Biological Significance of DNA Methylation on Testicular Tumorigenesis

CHEUNG, Hoi Hung

A Thesis Submitted in Partial Fulfilment

of the Requirements for the Degree of

Doctor of Philosophy

in

Biochemistry (Medicine)

The Chinese University of Hong Kong

April 2010

Thesis/Assessment Committee

Prof. FUNG Kwok Pui (Chair)

Prof. CHAN Wai Yee (Thesis supervisor)

Prof. TSUI Kwok Wing Stephen (Committee member)

Prof. CHO Chi Hin (Committee member)

Abstract

Change of DNA methylation is a hallmark of cancer. It is frequently associated with cancer progression. Testicular germ cell tumor (TGCT) is the most common malignant tumor in young males. Currently, only a limited number of genes are known to be epigenetically changed in TGCT. Genome-wide analysis of differential methylation in a previously established testicular cell line is documented here. A total of 35,208 differentially methylated regions (DMR) were identified. However, only a small number of DMRs mapped to gene promoters. Genome-wide analysis of gene expression revealed a group of differentially expressed genes that were regulated by DNA methylation. Several candidate genes (*APOLD1*, *PCDH10* and *RGAG1*) were found to be dysregulated in TGCT patients. Surprisingly, *APOLD1* was mapped to the TGCT susceptibility locus at 12p13.1, suggesting that it may be important in TGCT pathogenesis.

The majority of DMRs are located in introns or intergenic regions, but their functions are not well understood. Some of these DMRs were found to regulate non-coding RNAs (ncRNAs). In this study, differential methylation of 3 small nucleolar RNAs (snoRNA) and 3 microRNAs (miRNA) were identified. One of the miRNAs, miR-199a, is embedded in a conserved region in intron-14 of dynamin 3 at 1q24.3. Hypermethylation of miR-199a correlated with testicular cancer progression, and silencing of miR-199a. Re-expression of miR-199a in testicular cancer cells suppressed cell growth, cancer migration, invasion, and metastasis. miR-199a-5p, one of two mature miRNA species derived from miR-199a, is associated with cancer progression. An embryonal carcinoma antigen, podocalyxin-like protein 1 (PODXL), was identified to be a target of miR-199a-5p. PODXL is an anti-adhesive protein overexpressed in aggressive testicular cancer. Knockdown of PODXL suppressed cancer invasion. The inverse relationship between PODXL and miR-199a-5p expression suggests that PODXL is one of the downstream effectors mediating cancer invasion and metastasis. This study links DNA methylation, miR-199a dysregulation, and PODXL expression as a mechanism to explain testicular cancer progression.

論文題目 ： DNA 甲基化於睾丸癌的重要性
學生姓名 ： 張凱鴻
導師姓名 ： 陳偉儀 教授
修業學位 ： 哲學博士
學系　　： 生物化學（醫學院）
肄業日期 ： 二零一零年三月

摘要

DNA 甲基化的轉變是癌症的一個標誌。它經常與癌化有關係。睾丸生殖細胞瘤（簡稱睾丸癌或 TGCT）是年輕男性中常見的惡性腫瘤。目前，我們對於睾丸癌的表觀遺傳基因認識有限。使用已建立的睾丸癌細胞株，我分析了全基因組的甲基化差異，從而發現了共 35208 個甲基化差異區域（DMR）。然而，只有少數 DMR 位於基因的啟動子。全基因組基因表達分析顯示，有一組差異表達的基因是受 DNA 甲基化調控。在睾丸癌的病人中，有幾個基因（*APOLD1*，*PCDH10* 和 *RGAG1*）的表達調節失控。令人驚訝的是，*APOLD1* 正好位於睾丸癌易感區域 12p13.1 內，這表明它可能是睾丸癌發病機制的重要因素。

大多數的 DMR 位於內含子或基因間隔區內，但他們的功能不祥。我發現其中一些 DMR 調節非編碼核糖核酸 （ncRNA）。在這項研究中，我證實了 3 個 snoRNA 和 3 個 microRNA（miRNA）的甲基化差異。其中一個 miRNA（miR-199a）的位置在 1q24.3 內 *dynamin* 之第 14 個內含子中。我發現，miR-199a 的甲基化與睾丸癌癌化相關聯，並抑制 miR-199a 的表達。miR-199a 在睾丸癌細胞中重新表達可以抑制癌細胞生長、癌細胞轉移、侵襲和惡化。miR-199a-5p 是 miR-199a 的其中一個成熟 miRNA，它與癌症的發展有關聯。Podocalyxin-like（PODXL）是一個胚胎癌抗原蛋白。它被證實是 miR-199a-5p 的一個標靶。 PODXL 是一種抗黏蛋白，高度表達於惡性睾丸癌。抑制 PODXL 基因可以減低癌細胞的入侵。PODXL 與 miR-199a-5p 的表達相反。其相反關係暗示 PODXL 可能是控制癌細胞入侵和轉移的下游因子。本研究將 DNA 甲基化、miR-199a 調節失控、PODXL 過度表達等現象相聯繫，作為解釋睾丸癌症發展的其中一個機制。

Acknowledgements

I would like to thank Prof. Wai-Yee Chan for his supervision throughout the project. Prof. Chan was previously my mentor at the NIH and now my mentor at CUHK. He gave me a lot of support and inspiration on my research. He also provided me with many opportunities to learn and broaden my horizon. As an alumnus of CUHK, he gave HK students working overseas a lot of support and concerns.

I would also like to thank Dr. Owen M. Rennert for all his support and advice. Dr. Rennert is my NIH sponsor as well as my acting mentor at the NIH. He is a nice and knowledgeable scholar who gave me a lot of advice and suggestion on my project.

I like to thank my labmates at the Laboratory of Clinical Genomics for their kind help in many ways. I express my gratitude to Dr. Tin-Lap Lee, Dr. Shaoming Wu, Dr. Alan Pang, Ms. Vanessa Baxendale and Dr. Catherine Boucheron. I thank the postbaccalaureate students who assisted me in some of the experiments: Mr. Andrew Davis for some of the bisulfite sequencing and immunohistochemistry experiments; Ms. Diana Taft for some of the PCR experiment.

I thank Prof. Chris Lau and all the professors of the Examination Committee for reviewing my thesis. Their critical comments and suggestions help me tremendously in improving the quality of my thesis and are very appreciated.

Lastly, I thank all my friends at the NIH and Washington D.C. area for their supports. The research described in the thesis was support by the Intramural Research Program of the National Institutes of Health (NIH), Eunice Kennedy Shriver National Institute of Child Health and Human Development.

The content of this thesis have been described in the following manuscripts which have been either published or submitted for publication:

1. Cheung HH, Lee TL, Rennert OM, Chan WY. DNA methylation of cancer genome. *Birth Defects Res C Embryo Today*. 2009 Dec; 87(4):335-50.
2. Cheung HH, Lee TL, Davis AJ, Taft DH, Rennert OM, Chan WY. Genome-wide DNA methylation profiling reveals novel epigenetically regulated genes and non-coding RNAs in human testicular cancer. *Br J Cancer*. 2010 Jan 19; 102(2):419-27.
3. Cheung HH, Davis AJ, Lee TL, Pang AL, Nagrani S, Rennert OM, Chan WY. Methylation of an intronic region regulates testicular cancer invasiveness via miR-199a. Manuscript submitted.

Publications that are related to this work:

1. Lee TL, Cheung HH, Claus J, Sastry C, Singh S, Vu L, Rennert O, Chan WY. GermSAGE: a comprehensive SAGE database for transcript discovery on male germ cell development. *Nucleic Acids Res*. 2009 Jan; 37(Database issue):D891-7.
2. Lee TL, Li Y, Cheung HH, Claus J, Singh S, Sastry C, Rennert OM, Lau YF, Chan WY. GonadSAGE: a comprehensive SAGE database for transcript discovery on male embryonic gonad development. *Bioinformatics*. 2010 Feb 15; 26(4):585-6.

Table of Contents

List of Tables

List of Figures

List of Supplementary Materials

Abbreviations

%	Percent/Percentage
5-aza	5-aza-2-deoxycytidine
ANOVA	analysis of variance
bp	base pair
cDNA	complementary DNA
CGI	CpG island
ChIP	chromatin immunoprecipitation
Ct	threshold of cycle number
DMEM	Dulbecco's Modified Eagle Medium
DMR	differentially methylated region
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
FFPE	formalin-fixed, paraffin-embedded
GFP	green fluorescent protein
H&E	hematoxylin and eosin
IgG	immunoglobulin G
kb	kilobase
MeDIP	methylated DNA immunoprecipitation
miRNA	microRNA
ml	millilitre
µl	microlitre
µM	micromolar
mM	millimolar
µm	micrometer
ncRNA	non-coding RNA
ng	nanogram
NT2	Ntera-2 embryonal carcinoma
°C	degree Celsius
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
qPCR	quantitative PCR
RMA	robust multiarray average
RNA	ribonucleic acid
RNAi	RNA interference

rRNA	ribosomal RNA
RT	reverse transcription
s.e.m.	standard error mean
SDS	sodium dodecyl sulfate
shRNA	short hairpin RNA
snoRNA	small nucleolar RNA
TAS	Tiling Analysis Software
TGCT	testicular germ cell tumor
UCSC	University of California Santa Cruz
UTR	untranslated region
µg	microgram

Chapter 1

Introduction and a Review on DNA Methylation of Cancer Genome

1.1 Introduction

When normal cells are transformed into cancer cells, a series of genetic lesions and/or epigenetic disruptions that favor the uncontrolled growth of cells occur. Mutation of tumor suppressor genes, such as p53, leads to loss of function of the protein that is normally required for non-transformed cells. Epigenetic changes including global DNA hypomethylation and hypermethylation of tumor suppressor genes are frequently observed in cancer cells. Such changes cause genomic instability that increases mitotic recombination or silencing of tumor suppressor genes which play critical roles in the control of cell proliferation and transformation. In this chapter, I discuss the role of DNA methylation in cancer cells and summarize recent advancements of techniques that facilitate genome-wide study of the cancer epigenome.

1.1.1 DNA methylation as an important epigenetic modification of the genome

Methylation is the only known epigenetic modification of DNA. Other epigenetic marks of chromatins include different types of post-translational modifications of histones, which are highly diverse and some are closely correlated with DNA methylation (refer to the review by Kouzarides on histone modification and their function) (Kouzarides, 2007). DNA methylation is important as it is a well-known crucial regulator in dif-

ferent biological processes such as embryonic development, transcription, chromatin structure, X chromosome inactivation, genomic imprinting, genomic instability and carcinogenesis. Methylation of DNA occurs exclusively in 5-cytosine. In mammals, the majority of cytosine methylation is observed in CpG dinucleotides. Non-CpG methylation is rare and likely restricted to embryonic stem cells (Ramsahoye *et al*, 2000). Since transcriptionally active regions of the genome are usually CpG rich, methylation of CpG sites is one of the critical factors that affect gene transcription. Many regions of the genome contain an especially high frequency of CpG sites. These regions are called CpG islands and they represent approximately 70% of human promoters (Saxonov *et al*, 2006). In normal somatic cells, most of the CpG islands are unmethylated. Aberrant hypermethylation of some tumor suppressor genes is acquired during tumorigenesis. The reason of aberrant methylation is largely unknown. It might be caused by dysregulation of the methyltransferases of DNA or other chromatin binding proteins.

1.1.2 Molecular basis of DNA Methylation

The pattern of DNA methylation is dynamic during development but becomes relatively static in differentiated cells. This unique epigenetic code is heritable and thus, a mechanism for regulation of the methylome is required. Currently three DNA methyl-

transferases have been identified, namely, DNMT1, DNMT3A, DNMT3B, respectively. These developmentally regulated genes play critical roles in the establishment and maintenance of DNA methylation.

