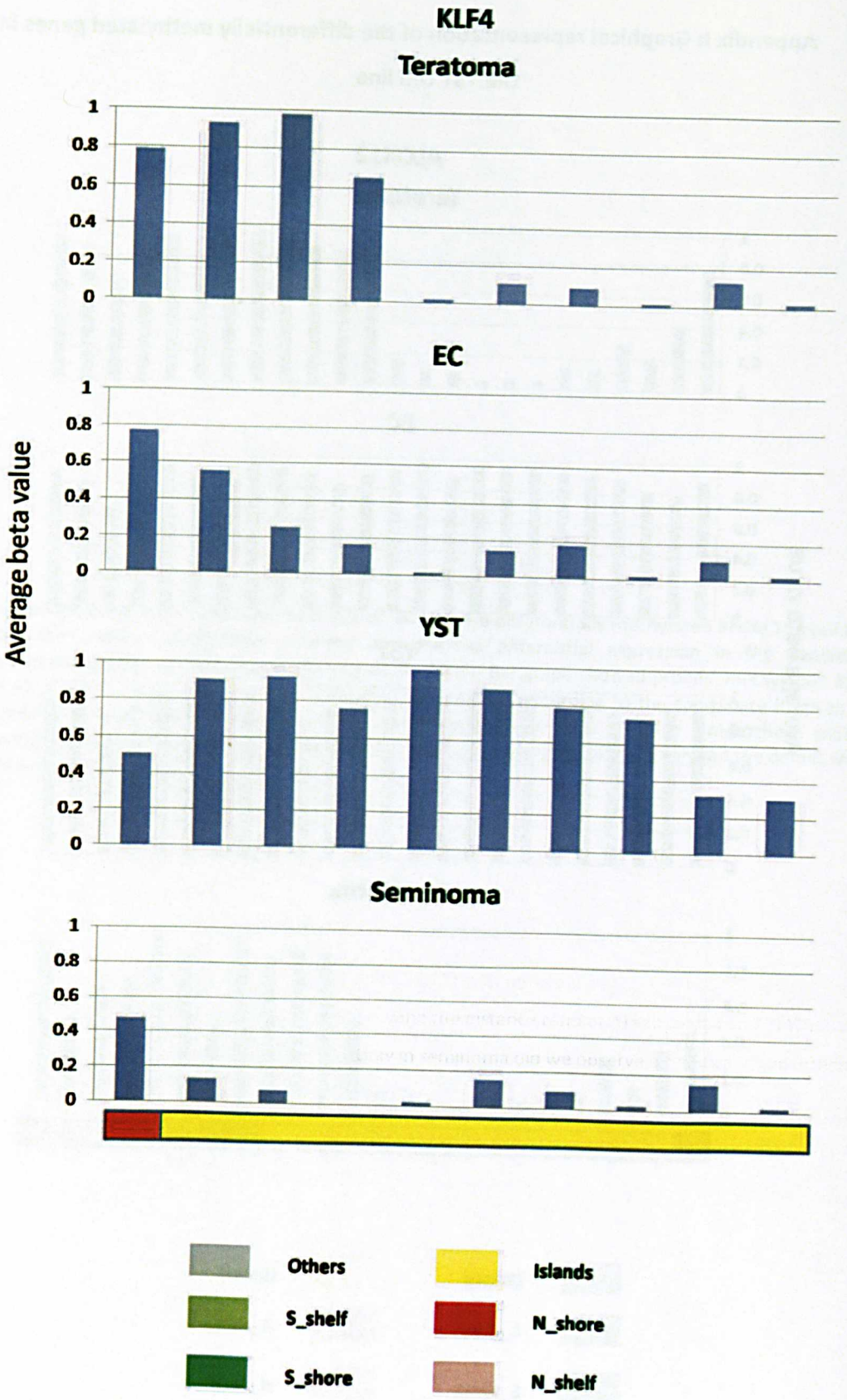
ZILLER, M. J., MULLER, F., LIAO, J., ZHANG, Y., GU, H., BOCK, C., BOYLE, P., EPSTEIN, C. B., BERNSTEIN, B. E., LENGAUER, T., GNIRKE, A. & MEISSNER, A. (2011) Genomic distribution and inter-sample variation of non-CpG methylation across human cell types. *PLoS Genet,* 7, e1002389.