DNMT1 is responsible for the maintenance of cytosine methylation. The epigenetic "code" is heritable. Methylation of cytosine is passed from parental cells to daughter cells if epigenetic marks have been stably established. As DNA replicates, DNMT1 methylates the newly synthesized, hemimethylated DNA in cooperation with MECP2. MECP2 is a methyl-CpG-binding protein that recognizes methylated CpG sites and, when associated with DNMT1, forms a complex to copy the parental DNA methylation to the daughter DNA strands during cell division (Kimura & Shiota, 2003). The function of DNMT1 is far more complicated than just methylation maintenance. DNMT1 interacts with a variety of proteins such as transcription factors (p53, STAT3 and HP1), histone modifiers (HDAC1, HDAC2) and ligands (DAXX) to specifically repress targeted genes (Esteve *et al*, 2005; Muromoto *et al*, 2004; Robertson *et al*, 2000; Rountree *et al*, 2000; Smallwood *et al*, 2007; Zhang *et al*, 2005a). Furthermore, DNA methyltransferases (DNMT1, DNMT3A and DNMT3B) interact with polycomb group (PcG) protein EZH2 to methylate EZH2-binding promoters, suggesting that the two major epigenetic repression systems are closely connected (Vire *et al*, 2006). Mutation of

Dnmt1 in murine embryonic stem (ES) cells causes reduction of two third of cytosine methylation in the genome and demethylation of endogenous retroviral DNA. Germline mutation of *Dnmt1* causes abnormal development and embryonic lethality (Li *et al*, 1992).

DNA methyltransferase-3 proteins are implicated in *de novo* methylation of CpG islands. DNMT3A is involved in parental imprinting. Imprinted genes are exclusively methylated in either parental allele and are therefore monoallelically expressed. Knockout of *Dnmt3a* or *Dnmt3b* in mice blocks *de novo* methylation and leads to lethality (Okano *et al*, 1999). However, conditional knockout of *Dnmt3a* in male germ cells causes impaired spermatogenesis and loss of paternal imprinting. Offsprings of *Dnmt3a* conditional mutant females die *in utero* due to the lack of maternal imprinting on *Peg3* and *Snrpn*. But *Dnmt3b* conditional mutants and their offspring show no apparent phenotype (Kaneda *et al*, 2004).

Unlike DNMT3A and DNMT3B, DNMT3L does not show any methyltransferation activity. It is a cofactor that enhances the *de novo* methylation activity of DNMT3A (Chedin *et al*, 2002). Disruption of *Dnmt3L* in mouse results in the failure of establish-

ment of maternal methylation imprints, indicating that this cofactor is as important as Dnmt3a and Dnmt3b in the acquisition of methylation imprinting (Bourc'his *et al*, 2001).

1.1.3 DNA Methylation as a repressive epigenetic mark

DNA methylation is an important regulator in many biological processes. In mammals, DNA methylation is essential for normal development. Defect in methylation causes diseases.

The mechanism of gene regulation in eukaryotic cells is more complicated than that in prokaryotic cells. Histone proteins provide an additional layer of gene regulation through epigenetic marks on histones or DNA. Double-stranded DNA wraps histone proteins to form chromatin. The state of chromatin can be either "active" or "silent", depending on the interaction between transcriptional factors and the cis-acting elements (promoters or enhancers) of the genes. It is well known that hypermethylated promoters are usually associated with gene repression. Inhibition of *de novo* methylation with methyltransferase inhibitors such as 5-azacytidine and 5-aza-2'-deoxycytidine can restore the expression of methylation silenced genes (del Senno *et al*, 1986). The mechanism by which the gain of methyl groups in CpG sites shuts down gene expression is not clear. The first proposed model for this mechanism is that methyl groups in

promoters provide a physical barrier to accessibility by transcription factors. Many transcription factors such as AP-2, c-myc, CREB/ATF, E2F, MLTF/USF and NF-kB are known to bind promoters with unmethylated CpG dinucleotides, but fail to bind methylated CpG sequences. However, transcription factor like CTF and Sp1 are insensitive to methyl-CpG, suggesting that DNA methylation only affects the transcription of a subset of methylated genes (Tate & Bird, 1993). The second model of methylation mediated gene repression involves a family of methyl-binding proteins. Complexes of methyl-CpG-binding protein-1 (MECP1) and protein-2 (MECP2) preferentially bind to methylated CpG sites and inhibit transcription (Boyes & Bird, 1991; Nan *et al*, 1993). These complexes contain several methyl-CpG-binding domain (MBD) proteins (MBD1, MBD2, MBD3, MBD4 and Kaiso) that bind to methylated CpG sites to suppress transcription initiation. Binding of MECP complexes to methylated promoters either prohibits access of transcription factors, or recruits histone deacetylase, another repressive epigenetic modification enzyme, to achieve gene silencing (Ng *et al*, 1999).

Another mode of transcription regulation involves the binding of the CTCF protein to Imprint Control Regions (ICR) of imprinted genes. The role of CTCF protein in the regulation of monoallelic expression of *H19/Igf2* locus has been well studied. In this model, the ICR is located between the *Igf2* and *H19* genes. The paternally methylated

ICR prevents binding of CTCF protein to the insulator sequence thereby permits the downstream enhancer to activate *Igf2* expression but suppress the expression of *H19* (Bell & Felsenfeld, 2000; Hark *et al*, 2000). The binding of CTCF protein is controlled by the methylation of the ICR. This is another illustration of DNA methylation mediated gene regulation.

1.1.4 Genome-wide demethylation and establishment of methylation during development

The pattern of DNA methylation in somatic cells changes during embryonic development until they fully differentiate and gain tissue-specific methylation. In germ cells, differential methylation between the male and female genome occurs at different stages of development.

In mammals, there are two waves of global demethylation during development. Soon after fertilization, the highly methylated gametes are actively demethylated, a process called reprogramming. However, demethylation is not synchronized between the male and female genomes. In the zygote, the highly methylated male genome is rapidly demethylated only hours after fertilization before the first round of DNA replication commences (Mayer *et al*, 2000; Oswald *et al*, 2000). Reprogramming of the male

genome is believed to be an active process that involves the demethylation of DNA and remodeling of sperm chromatin where the sperm-specific protamines are replaced by acetylated histones. Demethylation of the maternal genome is thought to be a passive process in which DNA replication dilutes the methylome in the absence of nuclear Dnmt1. Both parental genomes gain methylation during implantation, possibly with the participation of Dnmt3a and Dnmt3b. It should be noted that imprinted genes are protected from the first wave of global demethylation. The protection of imprinted genes from demethylation in the zygote ensures proper monoallelic expression of imprinted genes, many of which are important in the early stage of embryogenesis. The second wave of global demethylation occurs in primordial germ cells (PGC) prior to gametogenesis. Between 10.5 and 11.5 days post coitum (dpc), murine PGCs migrate to the genital ridge where they differentiate into gonocytes. A rapid and active erasure of DNA methylation of regions within imprinted loci commences between 10.5 and 13.5 dpc in both male and female embryos (Hajkova *et al*, 2002). During this period imprinted genes such as *H19* are demethylated in their differential methylated region (DMR) (Hajkova *et al*, 2002; Sato *et al*, 2003). Methylation in imprinted regions is acquired before birth on 13.5 dpc and continues after birth. The timing of re-establishment of different imprinted genes in the two sexes is different.

Although several methyltransferases have been found to be responsible for maintenance (Dnmt1) and establishment (Dnmt3a, Dnmt3b and Dnmt3L) of methylation, rapid demethylation of the zygote after fertilization and erasure of the methylated imprinted regions in PGCs suggest that there exists a temporally controlled demethylase for this active process. However, the existence of DNA demethylases is still controversial, although MBD2 is proposed to be a demethylase in addition to methyl-CpG-binding protein (Detich *et al*, 2002; Ng *et al*, 1999).

1.2 Aberrant methylation in cancers

The genome is subjected to a series of genetic and/or epigenetic alterations when normal cells are transformed to neoplasm. This can be caused by prolonged exposure to carcinogens, viral infection, imbalance of hormones, spontaneous mutation of tumor suppressor genes, or any disruption in the epigenome that favors the growth of tumor cells. Tumor cells gain survival advantage as their proliferation rate overcomes apoptosis. These cells become malignant cancer if they acquire the capability to invade adjacent tissues or further migrate to distant organs. Studies of cancer genomes reveal different molecular mechanisms that lead to tumorigenesis. These include the gain or loss of genetic materials (copy number variation), mutation of genes, or disruption of the epigenome that alters gene activity without changing the DNA sequence. Usually, cancers are formed as a consequence of multiple effects. Many cancers are found to be associated with changes in the epigenome that dysregulate normal transcriptome. Aberrant DNA methylation is frequently observed and considered to be a hallmark of cancers. Disruption of methylation can be global or localized. Global hypomethylation in repetitive DNA sequences destabilizes the chromosomes and increases the rate of genomic rearrangement. Alternatively, hypermethylation in CpG islands of tumor suppressor genes prevents these genes from inhibiting tumorigenesis.

1.2.1 Hypermethylation of tumor suppressor genes in cancers

Hypermethylation is more frequently reported than hypomethylation in cancers. CpG islands play an important role in the regulation of gene transcription. In normal somatic cells, most CpG islands are unmethylated. However, acquisition of methylation in particular CpG islands is observed in almost all types of primary tumors as compared to their normal counterparts. The mechanism of cancer hypermethylation is not fully understood. Several studies have shown that this might involve the interaction of the *de novo* methyltransferase DNMT1 and other DNA binding proteins. For example, DNMT1 forms complex with Rb, E2F1 and HDAC1 to repress transcription from promoters containing E2F-binding sites in cancer cells (Robertson *et al*, 2000). Moreover, DNMT1 interacts with p53 to repress p53 responsive genes *Survivin* and *Cdc25C* (Esteve *et al*, 2005). Since DNMT1 shows low sequence specificity, targeted methylation is possibly achieved through interaction between DNA binding proteins (which binds to DNA with a particular consensus sequence) and DNMT1, and probably other histone modifiers such as HDAC.

Numerous reports show that DNA hypermethylation can occur in many genes involved in different biochemical pathways that are related to tumor development or

progression. **Table 1.1** summarizes the most frequently reported genes that are silenced by DNA methylation; many of them demonstrate hypermethylation in CpG islands. These genes regulate a number of cellular processes including cell cycle (*CDKN2A/p16-INK4, CDKN2B/p15-INK4B, CCND2, RB1*), DNA repair (*MGMT, BRCA1, MLH1*), apoptosis (*DAPK, TMS1, TP73*), metastasis (*CDH1, CDH13, PCDH10*), detoxification (*GSTP1*), hormone response (*ESR1, ESR2*), Ras signaling (*RASSF1*), and Wnt signaling (*APC, DKK1*). Hypermethyation of some genes such as *CDKN2A/p16-INK4, RASSF1,* and *MGMT* is observed in many types of cancer while hypermethylation of others appears to be limited to a particular cancer type. These genes include *BEX1* and *BEX2* in glioma (Foltz *et al*, 2006), *PPP1R13B* in acute leukemia (Roman-Gomez *et al*, 2005b), and *PRSS21* in testicular germ cell tumors (Kempkensteffen *et al*, 2006). Certain cancer types appear to be more vulnerable to epigenetic disruptions. According to the cancer methylation database PubMeth, the most often reported cancers associated with DNA hypermethylation are lung, gastric, colorectal, leukemia, brain, liver, breast, and prostate cancers (Ongenaert *et al*, 2008). However, the prevalence of reports on hypermethylation in these major cancers does not indicate the infrequency of methylation disruption in other cancer types. Rare malignant tumors such as testicular germ cell tu-

mors have been known to be epigenetically changed as other major tumors, although many of the disrupted genes reflect the origin of the tumors (Lind *et al*, 2007).

Table 1.1 Most frequently reported genes that are hypermethylated in human cancers

Genes	Role in carcinogenesis	Most frequently reported cancer types	No. of papers to date	Key references
CDKN2A /p16-INK4	CDK inhibitor; induce cell cycle arrest in G1 and G2 phases	Lung, gastric, colorectal, liver, brain, leukemia, lymphoma	>450	(Nakata *et al*, 2006; Nosho *et al*, 2007; Oue *et al*, 2003)
RASSF1	Inhibits proliferation through negatively regulating cell cycle progression at G1/S phase transition by inhibiting accumulation of cyclin D1	Lung, ovarian, brain, liver, thyroid, cervical, kidney	>270	(Hesson *et al*, 2004; Kim *et al*, 2003; Teodoridis *et al*, 2005)
MGMT	Cellular defense against the biological effects of O6-methylguanine in DNA; involved in DNA repair and drug resistance	Brain, colorectal, lung, gastric	>210	(Hanabata *et al*, 2004; Ogino *et al*, 2007; Yu *et al*, 2004)
CDH1	Responsible for cell adhesion; downregulation results in increased cell motility and cancer progression and invasion	Gastric, lung, leukemia	>190	(Nakata *et al*, 2006; Oue *et al*, 2003; Roman-Gomez *et al*, 2005b)
DAPK1	Death-associated protein kinase which acts as a positive regulator of apoptosis	Lung, lymphoma, gastric, cervical, bladder	>150	(Chan *et al*, 2005; Kang *et al*, 2003; Kim *et al*, 2001)
MLH1	Responsible for DNA mismatch repair; also implicated in DNA damage signaling	Gastric, colorectal, endometrial	>140	(Nan *et al*, 2005; Ogino *et al*, 2007; To *et al*, 2002)
APC	Tumor suppressor which acts as an antagonist of the Wnt signaling pathway; also involved in cell migration and adhesion, transcriptional activation and apoptosis	Colorectal, lung, gastric, prostate, breast	>130	(Arnold *et al*, 2004; Sarbia *et al*, 2004; Suzuki *et al*, 2006)
CDKN2B /p15-INK4B	Interacts with CDK4 and CDK6 and inhibits CDK kinases; cell growth regulator that controls cell cycle G1 progression	Leukemia, lymphoma	>120	(Chim *et al*, 2003; Shiozawa *et al*, 2006)

GSTP1	Plays a role in detoxification by catalyzing the conjugation of many hydrophobic and electrophilic compounds with reduced glutathione; loss of GSTP1 increases susceptibility to cancer	Prostate, liver, lung	>110	(Woodson *et al*, 2003; Zhang *et al*, 2005b; Zochbauer-Muller *et al*, 2001)
RARB	Receptor for retinoic acid; limits its growth of many cell types by regulating gene expression	Lung, prostate, kidney	>100	(Dulaimi *et al*, 2004; Florl *et al*, 2004; Maruyama *et al*, 2004)
TIMP3	Inhibitor of the matrix metalloproteinases, induced in response to mitogenic stimulation	Brain, gastric, kidney, lung	>70	(Bachman *et al*, 1999; Dulaimi *et al*, 2004; Kang *et al*, 2003)
FHIT	Cleaves A-5'-PPP-5'A to yield AMP and ADP; possible tumor suppressor	Lung, oesophageal, ovarian, cervical, leukemia	>50	(Hong *et al*, 2005; Lee *et al*, 2006a; Nakata *et al*, 2006)
ESR1	Estrogen receptor for the regulation of eukaryotic gene expression and affects cellular proliferation and differentiation; commonly involved in pathology of breast cancer	Breast, leukemia, prostate, lung, thyroid	>50	(Garcia-Manero *et al*, 2002; Li *et al*, 2004; Widschwendter *et al*, 2004b)
TP73	Participates in the apoptotic response to DNA damage	Brain, leukemia, lymphoma	>50	(Roman-Gomez *et al*, 2004; Siu *et al*, 2002; Yu *et al*, 2004)

Defect of cell cycle control is one of the characteristics of cancer cells. This explains why suppression of genes involved in cell cycle control is so common. *RASSF1* is a tumor suppressor gene known to inhibit cell proliferation by negatively regulating cell cycle progression at G1/S phase transition through inhibiting accumulation of cyclin D1 (Shivakumar *et al*, 2002). Hypermethylation of *RASSF1* is prevalent in a wide variety of cancers, probably reflecting the intrinsic factors common to tumorigenesis (Yu *et al*, 2003). Aberrant methylation is also found in genes of signaling pathways. Hypermethylation of *SOCS-1*, for example, leads to the activation of the STAT3 pathways in head and neck squamous cell carcinomas (Lee *et al*, 2006b).

Cancer cells usually acquire aberrant methylation of multiple tumor-related genes that cooperate to confer survival advantage of neoplastic cells (Lee *et al*, 2002; Leung *et al*, 2001). Clinical studies must include a statistically significant sample size to reveal the frequency of aberrant methylation. A considerable variation of the frequency for a certain tumor suppressor gene is observed in different types of cancers, probably due to the different grades of cancers and different sample sizes.

1.2.2 Epigenetic reactivation of oncogenes by hypomethylation

The human cancer genome was first found to be hypomethylated in 1983 (Feinberg & Vogelstein, 1983). Global hypomethylation and the resulting genomic instability are regarded as hallmarks of cancers today. It is generally thought that global hypomethylation occurs early in tumorigenesis and predisposes cells to genomic instability and further genetic changes. Gene specific demethylation appears at a later stage. This allows tumor cells to adapt to their local environment and promote metastasis (Robertson, 2005). Hypomethylation has also been found to be correlated with tumor progression and cancer metastasis (Widschwendter *et al*, 2004a).

In contrast to hypermethylation that leads to gene silencing, hypomethylation of genes is usually accompanied with reactivation of transcription. In cancers, hypomethylation is often associated with oncogenes. *c-Myc*, a transcription factor that acts as an oncogene, is one of the widely reported hypomethylated genes in cancers. Hypomethylation of *c-Myc* was first found in cultured cell lines in 1984 (Cheah *et al*, 1984), and subsequently identified in other cancers such as hepatocellular carcinoma (Kaneko *et al*, 1985; Nambu *et al*, 1987), leukemia (Tsukamoto *et al*, 1992), and gastric carcinoma (Fang *et al*, 1996). Its methylation is also known to be associated with bladder and colo-

rectal cancer progression (Del Senno *et al*, 1989; Sharrard *et al*, 1992). The cancer-testis gene *MAGE* (*melanoma antigen*) is normally expressed in germ cells only, but reactivated in various tumor types. Reactivation by demethylation was observed during gastric cancer progression (Honda *et al*, 2004). Promoter hypomethylation and reactivation of *MAGE-A1* and *MAGE-A3* was also observed in colorectal cancer cell lines and cancer tissues (Kim *et al*, 2006). Moreover, hypomethylation of *P-cadherin* (*CDH3*) was found in colorectal cancinogenesis (Milicic *et al*, 2008) as well as in invasive breast carcinomas (Paredes *et al*, 2005). *c-Ha-Ras* is another hypomethylated oncogene involved in signal transduction by activating several cascades of kinases which lead to growth, differentiation, apoptosis or senescence. Hypomethylation of *c-Ha-Ras* was reported in gastric carcinoma (Fang *et al*, 1996). DNA hypomethylation of the oncogene *synuclein gamma* (*SNCG*) causes it to be over-expressed in breast and ovarian cancers (Gupta *et al*, 2003), gastric cancer (Yanagawa *et al*, 2004), and liver cancer (Zhao *et al*, 2006).

In addition, many other genes were found to be hypomethylated and reactivated in cancers, although their role in oncogenesis needs to be confirmed. These include *PSG* in testicular germ cell cancer (Cheung et al., unpublished observations), *WNT5A*, *CRIP1* and *S100P* in prostate cancer (Wang *et al*, 2007), *L1 cell adhesion mole-*

cule (*L1CAM*) in colorectal cancer (Kato *et al*, 2009), and the cancer/testis antigen gene *XAGE-1* in gastric cancers (Lim *et al*, 2005).

1.2.3 Global hypomethylation in repetitive sequence and their role in genomic instability

Although global hypomethylation was found in a wide variety of tumors, the role of hypomethylation is not fully understood. It raises the question that whether hypomethylation is the consequence of tumor transformation or the cause of tumorigenesis. The question could possibly be answered by genetic deletion of *Dnmt1*, the only known methyltransferase for methylation maintenance. However, since homozygous Dnmt1 knockout mice are lethal during gestation (Lei *et al*, 1996), a modified animal model is essential for studying hypomethylation *in vivo*. In a study, a hypomorphic allele of *Dnmt1* was combined with a null allele to generate the heterozygous mice in which the endogenous Dnmt1 level was reduced to 10%. Cells of the heterozygotes displayed genome-wide hypomethylation in all tissues. The mice developed T cell lymphomas and had a high frequency of chromosome 15 trisomy (Gaudet *et al*, 2003). These experiments suggest that DNA hypomethylation plays a crucial role in tumor development by promoting chromosomal instability.

Pericentromeric heterochromatin contains tightly packed repetitive DNA sequences (*LINE*, *SINE*, *IAP*, and *Alu* elements). In normal cells heterochromatin is highly methylated and epigenetically silenced to reduce transcriptional noise. In cancers, global demethylation is commonly observed. Methylation of *LINE-1* (long interspersed nucleotide elements) helps to maintain genomic stability and integrity. Loss of methylation increases genomic instability and results in a higher chance of mitotic recombination, both of which are frequently observed in tumor development.

Global hypomethylation of *LINE-1* is widely reported in different cancer types, including colorectal cancer (Estecio *et al*, 2007; Ogino *et al*, 2008), urothelial carcinoma (Jurgens *et al*, 1996), malignant germ cell tumors (Alves *et al*, 1996), ovarian cancer (Pattamadilok *et al*, 2008), cervical cancer (Shuangshoti *et al*, 2007), neuroendocrine tumors (Choi *et al*, 2007), prostate cancer (Cho *et al*, 2007), and chronic myeloid leukemia (Roman-Gomez *et al*, 2005a). In a study using pyrosequencing to determine the methylation status of *LINE-1* and *Alu* sequences in 48 primary non-small cell carcinomas, hypomethylation of the retrotransposable elements was found to correlate with genomic instability (Daskalos *et al*, 2009). It was therefore proposed as a surrogate marker for cancer-linked genome demethylation (Ogino *et al*, 2008).

1.2.4 Aberrant methylation in non-coding regions

Genome-wide methylation profiling reveals a large number of differentially methylated regions (DMRs) in cancer cells. However, a small proportion of DMRs are mapped to gene promoters (Cheung et al., 2010; Chapter 2). The majority of DMRs are located in intergenic regions or introns. It is yet a puzzle why the cancer genome displays differential methylation in these "non-regulatory" regions. One of the possible functions of intergenic and intronic DMRs is to regulate the expression of non-coding RNAs (ncRNA). Many ncRNAs such as miRNAs and snoRNAs are located in intergenic or intronic regions. Some are expressed through the action of independent promoters while others might be the splicing products of the host mRNAs (for intronic ncRNAs). It is estimated that half of the miRNAs are associated with CpG islands (Weber *et al*, 2007a). Several studies attempt to reveal the role of DNA methylation on regulation of miRNAs (Datta *et al*, 2008; Lujambio *et al*, 2008; Saito *et al*, 2006). Demethylation of cancer cell lines by 5-aza-2'-deoxycytidine restored expression of these miRNAs, indicating that like many tumor suppressor genes, miRNA is another class of ncRNAs that is epigenetically disrupted. In Chapter 2, I report that miR-199a and miR-184 were reactivated by 5-aza-2'-deoxycytidine treatment of embryonal carcinoma cells. Both miRNAs are hypermethylated in intronic and intergenic regions respectively. In another study,

miR-148a, miR-34b/c and miR-9 were found to be silenced by DNA methylation. These epigenetically regulated miRNAs act as tumor suppressors that contribute to suppression of cancer development and metastasis (Lujambio *et al*, 2008). Other hypermethylated miRNAs in cancers include miR-127 as a negative regulator of proto-oncogene *BCL6* (Saito *et al*, 2006), miR-124 as a negative regulator of *CDK6* (Lujambio *et al*, 2007), and miR-1 in hepatocellular carcinogenesis (Datta *et al*, 2008). It is anticipated that more DNA methylation regulated miRNAs will be identified by genome-wide analysis of cancer methylomes.

1.3 Genome-wide studies of cancer methylome

1.3.1 Introduction

The majority of current evidence linking DNA methylation, transcriptional regulation, and disease are derived from cancer research. Significant changes in global DNA methylation have been observed in cultured cancer cells and primary human tumor tissues. These changes include global DNA hypomethylation of centromeric repeats, repetitive sequences, and gene-specific hypermethylation of CpG islands (Lister & Ecker, 2009). Over the last decade the number of studies on the role of DNA methylation in cancer development has grown dramatically and "cancer epigenetics" is now the focus of many exciting and significant advances in cancer research. Diagnosis, prognosis and therapeutic regimes relating to DNA methylation are on the horizon. However, the understanding of the biological significance of aberrant DNA methylation in the cancer genome remains limited. This is largely due to the lack of high-throughput technologies and relevant genome information. In the past, DNA methylation analysis was usually performed on a single gene using qualitative or quantitative polymerase chain reaction (PCR)-based methods. Common ones include methylation specific PCR (MSP) (Licchesi & Herman, 2009), combined bisulfite restriction analysis (COBRA) (Xiong & Laird, 1997),

methylation sensitive single nucleotide primer extension (Ms-SNuPE) (Gonzalgo & Jones, 2002), small scale bisulfite sequencing (Frommer *et al*, 1992), and quantitative methylation-specific PCR (QMSP, also known as Methylight) (Jeronimo *et al*, 2001). Each method has its advantages and disadvantages (**Table 1.2**). It was costly and ineffective to survey whole-genome DNA methylation using these methods. In fact, only about 0.1% of the reported studies examined detailed DNA methylation in the genome (Schumacher *et al*, 2006).