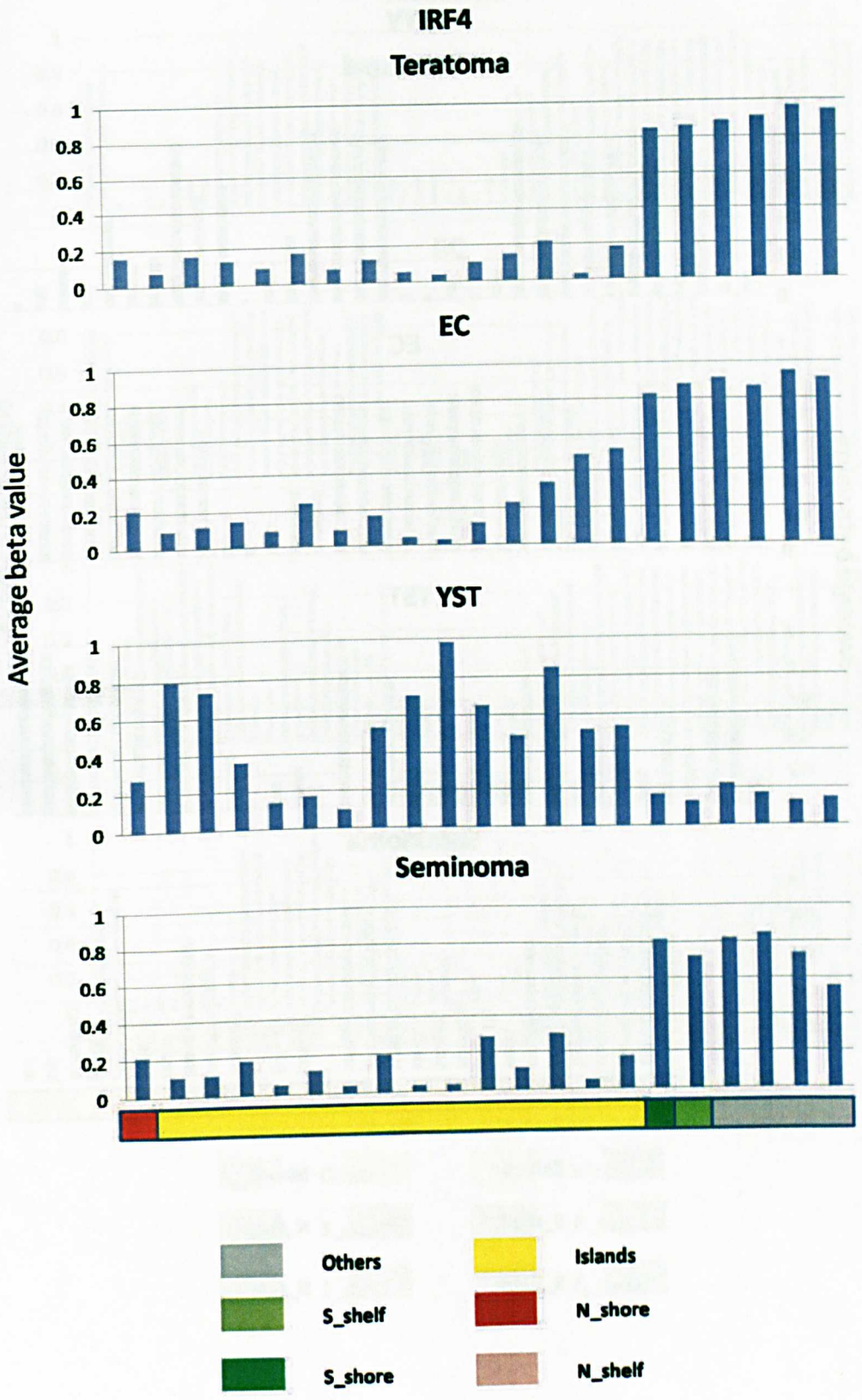
ZOU, H., ALLAWI, H., CAO, X., DOMANICO, M., HARRINGTON, J., TAYLOR, W. R., YAB, T., AHLQUIST, D. A. & LIDGARD, G. (2011) Quantification of methylated markers with a multiplex methylation-specific technology. *Clin Chem,* 58, 375-83.