With the completion of various genome projects and recent developments in high-throughput and whole-genome profiling techniques, large scale DNA methylation analysis has become feasible. Unlike whole genome transcriptome assays that are based on unified RNA sequence annotation, the design of whole genome methylome assays are more complicated due to the elusive and dynamic pattern of cytosine methylation in the genome. Such DNA modification, usually referred to as the "fifth base" (Bird, 1986), was not included in the original genome projects. There is no universal reference available for designing probes or assays to differentiate the "fifth base" from the unmethylated cytosine. Therefore, despite the wide availability of whole genome expression assays, identification of sites of DNA methylation throughout a genome has not been possible until recently. The full extent of the effect of global DNA methylation

on gene expression and chromatin structure remains largely unknown. The challenge has been overcome by recent availability of highly specific antibodies, high density microarrays, and massive parallel sequencing technologies. These technologies enable global mapping of this epigenetic modification at a very high or even single base resolution, providing new insights into the regulation and dynamics of DNA methylation in genomes. A number of global methylation methods are available. The differences are the resolution, features of DNA surveyed, and the qualitative or quantitative nature of the method.

Table 1.2 Methylation assays

Method	Feature	Disadvantages	Quantitaive / qualitative
Methylation specific PCR (MSP)	Rapid and sensitive, reduce false positives due to incomplete enzymatic digestion.	Primer design is not trivial. Not quantitative.	Qualitative
Combined bisulfite restriction analysis (COBRA)	Specific region methylation analysis by bisulfite conversion and restriction enzyme digestion	Bias to restriction targets	Semi-quantitative
Methylation sensitive single nucleotide primer extension (Ms-SNuPE)	Specific region methylation analysis by bisulfite conversion and radioactive incorporation	Radioactive, labor intensive	Quantitative
Quantitative methylation-specific PCR (QMSP)	Rapid and sensitive, reduce false positives due to incomplete enzymatic digestion. Use of real-time PCR	Primer design is not trivial. More costly compared to MSP	Quantitative
Bisufite sequencing	Single base-resolution, identification of exact location of methylcytosine	Costly and labor intensive. Clonal selection bias	Qualitative
Bisufite-single strand conformation polymorphism	Sensitive and high resolution	Requires minimal level of sequence alteration for single-base detection	Semi-quantitative

The procedure of whole genome DNA methylation profiling can be divided into two steps: the first step is to identify and enrich methylcytosines in the DNA sample (**Figure 1.1**). Common methods include: 1) restriction enzyme-based method; 2) chromatin immunoprecipitation (ChIP); and 3) bisulfite conversion. The second step involves capturing the enriched or chemically modified DNA by high-throughput and high resolution whole genome assays that use high density tiling microarrays or massive parallel sequencing.

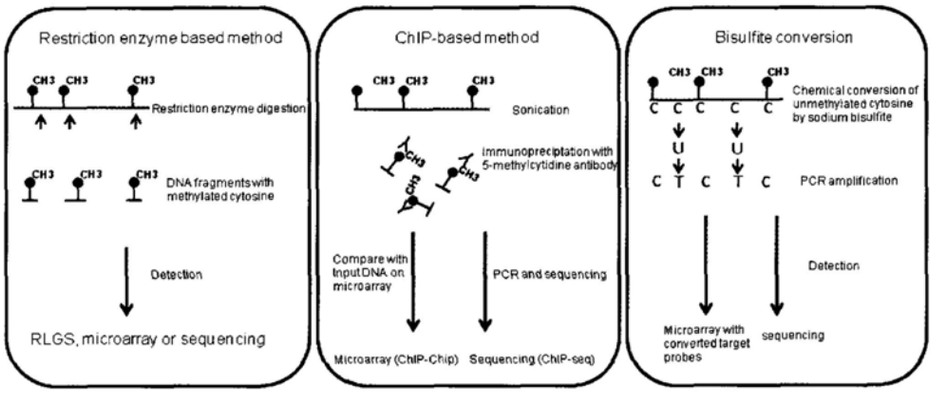

Figure 1.1 Methods of methylome analysis

1.3.2 First step in global methylome mapping

1. Restriction enzyme-based method

Digestion with methylation-sensitive restriction enzyme followed by Southern blot analysis was employed to examine the overall methylation status of CpG islands (Reilly *et al*, 1982). However, this approach does not provide information of methylcytosine in a specific sequence context. This approach is further hampered by the efficiency of restriction enzyme digestion and the amount of input DNA (> 5 μg) required. Replacing Southern blot analysis with PCR in subsequent modifications (e.g. COBRA) allows the application in small scale DNA methylation analysis. Restriction enzyme-based method can also be combined with other experimental approaches to gain global methylation information, including restriction landmark genomic scanning (RLGS) (Akama *et al*, 1997), array-based differential methylation hybridization (DMH)/Array-PRIMES (Huang *et al*, 1999) and HpaII tiny fragment enrichment by ligation-mediated PCR (HELP) (Khulan *et al*, 2006).

Restriction landmark genomic scanning (RLGS)

RLGS is a two-dimensional gel electrophoresis approach based on the use of methylation-sensitive restriction enzymes (e.g. *Not*I). Up to 2,000 end-labeled landmark

sites can be displayed in a single RLGS experiment. The labeling of the sites is based on incorporation of radionucleotides into the restriction site by DNA polymerase. Methylated sites are not digested and are not labeled; thus do not contribute to the two-dimensional pattern of RLGS fragments. Spots present in a normal profile, but absent in a tumor profile represent methylation of the landmark site. It allows quantitative global DNA methylation analysis in the context of CpG islands. This approach provides a platform for the simultaneous assessment of over 2000 CpG islands (Hatada *et al*, 1991; Okazaki *et al*, 1995).

The main strength of RLGS resides in its unbiased approach towards the analysis of CpG islands irrespective of their association with known genes, thus providing a unique tool for the discovery of novel hypermethylated sequences in mammalian genomes. In addition, it can be applied to any genome without prior knowledge of DNA sequence. RLGS has been used in the identification of novel imprinted genes and genes frequently hypermethylated (Blanchard *et al*, 2003; Costello *et al*, 2000; Dai *et al*, 2003; Fruhwald *et al*, 2001; Kuromitsu *et al*, 1995; Motiwala *et al*, 2003; Smiraglia *et al*, 2003; Song *et al*, 2005; Wang *et al*, 2008; Yamagata *et al*, 2009), and genomic hypomethylation (Konishi *et al*, 1996; Morey *et al*, 2006; Nagai *et al*, 1999) and methylation of 3'untranslated regions (Smith *et al*, 2007) in several types of cancers.

Despite its power in the systematic detection of epigenetic alterations due to DNA methylation, the identification of polymorphic spots is difficult with RLGS because the resulting spots contain very little target DNA and many non-labeled DNA fragments. Another major limitation of RLGS is that methylation can only be assessed in CpG islands which contain the sequence for the methylation-sensitive enzyme used in the assay. Sequence polymorphisms in any of the enzyme recognition sequences needed for RLGS or genomic deletions result in the effective loss of signal, which could be incorrectly interpreted as DNA methylation. Finally, the assay requires relatively large amounts of high molecular weight genomic DNA (> 1 μg), which makes this approach unsuitable for the analysis of samples when the amount of DNA available is low or when the DNA is highly fragmented.

Differential methylation hybridization (DMH)

Studies on global changes of DNA methylation at the CpG island level can also be achieved by the combination of restriction enzyme digestion and CpG island microarrays. DMH is the first successful attempt to build an array-based DNA methylation assay. It uses a methylation-insensitive restriction enzyme (MseI) to digest genomic DNA followed by ligation with DNA linkers. The ligation product is then digested with

methylation-sensitive restriction enzymes HpaII and BstUI. The product of the second round of enzyme digestion is amplified by PCR using primers complementary to the linker sequence. The PCR products are then labeled with fluorescent dyes (Cy3 or Cy5) and then hybridized to a CpG island microarray. Similar to other restriction enzyme-based methods, the specificity of DMH depends on the efficient digestion of genomic DNA by methylation-sensitive restriction enzymes. Incomplete digestion could lead to the generation of false-positive results. The technique had been used to successfully identify epigenetic alterations in cancers including breast (Fan *et al*, 2006; Huang *et al*, 1999; Yan *et al*, 2000; Yan *et al*, 2006), ovary (Balch *et al*, 2005), colon (Paz *et al*, 2003), and brain cancers (Felsberg *et al*, 2006; Vladimirova *et al*, 2009; Waha *et al*, 2007).

*Hpa*II tiny fragment enrichment by ligation-mediated PCR (HELP)

HELP assay interrogates cytosine methylation status on a genomic scale (Khulan *et al*, 2006; Oda & Greally, 2009). In this assay, two restriction enzymes (HpaII and MspI) are used. HpaII only cleaves sites where the cytosine in the CpG is not methylated. Resulting DNA fragments after digestion with each of these enzymes are separately amplified by PCR and labeled with different fluorescent dyes. The particular PCR process used in the HELP assay will produce DNA fragments with a size of 200 bp to 2000 bp

known as HTFs (HpaII tiny fragments). Comparison of the quantity of HTFs derived from MspI and HpaII treatment will reveal the methylation state of the different genomic sites. The relative amounts of MspI and HpaII fragments are compared by hybridizing to tiling microarray. Beside CpG island methylation, it will also provide insights into the distribution of cytosine methylation in other genomic regions.

2. Chromatin immunoprecipitation (ChIP)-based methods

Chromatin immunoprecipitation (ChIP) allows one to investigate interactions between proteins and DNA. It was first applied to study the regulation of *Hsp70* genes in Drosophila (Solomon *et al*, 1988). The technique has also been applied extensively in cancer research (Neff & Armstrong, 2009; Ren & Dynlacht, 2004; Wang, 2005). The procedure involves cross-linking of chromatin proteins-DNA complex by formaldehyde and the generation of short random fragments of the chromatin by sonication. Using antibodies directed against the protein of interest, cross-linked chromatin fragments are immunoprecipitated. The isolated antibody-chromatin-complexes and the input or non-immunoprecipitated materials are treated to remove the crosslink and the DNA is purified. Both control and immunoprecipitated samples are amplified by quantitative PCR using primers specific for the genomic region of interest. With different antibody

combination, ChIP allows for profiling chromatin-associated factors, histone modifications, histone variants as well as local nucleosome density. When ChIP is combined with DNA microarray technology (ChIP-chip), it can be applied in the identification of DNA binding sites for transcriptional factors (Jiang & Pugh, 2009; Rodriguez & Huang, 2005; Wu *et al*, 2006). Combining ChIP with genomic tiling array hybridization or massive-parallel sequencing (ChIP-seq) allows whole genome studies, including global methylome analysis.

ChIP-Chip

Although RLGS has been proven useful in identifying differential methylated regions in a variety of tumors, it is limited to detecting methyl groups at defined restriction sites and the data obtained are limited by the frequency of the restriction enzyme recognition sequence (Smiraglia & Plass, 2002). ChIP-Chip provides an alternative solution to RLGS. Methylated DNA immunoprecipitation (MeDIP or mDIP) (Keshet *et al*, 2006; Mohn *et al*, 2009; Sorensen & Collas, 2009; Thu *et al*, 2009; Weber *et al*, 2005) is a ChIP-chip based method that uses antibody against 5-methylcytosine to capture methylated DNA fragments. Enriched fragments are then detected by hybridizing to genomic tiling microarrays. It is suitable for unbiased interrogation of whole genome me-

thylation to uncover non-CpG island methylation regions. Using MeDIP approach, Weber *et al* showed that only a small set of promoters was methylated differentially, suggesting that aberrant methylation of CpG island promoters in malignancy might be less frequent than previously speculated (Weber *et al*, 2005). Follow-up study also demonstrated CG-depleted regions to be strikingly hypomethylated, manifesting a degree of change greater than those at the CpG tested islands in the same experiment (Weber *et al*, 2007b).