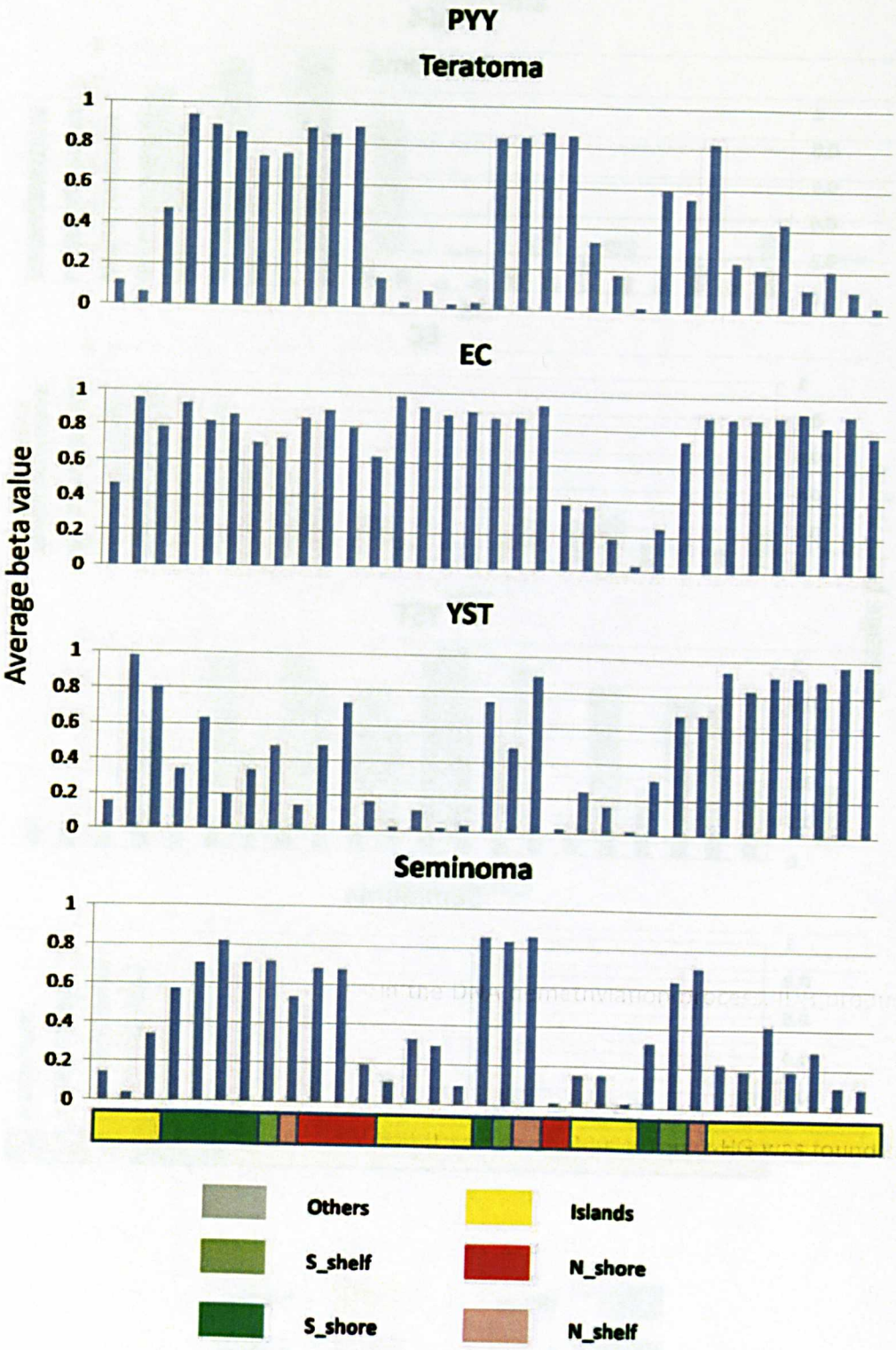
Appendix I: Graphical representation of genes found to be differentially methylated in the YST cell line from the Infinium methylation analysis and showed differential expression in the paediatric seminoma samples as reported by (Palmer et al., 2008). Bar graph showed probes' methylation level for all of these genes in all four cell lines. They were ranked