ChIP-seq

ChIP-seq is an alternative method for reading ChIP results by using high-throughput sequencing technologies (Barski & Zhao, 2009; Hoffman & Jones, 2009; Neff & Armstrong, 2009). Similar to MeDIP/mDIP procedure, the methylated DNA is immunoprecipitated with antibody against 5-methylcytosine. The 5' ends of the enriched DNA fragments are sequenced in parallel. Depending on the technology, the sequences are read in short or long fragments known as tags. The tags are assembled and mapped to the reference genome using alignment algorithms (Pettersson *et al*, 2009). The ChIP-seq data provides single base resolution information on methylation and the digital nature of sequencing data allows comparison between different ChIP-

seq experiments directly. The drawbacks of the ChIP-seq approach include high cost, long experiment time, and extensive sequencing required. Significant amount of non-relevant methylation signals from repetitive DNA elements are also included in the dataset.

3. Bisulfite conversion method

Genomic DNA is treated with bisulfite to convert unmethylated cytosine to uracil. Methylated cytosine is not affected by this treatment. This procedure is sensitive and is independent of the presence or absence of restriction enzyme recognition sequence. Similar to ChIP, the chemically modified DNA can be detected by microarrays containing bisulfite-modified targets (Zhou *et al*, 2006) or direct sequencing (Cokus *et al*, 2008; Lister *et al*, 2008; Meissner *et al*, 2008). Unlike classic whole genome sequencing, the Watson and Crick strands of bisulfite-treated sequences are not complementary to each other because bisulfite conversion occurs on cytosine only. As a result, there will be four distinct strands after PCR amplification: BSW (bisulfite Watson), BSWR (reverse complement of BSW), BSC (bisulfite Crick), and BSCR (reverse complement of BSC). This increases the amount of work in the alignment step. It also requires an effective

method in asymmetric C/T matching. Mapping of millions of bisulfite reads to the reference genome remains a computational challenge.

1.3.3 Second step in global methylome mapping

1. Microarray technology

A microarray is a solid support on which DNA of known sequence is deposited. The DNA may take the form of oligonucleotides, cDNA or clones and act as probes to detect sequences present in the sample through hybridization. Depending on resolution, a whole genome human microarray chip could contain more than two millions probes. DNA microarrays were originally developed for high-throughput gene expression analysis. The fast, comprehensive and flexible nature makes it an indispensable tool in the post-genomic era.

Tiling microarrays are high-resolution microarrays made of probes ranging from 5 bp to 60 bp. In contrast to classic microarray design where probes are biased to the annotated gene regions, the probe sequences in tiling microarrays tile along the genome without considering sequence features. The design allows unbiased interrogation of the whole genome. The use of tiling arrays has unveiled that large portion of the human genome is transcribed (Johnson *et al*, 2005; Willingham & Gingeras, 2006). They

are useful in splice variant analysis and the detailed examination of gene structure (Finocchiaro *et al*, 2007). This research so far has challenged our notion on gene definition.

2. ***Massive parallel sequencing technology (the next-gen sequencing)***

The capillary sequencer was the main workhorse of the Human Genome Project. It does not require radiation and polyacrylamide gel electrophoresis as initially invented by Frederick Sanger in the 1970s (Sanger *et al*, 1977; Sanger *et al*, 1992). However, it is still cumbersome and slow, with relatively high cost to run ($0.10 per 1000 bases). This situation was changed in 2005 with the introduction of the 454 sequencer and later the other new players such as Illumina and SOLiD. These sequencing technologies are referred to as "next-gen" sequencing (**Table 1.3**) (Morozova & Marra, 2008).

Table 1.3 The evolution of sequencers

Sequencing technology	Read length (bases)	Runtime (days per gigabase)	Cost ($ per 1000 bases)
Capillary	1000	500	$0.10
454	450	2	$0.02
Illumina (Solexa)	75	0.5	$0.001
SOLiD	50	0.5	$0.001

Source: Wellcome Trust (http://www.wellcome.ac.uk/News/2009/Features/WTX056032.htm)

454

Founded by Jonathan Rothberg, the technology of 454 sequencing (http://www.454.com) was developed by 454 Life Sciences, a Roche company. The method relies on tiny resin bead to anchor the DNA fragments, which are amplified and denatured to single stranded form. The beads are then put into wells on a plate along with enzyme beads. The polymerase and primer attach to the DNA fragment to initiate the sequencing reaction. As the nucleotides are incorporated into the DNA strand, light is given off. Light intensity is proportional to the number of A's, T's, C's or G's incorporated. The latest 454 machine is able to read one gigabase of DNA sequence within days, at a cost of $0.02 per 1000 bases.

Illumia

In 2006, Solexa debuted a new sequencing technology. Instead of using beads for DNA fragment capture, DNA fragments are amplified in dense clusters on a slide to provide stronger fluorescence signals. Fluorescence signals specific to A, T, C and G are read as the bases are incorporated into the DNA fragment template in each cluster. The platform made its mark delivering the first African, Asian and cancer patient genomes. It was acquired by Illumia (http://www.illumina.com) in 2006.

SOLiD

Applied Biosystems rolled out the SOLiD (Sequencing by oligonucleotide ligation and detection) sequencing technology in 2007. Unlike 454 and Illumia platforms that rely on DNA polymerase for replicating new DNA strand a base at a time (sequencing through synthesis), SOLiD sequences by ligation, hybridizing a range of probes to the DNA template. The advantage of this sequencing method is that each base is read twice. This increases the confidence level in genome-wide SNP analysis.

Compared to 454, both SOLiD and Illumina sequence DNA around 20 times cheaper, at about $0.001 per 1000 bases and take just half a day to read one gigabase. They also have the advantage of being able to handle more samples simultaneously.

1.3.4 Conclusion and future direction

Epigenetic changes have been recognized as one of the most important molecular signatures of human tumors in recent years. Aberrant promoter hypermethylation is now considered to be a *bona-fide* mechanism for transcriptional inactivation. Promoter hypermethylation at the CpG islands of certain tumor suppressor genes could lead to the disruption of multiple pathways. Increasing number of hypermethylated genes are implicated to correlate with malignant potential and prognosis in cancer.

The development of DNA methylation markers for early cancer detection holds the promise of being accurate, sensitive, and cost-effective for risk assessment, early diagnosis and prognosis. DNAs from body fluids, blood, serum or tissue samples can be readily obtained by noninvasive or minimally invasive techniques (Cairns, 2007; Chan *et al*, 2002; Lee *et al*, 2002). A panel of markers can be applied to increase the sensitivity and provide a potentially powerful system of biomarkers for developing molecular detection strategies for virtually every form of human cancer. This non-invasive approach will promote epigenetics into one of the most exciting areas in cancer management and translational cancer research.

What makes DNA methylation even more exciting than traditional genetics is that this inheritable change is reversible. Unlike genetic alterations, which are almost impossible to revert, DNA methylation is a reversible event. The epigenetic effect due to DNA hypermethylation can be reversed by using demethylating agents such as DNA methyltransferase (DNMT) inhibitors 5-aza-2'-deoxycytidine. DNA demethylating agents could be potentially developed into standard regiments for cancer therapy. Drugs such as decitabine have shown promising results in clinical trials in solid and liquid tumors (Jabbour *et al*, 2008). 5-azacytidine and 5-aza-2'-deoxyazacytidine have recently been approved for clinical use in the treatment of myelodysplastic syndrome

(MDS) of all types and chronic myelomonocytic leukemia (CMML) (Griffiths & Gore, 2008). In addition, over-expression of both HDAC and DNMT has been demonstrated to be associated with epigenetic inactivation of tumor suppressor genes, as well as cell cycle and apoptosis regulators. The HDAC and DNMT inhibitors possess direct cytotoxic properties, and can sensitize tumor cells to conventional radiotherapy and chemotherapy (Fandy, 2009; Miremadi *et al*, 2007). Preliminary clinical studies have found the combined effects of DNMT and HDAC inhibitors led to complete or partial responses in patients with hematological malignancies (Fabre *et al*, 2008; Griffiths & Gore, 2008; Schneider-Stock & Ocker, 2007). However, due to the non-specific nature of nucleotide analogs, it is critical to monitor the effects in both tumor and normal tissues to ensure that no long-term damage is inflicted. Nevertheless, the use of these inhibitors will open up new and promising possibilities for cancer patient management and treatment.

Despite increasing number of candidate genes affected by DNA methylation in cancer being identified, there are still numerous targets waiting to be discovered. Our understanding of the peculiarities of DNA methylation and its biological effects in the human cancer genome is yet very limited. With the completion of the human genome sequence and the application of high-throughput techniques, various cancer methylomes can be expected to be unmasked in the near future. Emerging evidences from

various methylome studies are striking. They suggest the majority of DMRs are either located outside the CpG islands, or genomic regions without annotations and gene evidence (Keshet *et al*, 2006; Ordway *et al*, 2007; Smith *et al*, 2007; Weber *et al*, 2005; Weber *et al*, 2007b). These observations implicate that non-promoter non-CpG island methylation could play an active role in epigenetic alteration. It is not clear whether DNA methylation changes in these intergenic regions have functional consequences in terms of gene expression or other outcomes. Nevertheless, the data will further provide clues in elucidating the molecular mechanisms of DNA methylation in cancer during neoplastic transformation.

1.4 Hypothesis and project design

As methylation change is common in cancers, I aim at revealing these alterations in human testicular cancer, and studying the biological consequence of such changes. I hypothesize that, like other forms of cancer, the methylation signature of testicular cancer genome can help us understand the epigenetic defects for the disease, providing data for the the elucidation of the molecular mechanism of testicular cancer tumorigenesis.

With the evolution of techniques for global methylation analysis and the emergence of high resolution tiling microarrays, genome-wide profiling of DNA methylation is possible. This project adopted the recently developed MeDIP technique (Weber *et al*, 2005), combined with whole genome microarray hybridization, as a tool to unmask the methylation changes in human testicular cancer. The data obtained are informative and guide us to understand the epigenetic changes in testicular cancer.

In Chapter 2, I document the use of MeDIP with tiling microarray to reveal the global DNA methylation changes in testicular cancer. A large number of DMRs were identified. Many genes and non-coding RNAs (ncRNA) were found to be differentially

methylated. In particular, some genes or ncRNAs are novel and for the first time known to be dysregulated in testicular tumorigenesis.

In Chapter 3, I document the role of miR-199a, one of the ncRNAs identified, in testicular cancer progression. In-depth investigation on miR-199a found that it is a microRNA (miRNA) associated with cancer progression. It regulates cancer invasiveness and metastasis. DNA hypermethylation is a mechanism for dysregulation of this miRNA. A target known as *podocalyxin* (*PODXL*) is regulated by miR-199a. *PODXL* correlates with cancer progression and therefore, is likely a downstream target of miR-199a for testicular cancer metastasis.

In Chapter 4, I summarize all the results and the conclusion of the project, and outline potential future studies.

Chapter 2

Identification of Novel Epigenetically Regulated Genes and Non-coding RNAs in Human Testicular Cancer

2.1 Introduction

Testicular germ cell tumor (TGCT) is an invasive germ cell neoplasm histologically classified as seminoma and non-seminoma. Non-seminoma can be further subclassified into embryonal carcinoma, teratoma, choriocarcinoma, and yolk-sac carcinoma. Most non-seminomatous tumors include multiple cell types. Embryonal carcinoma is the most frequent non-seminomatous tumors. It represents about 87% of non-seminoma (Bosl & Motzer, 1997). Few seminomatous cell lines have been identified to date; several embryonal carcinoma cell lines have been established and shown to be useful for pathobiological and clinical studies (Andrews *et al*, 2005). Ntera2 (NT2) is one of the established pluripotent human testicular embryonal carcinoma cell lines. This cell line has been extensively used in research on TGCT (Burger *et al*, 1998; Koch *et al*, 2003; Skotheim *et al*, 2005). In this study, I used NT2 as a cell model to study differential methylation in embryonal carcinoma.