according to the coordinate ID given by Illumina Inc from smallest to the largest coordinate (left to right). Regions which these probes correspond to were represented by colour-coded blocks at the bottom of the graph and the details were listed in the legends.

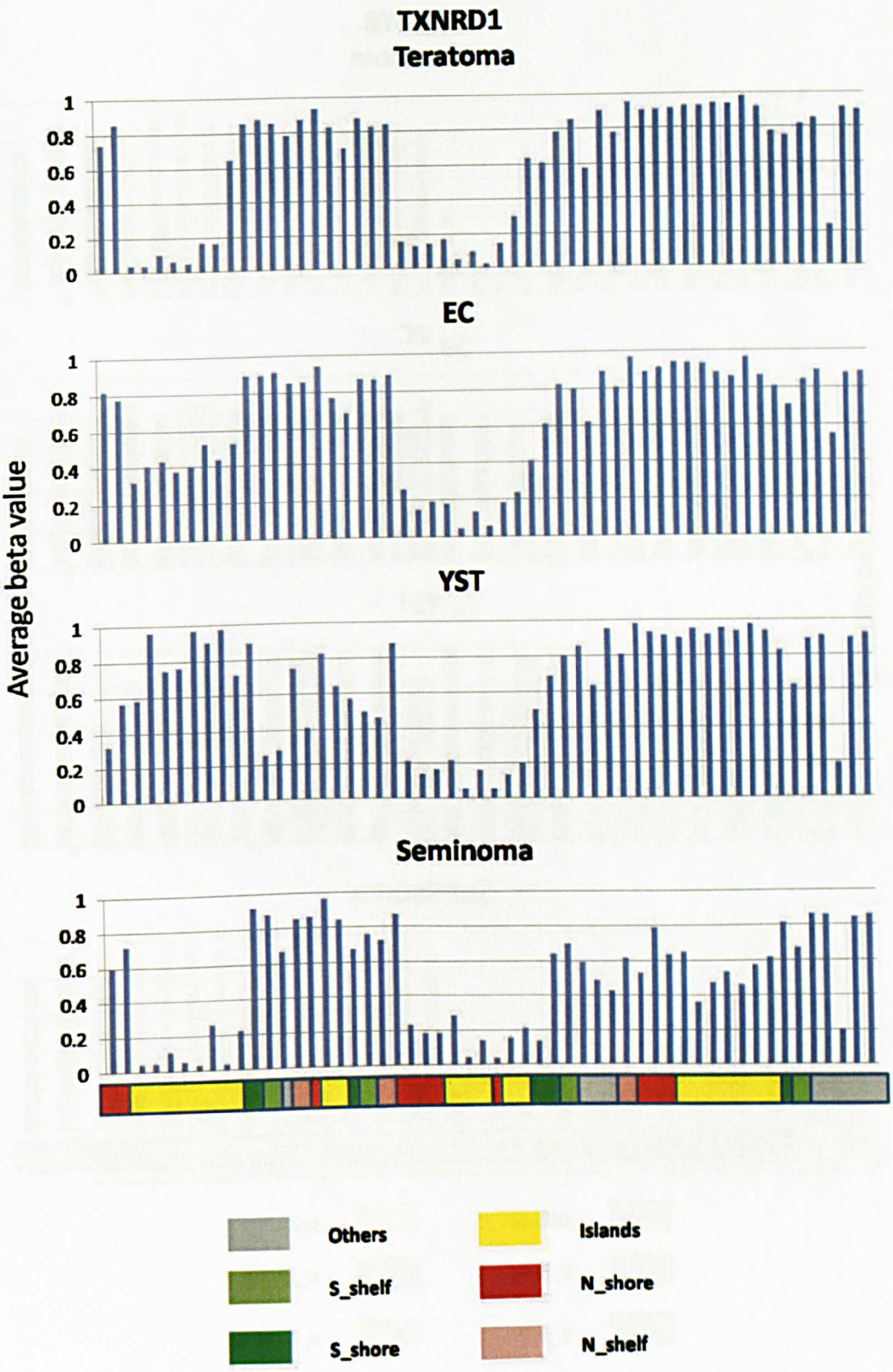
Appendix I: Graphical representation of the differentially methylated genes in the YST cell line

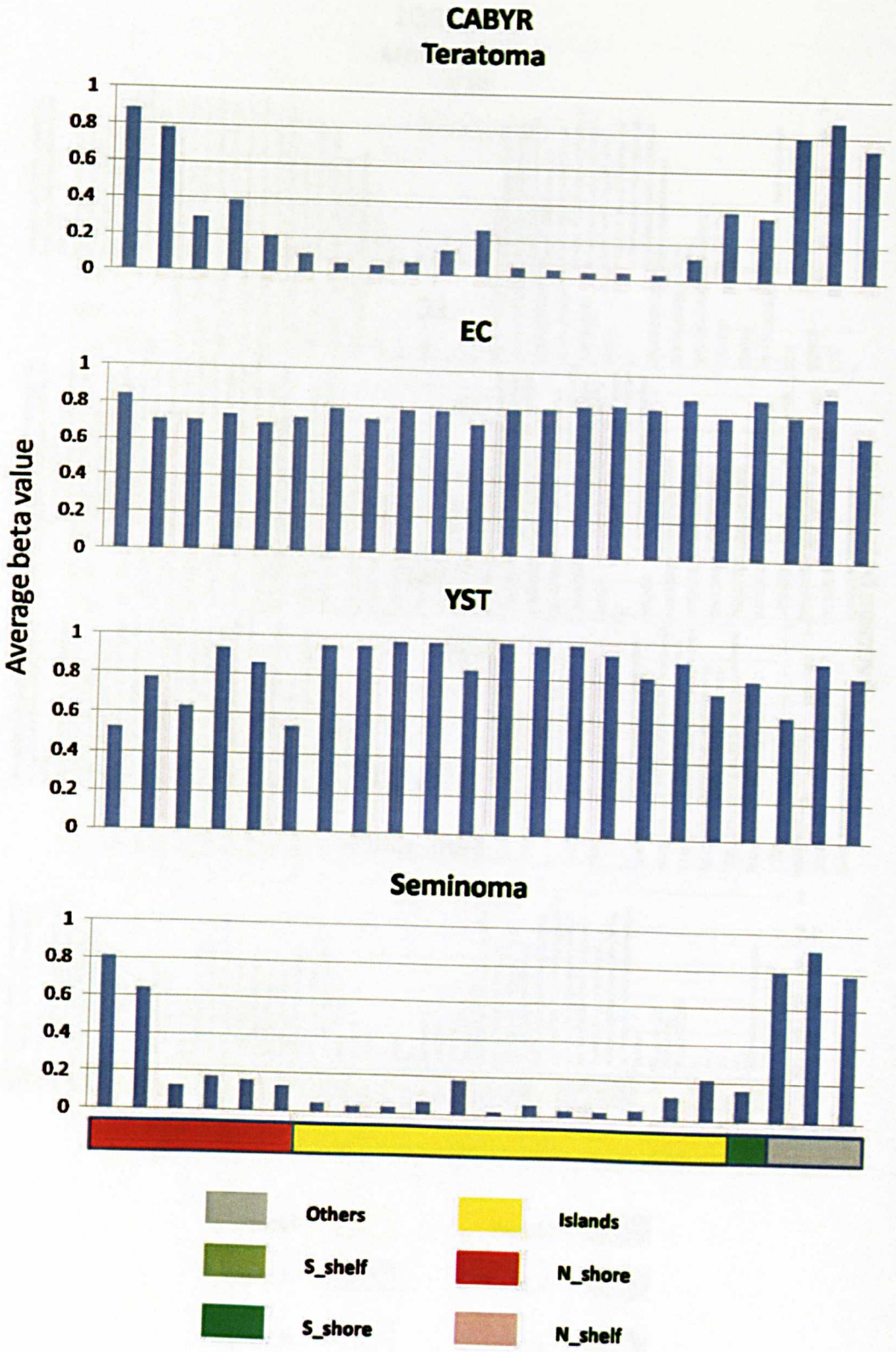


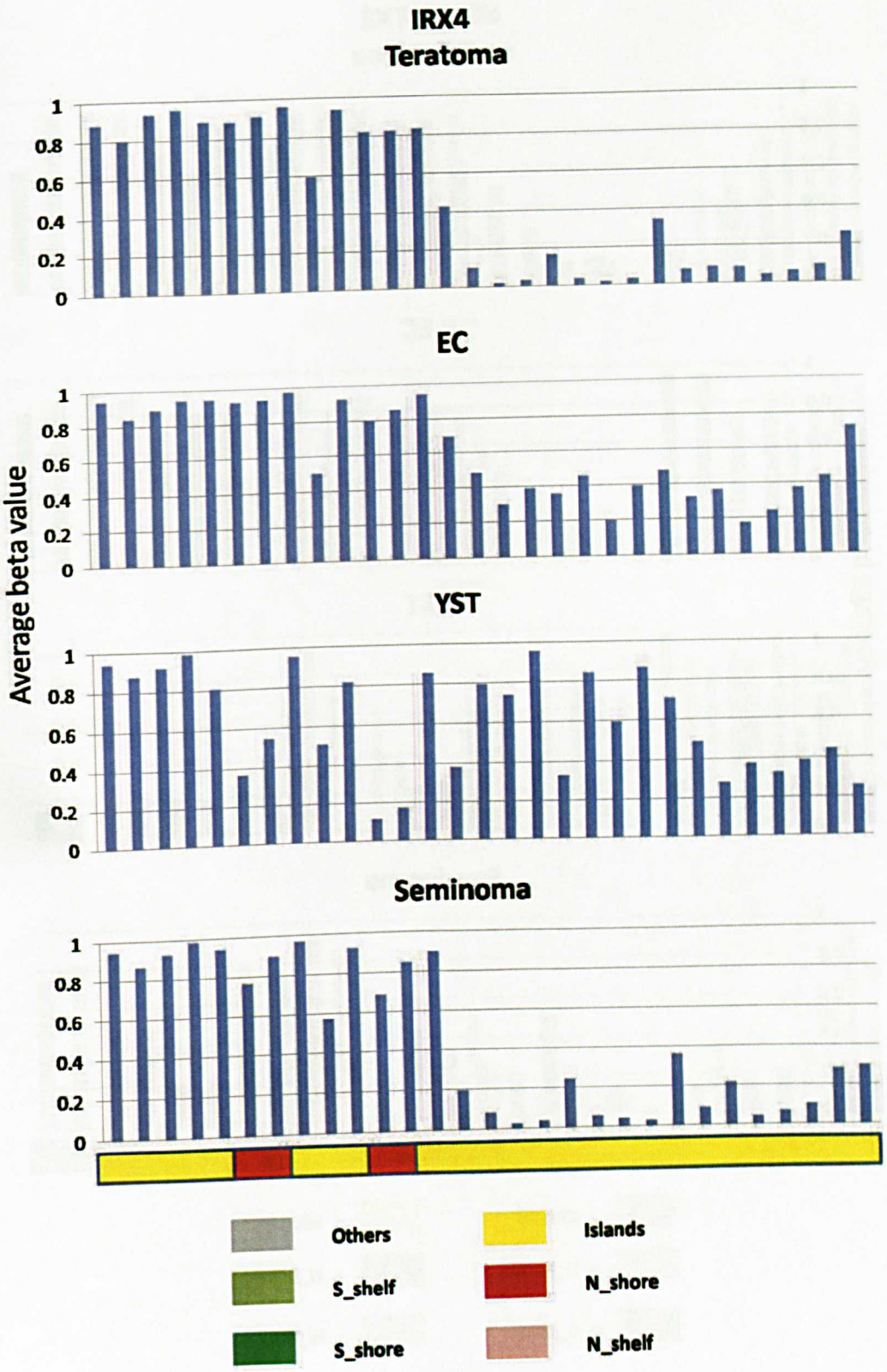

KLF4
Teratoma
EC
YST
Seminoma
Average beta value
1
0.8
0.6
0.4
0.2
0
Others
S_shelf
S_shore
Islands
N_shore
N_shelf