Unlike many cancers which peak during old age, TGCT is common in young males. Risk factors, including cryptorchidism, prenatal exposure to diethylstilbestrol and genetic factors at locus Xq27, increase susceptibility to develop TGCT (Horwich *et al*, 2006; Rapley *et al*, 2000). DNA mutation may be one cause of TGCT; however, accumulating information suggests a more prominent role for epigenetic alteration as a

factor in tumorigenesis including TGCT (Esteller, 2007; Feinberg *et al*, 2006). Previous reports on aberrant methylation of tumor suppressor genes/oncogenes provided information for an epigenetic role in tumor development. Many studies focused on individual target genes. The first genome-wide study of DNA methylation in TGCT used the technique of restriction landmark genome scanning (RLGS) (Smiraglia *et al*, 2002). However, no report of global high-resolution analysis of methylation changes in TGCT has been published. Tiling array technology permits elucidation of differentially methylated regions (DMR) of the whole genome (Cokus *et al*, 2008; Weber *et al*, 2005; Zhang *et al*, 2006) by the ChIP-Chip approach. A popular ChIP-Chip based method employed is methylated DNA immunoprecipitation (MeDIP), where methylated DNA is enriched by use of antibodies directed against 5-methylcytidine and hybridized to custom arrays such as promoter arrays or CpG island microarrays (Irizarry *et al*, 2008; Jacinto *et al*, 2007; Yan *et al*, 2002) . These whole genome approaches are powerful tools for identification of differentially methylated genes that may be important in tumorigenesis.

In this Chapter I utilized MeDIP in combination with human tiling microarrays (MeDIP-chip) covering the entire human genome, to elucidate DMRs. This approach allows identification of not only differentially methylated promoters and gene-associated CpG islands, but also differentially methylated non-coding RNAs (ncRNA)

such as microRNAs (miRNA). Increasing number of reports suggest miRNAs may play pivotal roles in tumor progression and development including the regulation of neoplastic transformation and metastasis (Huang *et al*, 2008; Ma *et al*, 2007; Varambally *et al*, 2008). Some miRNAs are epigenetically silenced in cancer cells as a result of cancer-specific hypermethylation (Han *et al*, 2007; Lujambio *et al*, 2008; Toyota *et al*, 2008). Since most miRNAs are located in intergenic or intronic regions, they were not identified in previous studies using promoter or CpG island arrays. To validate the clinical utility of this approach I documented methylation and expression changes of 3 novel genes and a miRNA in normal and tumorous testicular tissue. Our genome-wide approach demonstrates the use of MeDIP-Chip integrated with expression profiling as a tool for discovery of methylation-regulated genes and ncRNAs that might be important in diseases.

2.2 Materials and methods

Primary tumor specimens, cell cultures and drug treatment

Genomic DNA (17 cases) and RNA (18 cases) samples of TGCT patients were purchased from Oncomatrix (San Marcos, CA, USA). Normal testicular DNA (6 cases) and RNA (8 cases) were purchased from Biochain (Hayward, CA, USA) and Zyagen (San Diego, CA, USA). RNAs of tumor and normal adjacent tissues of other tumor types were purchased from Ambion (Austin, TX, USA). Each RNA sample was isolated from a single individual. Cell culture system Ntera2 (NT2, ATCC#: CRL-1973), Tera-1 and normal human testis cell line CRL-7002 (HT) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS and incubated in 37°C humidified incubator supplied with 5% CO_2. For demethylation analysis, 1 x 10^5 NT2 cells were seeded for 24 hours and treated with 1-5 μM of 5-aza-2-deoxycytidine (Sigma, St Louis, MO, USA) for 72 hours.

MeDIP and microarray hybridization

Methylated DNA immunoprecipitation (MeDIP) was performed as previously described (Weber *et al*, 2005). Briefly, genomic DNA was sheared by sonication on ice to generate random fragments of 100-500 bp. Five μg of sonicated DNA were used for immunoprecipitation (IP). Heat denatured DNA was incubated with 10 μl of mouse anti-5-

methylcytidine monoclonal antibody (Eurogenetec, San Diego, CA, USA) in 1X IP buffer (10mM Na-Phosphate pH7.0, 140mM NaCl and 0.05% Triton X-100) with periodic shaking for 2 hours at 4°C. Sheep anti-mouse IgG conjugated Dynabeads (Invitrogen, Carlsbad, CA, USA) were added to the IP and incubated for additional 2 hours. The beads were washed 3 times with 700 μl 1X IP buffer and then resuspended in 250 μl digestion buffer (50mM Tris, pH8.0, 10mM EDTA, 0.5% SDS). The antibodies were digested with 80 μg of proteinase K for 3 hours at 50°C. DNA was extracted with phenol/chloroform and precipitated with ethanol. Precipitated DNA was resuspended in water and used for real-time qPCR (for validation of IP efficiency) or microarray hybridization. Several positive and negative control loci were used for confirmation of IP efficiency before hybridizing to microarrays (**Figure 2.1A**). The immunoprecipitated DNA was amplified, labeled, and hybridized to Human Tiling Array 2.0R Chips (Affymetrix, Santa Clara, CA, USA) sequentially, as suggested by Affymetrix ChIP-chip protocol. Triplicate sets of hybridization were performed from 3 independent MeDIP experiments for each cell line. Both tiling and expression arrays were washed and stained on the Affymetrix Fluidic Station 450 and Chips were scanned on GeneChip Scanner GCS3000 (Affymetrix, Santa Clara, CA, USA).

Tiling array data analysis

The raw CEL data files from tiling array experiments were analyzed by Tiling Analysis Software (TAS) (Affymetrix, Santa Clara, CA, USA). Arrays from each group (cancer versus normal) were quantile-normalized and differential methylation between groups of cancer and normal was compared by choosing the "two-sample comparison analysis" option in TAS. A two-sided test was performed to evaluate both hypermethylation and hypomethylation. A bandwidth was set at 275 such that the sliding window (2*bandwidth+1) of the analysis is 551. Transfrags (or DMRs) were generated by Interval Analysis with a *P* value cutoff at 20 ($P < 0.01$), maximum gap to be 250 and minimum run to be 50. Transfrags generated by *P* value cutoff with a positive signal difference were defined as hypermethylation while those of negative difference were defined as hypomethylation. Genomic bisulfite sequencing was performed to confirm the sensitivity of the observed DMRs (**Figure 2.1C**). Mapping of DMRs to Refseq, CpG island, promoter, miRNA and snoRNA was performed by using the Table Browser function embedded in UCSC Genome Bioinformatics (Santa Cruz, CA, USA; http://genome.ucsc.edu/cgi-bin/hgTables?command=start) or by our customized web-based tool TileMapper (http://tilemapper.nichd.nih.gov/tilemapper) designed specifically for transfrag mapping. Promoter annotation was retrieved from Genomatix (San Jose, CA, USA; http://www.genomatix.de) and the coordination of each promoter was

stored in BED files. Annotation of Refseq, CpG island, miRNA and snoRNA were retrieved from the UCSC Genome Browser. All analysis was based on human genome Build 35.1.

Expression array hybridization and data analysis

Total RNA was extracted from NT2 and HT cells with Trizol Reagent and analyzed by Bioanalyzer (Agilent, Santa Clara, CA, USA). 3 μg of DNaseI treated RNA were amplified and the resulting cRNA was biotin-labeled and hybridized to Human Genome U133 Plus 2.0 Arrays (Affymetrix, Santa Clara, CA, USA). Triplicate sets of hybridization were performed for each cell line and the raw data were normalized by robust multiarray average (RMA) algorithm and analyzed in Partek Genomics Suite Software (Partek, St Louis, MO, USA). Differential gene expression was evaluated using one-way ANOVA. Expression fold change of differentially methylated genes was represented by the probe of most significant *P*-value. Differentially expressed genes were confirmed by real time PCR (**Table 2.1**).

Genomic bisulfite sequencing and methylation-specific PCR (MSP)

400 ng of genomic DNA was treated with sodium bisulfite using the EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA, USA). 80 - 100 ng of bisulfite-treated DNA was used for PCR amplification. For bisulfite sequencing, the PCR product was TOPO-

cloned into the pCR4 vector (Invitrogen, Carlsbad, CA, USA) and 5-10 positive clones were sequenced. Graphics of CpG methylation were generated by CpGviewer (Carr *et al*, 2007). For MSP, methylated and unmethylated specific primers were designed in the same genomic region as in bisulfite sequencing. MSP products were resolved in 2.5% agarose gel.

Quantitative real-time RT-PCR

1 μg of total RNA was primed by random hexamers and converted into cDNA by Super-Script III (Invitrogen, Carlsbad, CA, USA). SYBR green based real-time PCR was performed in an Applied Biosystems 7500 Fast Real Time PCR system (Applied Biosystems, Foster City, CA, USA) and the level of gene expression was normalized by 18S rRNA. For real-time quantification of miRNAs, total RNA was extracted with mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA). cDNA was synthesized from 1 μg of total RNA using miRNA-specific primers with TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and normalized by hsa-mir-191. All PCR primers are listed on **Supplementary Table 2.3**.

Statistical analysis

The *P* value of tiling array analysis was computed by TAS, which uses a Hodges-Lehmann estimator associated with the Wilcoxon rank-sum test to compute the fold

enrichment between treatment (cancer) and control (normal) groups. *P* value less than 0.01 is considered to be statistically significant. The *P* value of expression microarray analysis was determined by one-way ANOVA by comparing triplicate sets of normalized normal and cancer cells. Differential expression of *APOLD1*, *PCDH10*, *RGAG1* and hsa-mir-199a-2 in TGCT patients as determined by qPCR was analyzed by two-tailed Student's *t*-test. $P < 0.05$ was considered statistically significant.

2.3 Results

2.3.1 Validation of microarray data

To validate the efficiency of MeDIP, relative real-time qPCR was used to determine the fold of enrichment for positively methylated loci (*RASSF1* and *NPY*) and a negative locus (*ACTB*) in the cancer geome. The results showed that MeDIP enriched loci of *RASSF1* and *NPY* by 80-100 fold as compared to *ACTB* (**Figure 2.1A**). The MeDIP product was subsequently amplified by PCR method to generate sufficient amount of DNA for microarray hybridization. To show that the PCR does not create any bias on the DNA content, MeDIP product after amplification was examined for enrichment with those control loci. The result showed a consistent pattern of methylation enrichment for *RASSF1* and *NPY* (**Figure 2.1A**). These experiments demonstrate that MeDIP is efficient in capturing methylated loci.

Principal Component Analysis (PCA) was used to determine whether the cancer and normal methylomes are different. The distinct distribution between the two groups indicates that global methylation is different (**Figure 2.1B**).

The microarray hybridization generated a large number of differentially methylated regions (DMR). To experimentally validate these DMRs, eight loci were randomly picked for bisulfite sequencing. These loci include *EBNA1BP2*, *PQLC2*, *HOXC10*, *HOXA7*,

OSR1, *GAD1*, *ZSWIM2* and an intergenic region from different chromosomes. The results of bisulfite sequencing confirmed tiling array data and documented it to be a sensitive and reliable tool to detect DMRs with a *P* value cutoff at 0.01 (**Figure 2.1C**).

Besides tiling arrays for methylation profiling, expression microarrays were used for expression profiling. The relative fold change of genes determined in expression microarray was confirmed by real-time RT-qPCR (**Table 2.1**). Results of qPCR were consistent with microarray data with fold change greater than 2. Variation of fold change was observed between the two methods if the fold change from microarray is between -2 and 2. Such variation may reflect the false negative result from microarray experiments. Thus, only those genes with fold change greater than 2 were further analyzed.