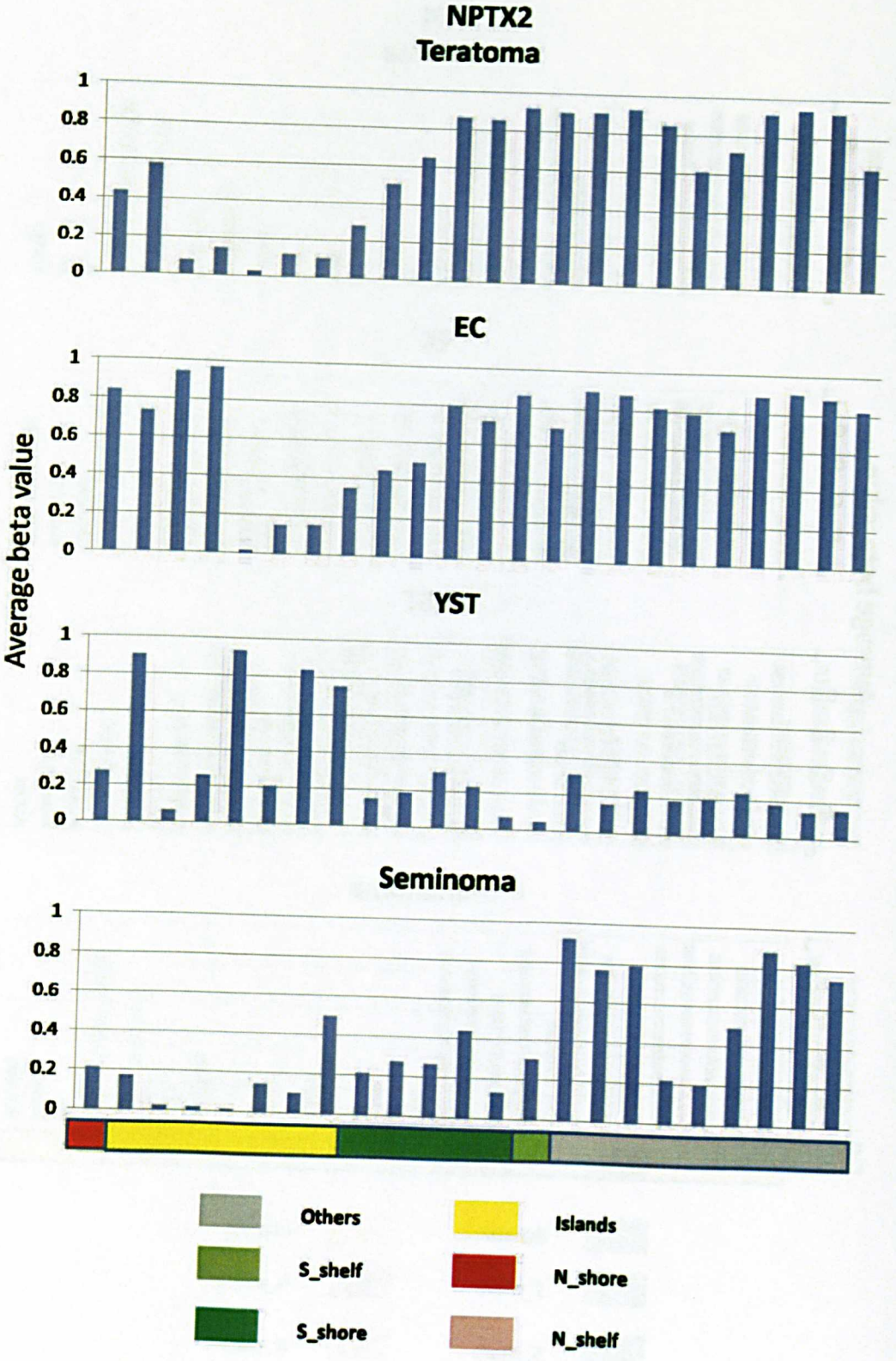

IRF4
Teratoma
EC
YST
Seminoma
Average beta value
1
0.8
0.6
0.4
0.2
0
Others
Islands
S_shelf
N_shore
S_shore
N_shelf