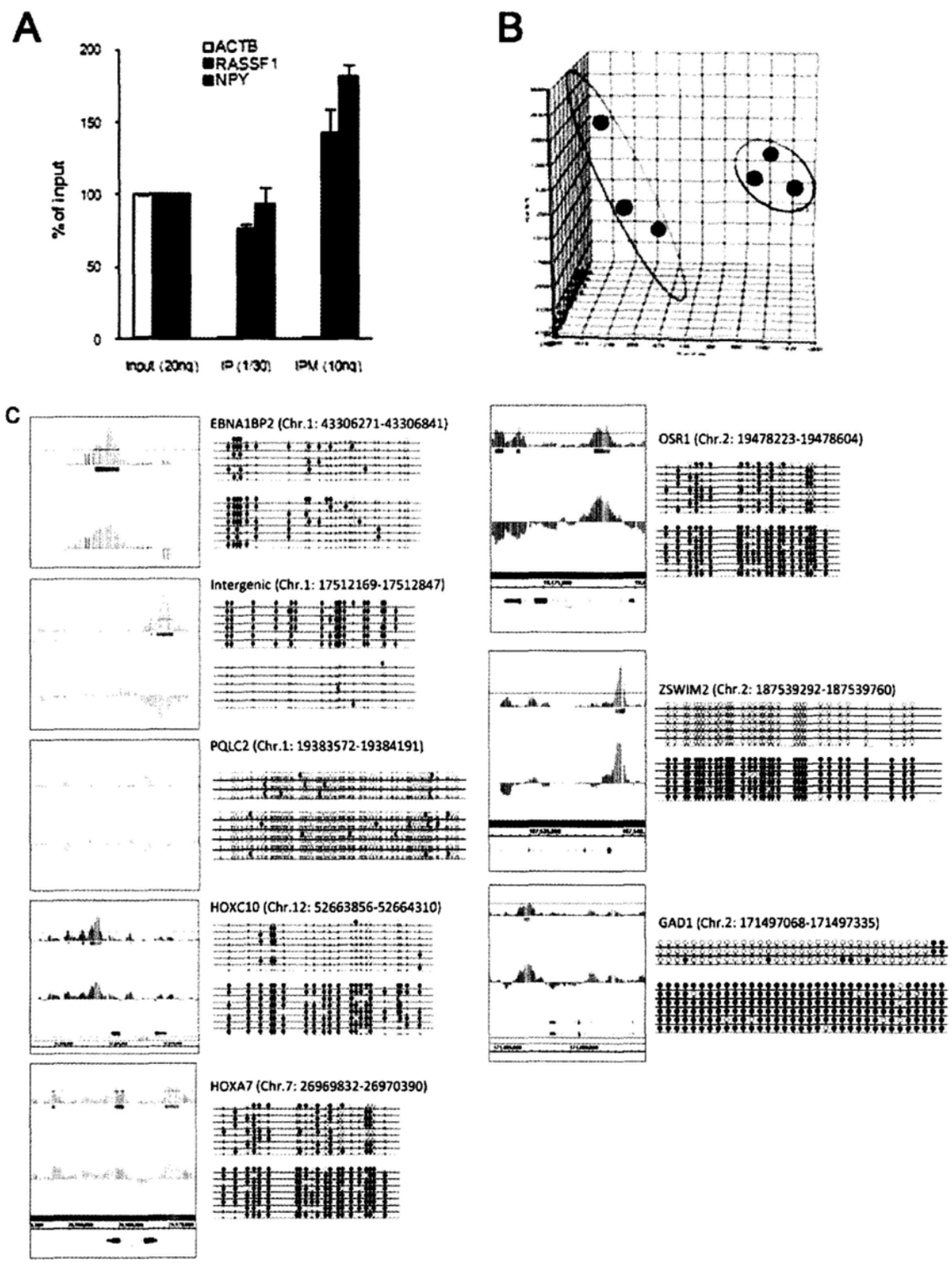
A
ACTB
RASSF1
NPY
% of input
200
150
100
50
0
Input (20ng)
IP (1/30)
IPM (10ng)
B
C
EBNA1BP2 (Chr.1: 43306271-43306841)
Intergenic (Chr.1: 17512169-17512847)
PQLC2 (Chr.1: 19383572-19384191)
HOXC10 (Chr.12: 52663856-52664310)
HOXA7 (Chr.7: 26969832-26970390)
OSR1 (Chr.2: 19478223-19478604)
ZSWIM2 (Chr.2: 187539292-187539760)
GAD1 (Chr.2: 171497068-171497335)

Figure 2.1 Validation of MeDIP-Chip result and confirmation of DMRs by bisulfite sequencing. (**A**) Validation of MeDIP. Real-time qPCR analysis of *ACTB*, *RASSF1* and *NPY* on immunoprecipitated DNA (IP) and immunoprecipitated DNA with PCR amplification (IPM). Input DNA serves as background control. *RASSF1* and *NPY* are two positive methylation controls while *ACTB* is a methylation negative control. Error bars indicate s.e.m. of triplicate experiments. (**B**) Principal Components Analysis (PCA) of triplicate sets of array hybridization of the normal (red) and cancer (blue) cell lines. The distinct distribution between the two groups indicated that global methylation was different. (**C**) Different loci (shaded regions) from different chromosomes (*EBNA1BP2* and *PQLC2* on chromosome 1, *HOXC10* on chromosome 12, *HOXA7* on chromosome 7, *OSR1*, *GAD1*, and *ZSWIM2* on chromosome 2, and an intergenic region on chromosome 1) as printed in different tiling array chips were selected and differential methylation was confirmed by bisulfite sequencing.

Table 2.1 Validation of microarray expression data by real-time qPCR

Hypermethylated genes

Gene	Array *P*-value (ANOVA)	Array fold change	qPCR fold change
PCDH10	0.000000825	-53	-1340
RBMS3	0.000000271	-8.6	-11.9
MAN2B2	0.0000122	-4.9	-5.8
H2AFJ	0.0000226	-3	-1.4
APOLD1	0.00469257	-1.4	-11.9
ZSWIM2	0.0109417	-1.3	-4.1
XAGE1D	0.104124	-1.3	1.5
NLRP3	0.188484	-1.3	-1.8
CDX4	0.0242089	-1.2	5.7
C20orf85	0.903475	-1	-1.2
TMEM29	0.22711	1.1	1.6
NPY	0.28104	1.1	-2.8
MNS1	0.00118783	1.7	-1.7
TIAM1	0.0000229	10.2	23.6
EOMES	0.000320088	33.2	66.4

Hypomethylated genes

Gene	Array *P*-value (ANOVA)	Array fold change	qPCR fold change
EIF2C1	0.000000214	11.4	7.4
ZNF480	0.000724322	3.7	3
ZNF780B	0.0114908	2.2	2.4
ZNF615	0.000595708	2.1	2.9
VBP1	0.00403041	1.1	-1.1
MAB21L2	0.132716	-1.1	1.4
SLC40A1	0.00484154	-1.6	2
CCDC82	0.000326356	-2.2	-1.9
ZFHX4	0.000000409	-12.7	-25.6

2.3.2 Identification of differentially methylated regions in NT2 cells

The pattern of DNA methylation changes substantially when cells become cancerous. To better understand the global change of DNA methylation and its effect on transcription, genome-wide methylation and expression were examined in an *in vitro* pluripotent cell model NT2, which is an embryonal carcinoma derived from a testicular cancer patient, and normal testis cells (HT) (Andrews, 1998). Methylated DNA fragments in the genome of each sample were enriched by MeDIP, followed by whole genome interrogation by hybridizing to tiling microarrays that cover the entire non-repetitive human genome.

To highlight the aberrant methylated regions in the NT2 cells and allow downstream processing and analyses, DMRs were compiled based on the *P* value cutoff ($P < 0.01$). As a result, 22,452 hypermethylated and 12,756 hypomethylated DMRs in the cancer genome were identified.

Next, global distribution of DMRs were analyzed. The chromosomal distribution of hypermethylation and hypomethylation, as represented by the percentage of the total length of DMRs per 500 kb interval, was plotted against the genome. As anticipated, DMRs were not evenly distributed in the genome. Some chromosomal regions were preferentially methylated or demethylated. For example, chromosomes 1p34.3,

1q43-4, 7q36.2-3, 16p13.2, and 21q22.2-3 were intensively hypermethylated, whereas chromosomes 5q13.2, 18q11.2-12.1 and 19q13.31 were more hypomethylated. Some chromosomes, such as chromosome 3, 10, 13, 14, and Y, exhibited fewer DMRs (**Figure 2.2**).

Aberrant promoter methylation is usually linked to transcriptional gene silencing. To determine whether DMRs preferentially occurred in promoters, genome-wide mapping of DMRs was performed. Intriguingly, most of the DMRs (92.9% of the hypermethylated and 88.2% of the hypomethylated DMRs) were mapped to genomic regions without any gene annotation (intergenic). Only 5.2% of hypermethylated and 9.5% of hypomethylated DMRs were mapped to annotated Refseq including exons and introns. A low percentage of DMRs (1.9% of hypermethylated and 2.3% of hypomethylated DMRs) were mapped to promoter regions of known genes (**Figure 2.3A** and **Supplementary Table 2.1**). Thus, various epigenetic hotspots were found in gene bodies, promoters, CpG islands and intergenic regions. The consequence of these DMRs will be discussed below.

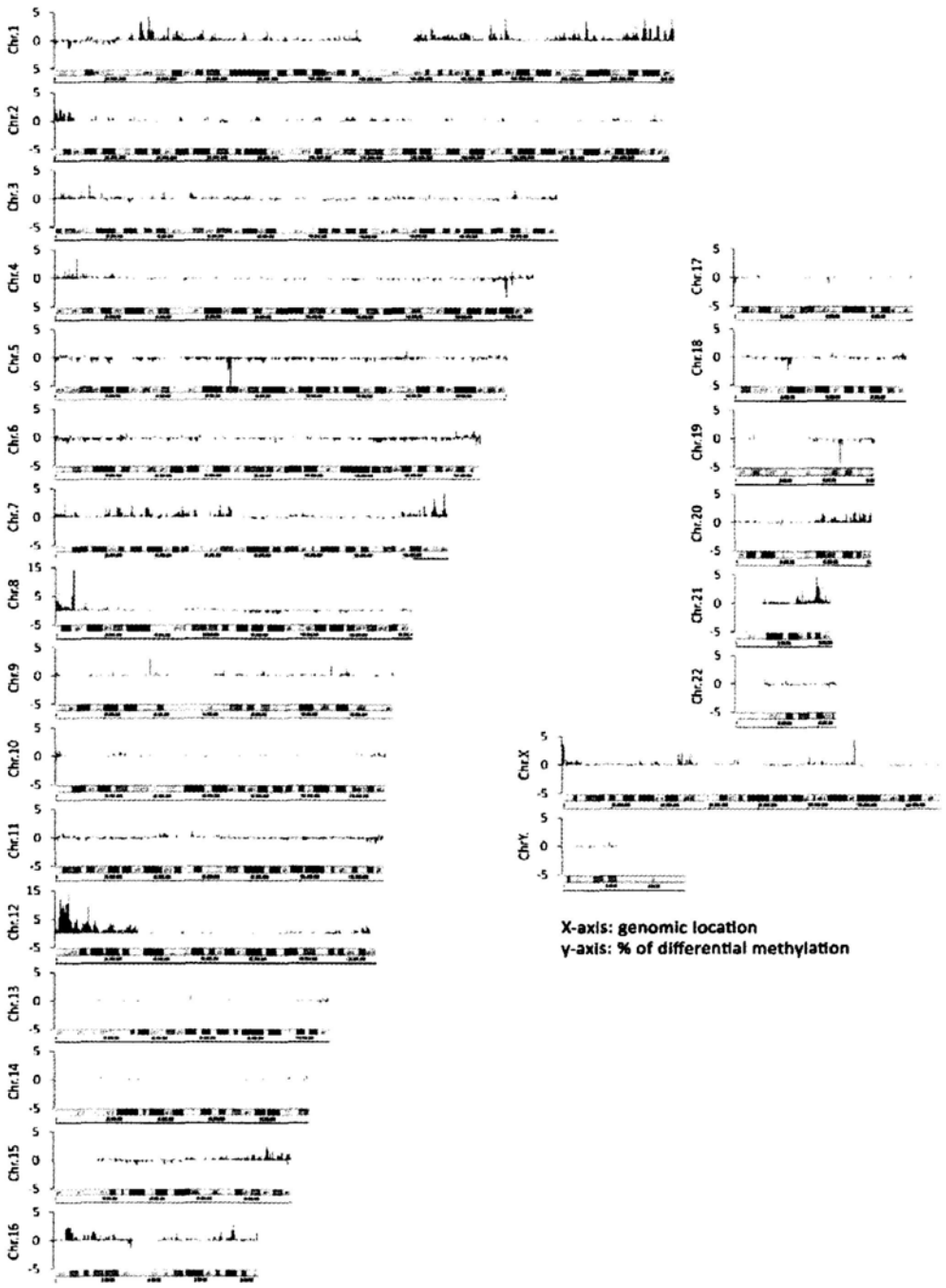

Figure 2.2 Distribution of hypermethylation (blue peaks) and hypomethylation (red peaks) in all chromosomes. Differential methylation is represented as percentage of the total length of hypermethylated or hypomethylated DMRs in a 500 kb interval and plotted across the genome.