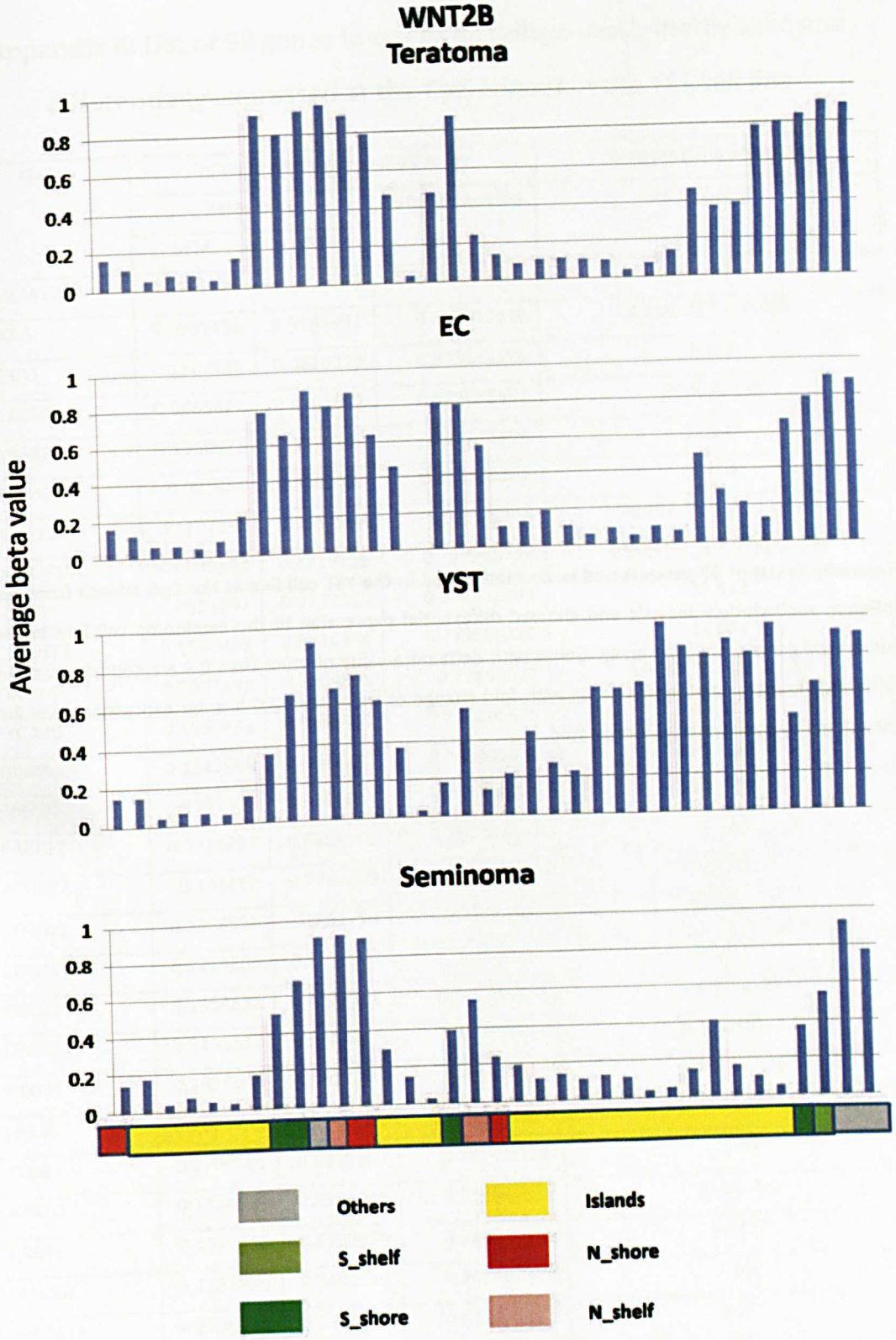

PYY
Teratoma
EC
YST
Seminoma
Average beta value
1
0.8
0.6
0.4
0.2
0
Others
Islands
S_shelf
N_shore
S_shore
N_shelf


TXNRD1
Teratoma
EC
YST
Seminoma
Average beta value
1
0.8
0.6
0.4
0.2
0
Others
Islands
S_shelf
N_shore
S_shore
N_shelf


CABYR
Teratoma
1
0.8
0.6
0.4
0.2
0
EC
Average beta value
YST
Seminoma
Others
Islands
S_shelf
N_shore
S_shore
N_shelf


IRX4
Teratoma
EC
YST
Seminoma
Average beta value
1
0.8
0.6
0.4
0.2
0
Others
S_shelf
S_shore
Islands
N_shore
N_shelf


NPTX2
Teratoma
1
0.8
0.6
0.4
0.2
0
EC
Average beta value
YST
Seminoma
Others
Islands
S_shelf
N_shore
S_shore
N_shelf


WNT2B
Teratoma
EC
YST
Seminoma
Average beta value
1
0.8
0.6
0.4
0.2
0
Others
Islands
S_shelf
N_shore
S_shore
N_shelf

Appendix II: List of 98 genes found to be methylated in the YST cell line at the CpG islands from the Infinium methylation analysis and showed differential expression in the seminoma cell line in the Affymetrix gene expression array. Genes with delta beta-value of more than 0.3 are considered to be differentially methylated while genes with fold change of more than 2.0 in their expression level are considered to be differentially expressed.

Appendix II: List of 98 genes found to be differentially methylated and differentially expressed at the CpG Islands in the YST cell line

Genes	Infinium Methylation450K Array			Affymetrix gene expression
	Beta-value		Delta beta-value	Fold change
	SEM	YST		
TMEM179B	0.0798845	0.9611163	0.881231782	5.288
NAAA	0.0969456	0.9663992	0.869453578	4.216, 3.551, 0.938
RCSD1	0.1167558	0.9619279	0.845172125	4.492
LGALS3	0.0465027	0.8701882	0.823685457	27.934, 1.0
CYB5R2	0.1060973	0.9278543	0.82175694	17.977
PLEKHG4	0.086451	0.9003403	0.813889264	7.164
C8orf42	0.1107351	0.9133594	0.8026243	26.467, 21.137, 13.038
TNFRSF10C	0.0839552	0.8710159	0.787060766	6.821, 2.124, 1.686, 1.035
DDX43	0.0984091	0.8727305	0.774321444	22.838
C13orf27	0.1578432	0.9314394	0.773596226	16.081
CXCL14	0.1093593	0.8804395	0.77108022	16.011, 10.608, 1.048
PYCARD	0.1563432	0.9265451	0.770201975	19.669
TRIM59	0.1342966	0.8947287	0.760432096	26.334, 2.112
AMPD3	0.1701624	0.9285633	0.758400967	2.07, 1.142
PARP12	0.0719291	0.8297166	0.757787533	4.293
ABHD14A	0.143157	0.8950109	0.751853917	2.084
ECHDC2	0.1368952	0.881794	0.74489879	16.349, 1.0
C8orf41	0.1214805	0.8650907	0.743610235	2.022
OVOL1	0.1354512	0.878592	0.743140737	8.465, 2.08
C8orf84	0.2156512	0.9554388	0.739787541	3.005, 2.431, 1.0
HCG11	0.1836098	0.917161	0.73355111	2.410, 1.674
HYLS1	0.1507745	0.8780493	0.727274774	15.174
PLD6	0.1700991	0.8915967	0.721497568	2.644
RBMXL2	0.1304589	0.8504149	0.719955922	43.16, 1.0
FABP5	0.1015446	0.8180639	0.716519285	32.377
MYD88	0.1391926	0.8406829	0.701490237	3.357
PRDM14	0.2030171	0.8984982	0.695481061	3.187
ACOT4	0.134381	0.810107	0.67572599	3.192
TTYH2	0.2547973	0.9260941	0.67129675	2.630, 1.117
ELMO3	0.2689428	0.9246207	0.65567793	2.188

TNFRSF10D	0.1472915	0.7953831	0.648091614	18.861, 1.114
GUCA1A	0.1933914	0.8300115	0.636620043	11.572, 3.991, 1.098
CST6	0.2872983	0.9233598	0.636061457	4.814, 1.207
TDRD12	0.3292812	0.9531206	0.62383946	84.861
SULT1A1	0.0861473	0.7074728	0.621325497	2.317, 2.150, 1.236
MEST	0.1683002	0.7882772	0.619977051	2.203
AIFM2	0.2132587	0.8161559	0.602897136	7.24, 1.765
OXCT1	0.1427753	0.7452948	0.602519459	14.948, 1.0
PPP1R14A	0.0831851	0.6819852	0.598800092	3.29
CLDN7	0.1059024	0.7034244	0.597521998	15.216
TNFRSF10A	0.1634931	0.758585	0.595091897	7.703, 2.294, 1.631
RASD1	0.0932478	0.6784343	0.585186488	44.246
B3GNT7	0.3716875	0.9450635	0.57337601	12.449, 8.771, 1.0
SHISA3	0.0784321	0.6192666	0.540834478	42.467
LTBP2	0.3526378	0.8770516	0.524413841	3.995, 2.111
ARPC1B	0.1346822	0.6525514	0.517869214	2.599
FAM114A1	0.41635	0.9255086	0.5091586	2.23, 2.049
UTF1	0.1665678	0.6748036	0.508235745	4.867
PPP1R14C	0.129667	0.6170103	0.487343291	5.908
HAS3	0.3837761	0.8696021	0.485825999	6.372, 0.921
ME1	0.1028691	0.574757	0.471887869	4.122, 2.121, 1.0
UHRF2	0.1194116	0.5886272	0.46921561	3.263
NPEPL1	0.4497619	0.9164939	0.466732	6.374, 2.29, 1.471, 1.073
BNC1	0.1602997	0.6189293	0.458629586	2.256, 1.178
MDFI	0.1684365	0.6150566	0.446620102	2.697
INSL6	0.3001802	0.7433963	0.44321615	3.058
PLEKHG1	0.0926288	0.5329287	0.44029989	3.598
FAM150B	0.1410692	0.579992	0.438922883	2.976
ACADL	0.0815099	0.5183729	0.43686293	3.481, 1.388
CYP11A1	0.0716475	0.5045341	0.432886587	2.151
ACOXL	0.1863722	0.6179977	0.43162557	4.193, 1.0
ABCA13	0.0656874	0.4961143	0.430426878	7.431, 2.218, 1.0
TMEM163	0.1017777	0.5205416	0.418763878	5.27, 3.377
GAL	0.3242434	0.7337861	0.409542702	3.976, 1.0
NAPRT1	0.5283096	0.9374048	0.409095171	3.985, 0.884
C1orf59	0.1263527	0.5338886	0.407535901	8.062

FAM162B	0.1573661	0.5601927	0.402826563	37.913
MEIS1	0.2153709	0.6169777	0.401606798	2.543, 3.852, 1.174
FHL2	0.1263811	0.52632	0.399938986	6.98
PLBD1	0.1163957	0.5158228	0.399427155	200.722, 1.131
C6orf132	0.3827052	0.7733776	0.390672384	4.84, 3.14
MTL5	0.0904993	0.479908	0.389408718	3.406, 1.0
AIM1	0.1596687	0.5404776	0.380808871	9.464
SECTM1	0.3139193	0.6940444	0.380125055	2.59
DLX3	0.125641	0.4989429	0.373301881	2.015
ADM	0.1110315	0.4784956	0.367464051	33.594
CPM	0.1804917	0.5473312	0.366839453	6.507, 2.937, 2.282, 1.249, 1.0775, 1.0
NSD1	0.3131123	0.6779757	0.364863373	3.871, 2.663, 2.357, 1.897
GRHL2	0.1006928	0.4561325	0.355439643	13.451
RASGEF1A	0.1152567	0.47041	0.35515328	59.554, 8.652
CFD	0.2801656	0.6299344	0.349768867	2.453
NRIP3	0.1220335	0.47116	0.349126458	7.357, 3.904
BASP1	0.3352765	0.6838573	0.348580787	41.935
SFN	0.5985111	0.9465868	0.3480757	15.734, 5.463, 1.547
ATP6V1B1	0.091879	0.437402	0.345523073	2.027, 0.999
NFE2L3	0.1316547	0.4658663	0.334211638	28.889, 20.526
CHODL	0.0760279	0.4087888	0.332760883	34.072
PGCP	0.0903661	0.4225412	0.332175078	5.643, 3.188
THNSL2	0.111158	0.4407438	0.329585785	2.732, 1.468
RAB11FIP4	0.1973342	0.5250608	0.327726567	3.193, 2.058, 1.636
AP1M2	0.1327458	0.4604457	0.327699879	3.119, 1.616
NACAD	0.33309	0.6603458	0.327255808	2.944
WNT2B	0.126284	0.4524965	0.32621242	2.339, 1.0
MAP7	0.2428566	0.5678175	0.324960939	36.396, 5.695, 5.561
C1QTNF4	0.4640128	0.7883627	0.324349896	3.023
LPAR2	0.273576	0.5902439	0.316667924	3.243, 1.589
CDH1	0.1036067	0.4076812	0.304074501	322.784, 2.073
BSPRY	0.0792668	0.3833141	0.304047265	3.361, 2.043
FAM84B	0.1138834	0.4163967	0.302513294	3.179, 1.412