2.3.3 Differentially methylated CpG islands and promoters

While the effect of DNA methylation in intergenic regions is less clear, aberrant methylation in promoter regions is frequently linked to altered transcriptional activity. About half of the known human gene promoters are associated with CpG islands (Larsen *et al*, 1992). These CpG islands are protected from *de novo* methylation in normal tissues, but often acquire methylation in cancer cells that leads to gene silencing. Among the 35,208 DMRs identified in my study, 410 (295 hypermethylated DMRs and 115 hypomethylated DMRs) overlapped with CpG islands (**Supplementary Table 2.2**). However, only 79 (~27%) hypermethylated CpG islands and 13 (~13%) hypomethylated CpG islands were coupled with gene promoters (**Figure 2.3B**). The other differentially methylated CpG islands resided either inside genes or in non-genic regions. For the promoter-associated CpG islands, a number of them, including those of *NTF3, FGF, OSR1, HOXA6, NPY* and *WT1* have previously been reported as differentially methylated in other cancer types (Bibikova *et al*, 2006; Houshdaran *et al*, 2007; Illingworth *et al*, 2008; Mares *et al*, 2001; Oka *et al*, 2006). This study also identified many CpG islands that were not previously shown to be differentially methylated, such as *CXCL5*, *EID1* and *TRHDE*; expression of these genes were downregulated in NT2 cells.

Previous studies suggested that many genes, such as *Oct-4* and *Il2*, lacked CpG islands in their promoters but were regulated by CpG methylation (Bruniquel & Schwartz, 2003; Hattori *et al*, 2004). I undertook a more comprehensive DMR mapping strategy including all promoters, not limited to the presence of CpG islands. A total of 693 genes (414 are hypermethylated and 279 are hypomethyalted) were differentially methylated in promoters (**Figure 2.3C** and **Supplementary Table 1**). Compared to the result restricted to CpG islands, more genes exhibited differential methylation in promoters, although some were not coupled with CpG islands. Aberrant promoter methylation is, thus, not restricted to CpG islands.

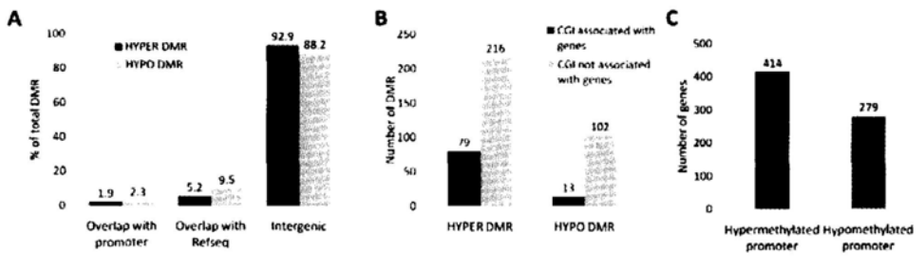

Figure 2.3 Genome-wide analysis of DMRs. (**A**) Distribution of DMRs. Most of the identified DMRs (88-93%) are mapped to intergenic regions. Promoter DMRs only represent 2% of total. (**B**) Number of differentially methylated CpG islands that are associated with or without genes. (**C**) Number of differentially methylated promoters.

2.3.4 Variability in the expression of differentially methylated genes

To assess the effect of methylation on transcriptional activity in cancer cells, a genome-wide analysis of gene expression by microarray was performed. The expression data were then compared to the DMR data. Based on the relative expression level, genes with differentially methylated promoters could be divided into three groups (**Figure 2.4A**). Group A, 19% of hypermethylated genes showed more than a 2-fold downregulation in gene epression while 20% of hypomethylated genes showed more than 2-fold upregulation. Group B, 25% of hypermethylated genes were upregulated more than 2-fold, while 22% of hypomethylated genes were downregulated by more than 2 fold. Group C, which accounts for 56% of hypermethylated and 58% of hypomethylated genes, the change of expression was marginal (fold change ranges from -2 to 2). The expression of genes in this group appeared to be independent of promoter methylation.

To confirm the effect of CpG methylation on gene expression, I randomly selected 8 genes from group A and 9 genes from group B, and assessed whether treatment with the demethylating agent 5-aza-2-deoxycytidine (5-aza) would restore transcription. For group A genes, 5-aza treatment restored expression of 8 of 9 selected genes (**Figure 2.4B**). For group B, expression of only 2 of the 8 selected genes was res-

tored by 5-aza treatment (**Figure 2.4C**). Transcription of most of the genes in Group A, but not Group B, suggests a potential functional role for DNA methylation. The effect of demethylation by 5-aza on gene expression appeared to be independent of the presence of CpG islands.

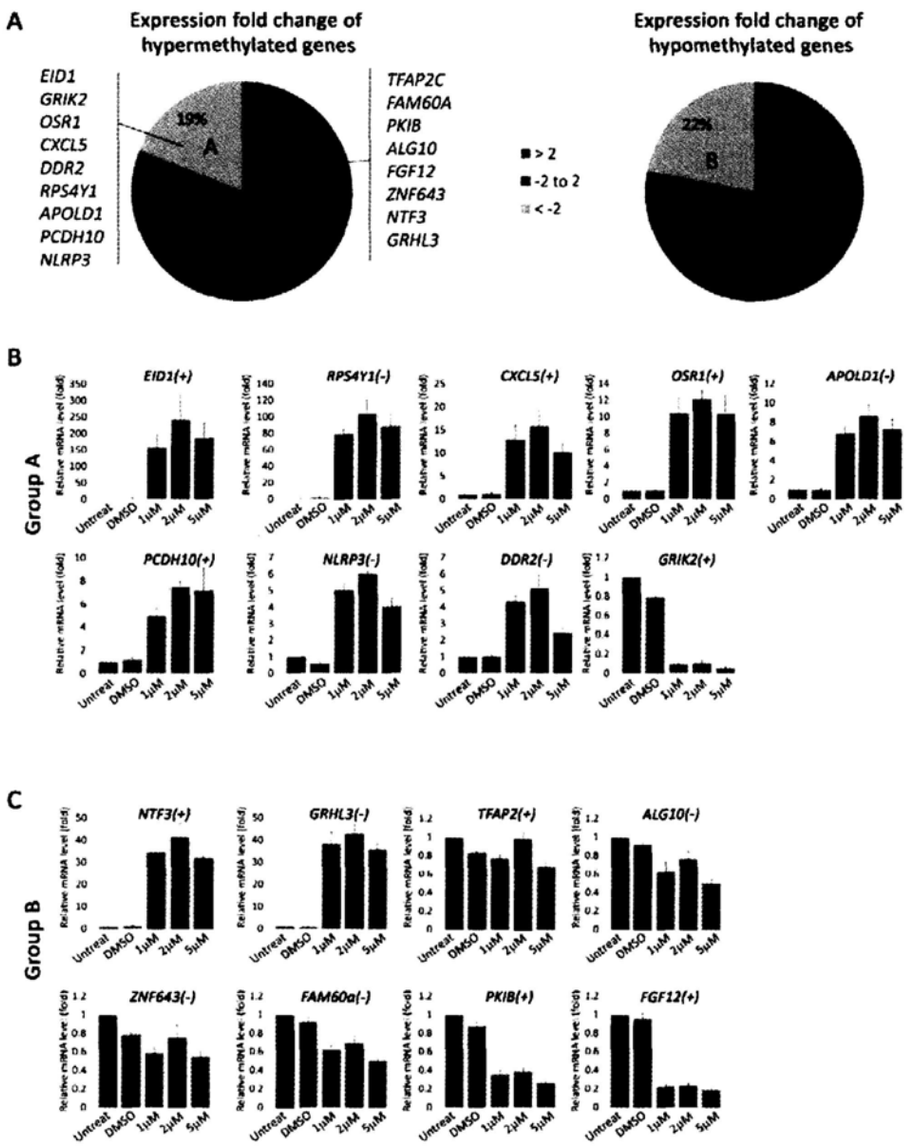

Figure 2.4 Gene expression of differentially methylated genes. (**A**) Expression of hypermethylated and hypomethylated genes. Genes are divided into 3 groups based on their expression. Group A: hypermethylated genes (19%) are downregulated (fold change >2) whereas hypomethylated genes (20%) are upregulated (fold change < -2). Group B: hypermethylated genes (25%) are upregulated whereas hypomethylated genes (22%) are downregulated. Group C: expression fold change of the differentially

methylated genes is marginal (fold change between -2 and 2). 9 genes of group A and 8 genes of group B are randomly selected and the effect of demethylation is examined, as shown in (**B**) and (**C**). (**B**) Effect of 5-aza treatment on the expression of 9 Group A genes. NT2 cancer cells are treated with 1-5 μM 5-aza for 72 hours. (**C**) Effect of 5-aza treatment on the expression of 8 Group B genes. "+" indicates the association of promoters with CpG islands. "-" indicates the absence of CpG islands in the promoters. Error bars indicate s.e.m. of triplicate experiments.

2.3.5 Identification of novel aberrantly methylated genes in primary TGCT

The testicular embryonal carcinoma NT2 cell is one of the well studied testicular germ cell neoplasms (Andrews, 1998). Based on the DMR data I identified several novel hypermethylated candidate genes that might be important for tumorigenesis of TGCT. Candidate genes were selected based on the following criteria: first, genes with hypermethylated promoters (**Figure 2.5A**); second, expression of genes that are downregulated and demethylation by 5-aza restored gene expression (**Figure 2.4B**); third, a biological role in testicular cancer was not previously described. The candidate genes were validated in normal testis biopsies and primary TGCT samples. Based on these criteria, three candidate genes were identified, namely, *APOLD1*, *PCDH10* and *RGAG1* for further investigation in primary TGCT tissue. Promoters of *APOLD1* and *PCDH10* were associated with CpG islands. In contrast, *RGAG1* lacks any CpG island in its promoter region. Hypermethylation of the promoters of these three genes in NT2 cells was confirmed by bisulfite sequencing (**Figure 2.5A**). Additionally I examined the methylation status of these genes in another testicular embryonal carcinoma Tera-1. Analogous to NT2 cells, hypermethylation of the 3 genes in Tera-1 cells was observed (**Figure 2.6**). In addition, the methylation status of the genes in cultured normal testicular cells was similar to that of normal testis tissue, indicating that methylation of these loci was not

changed throughout cell culture (**Figure 2.5A** and **Figure 2.6**). We explored whether gene expression was altered in primary TGCT tissue. The expression of these three genes, similar to the results observed in cell culture, was significantly downregulated in both seminoma (n = 8; *APOLD1*: $P<0.005$; *PCDH10*: $P<0.05$; *RGAG1*: $P<0.001$ by 2-tailed Student's *t*-test) and embryonal carcinoma (n = 9; *APOLD1*: $P<0.005$; *PCDH10*: $P<0.05$; *RGAG1*: $P<0.0005$ by 2-tailed Student's *t*-test) and a case of yolk sac tumor (n = 1) as compared to normal testicular tissue (n = 8) (**Figure 2.5B**).

Among the candidate genes, hypermethylation of *PCDH10* had been implicated in other cancers such as nasopharyngeal, esophageal, breat, colorectal, cervical, lung and hepatocellular carcinomas (Ying *et al*, 2007; Yu *et al*, 2009). The present result supports the role of this putative tumor suppressor gene in testicular cancer. *APOLD1* is an uncharacterized gene and its biological function is currently unknown. To examine whether aberrant hypermethylation of *APOLD1* is also observed in primary TGCT, the methylation status of the promoter of *APOLD1* was measured by methylation-specific PCR (MSP) (**Figure 2.5C**, upper panel). Hypermethylation of the *APOLD1* promoter was confirmed in 71% (n = 17) of TGCT specimens. The *APOLD1* promoter was unmethylated in all cases of normal testicular tissue (n = 6). To validate the result of MSP, a pair of tumor and normal tissues was selected and analyzed by bisulfite sequencing (**Figure**

2.5C, lower panel). Consistent with the MSP result, bisulfite sequencing showed that this gene was almost unmethylated in normal testicular tissues, but exhibited partial methylation in primary tumors. The observations of hypermethylation and downregulation of *APOLD1* in primary TGCT tissues suggest DNA methylation plays a crucial role in silencing this gene. In a preliminary screen of various primary tumors, the expression of *APOLD1* was downregulated in tumors of not only testis, but also those of ovary, lymphoma, kidney, bladder and cervix (**Figure 2.5D**). The RNA samples of each tumor type and the corresponding normal adjacent tissue were collected from a single individual; therefore, the role of *APOLD1* as a tumor suppressor gene awaits further confirmation with examples of more tumor specimens.

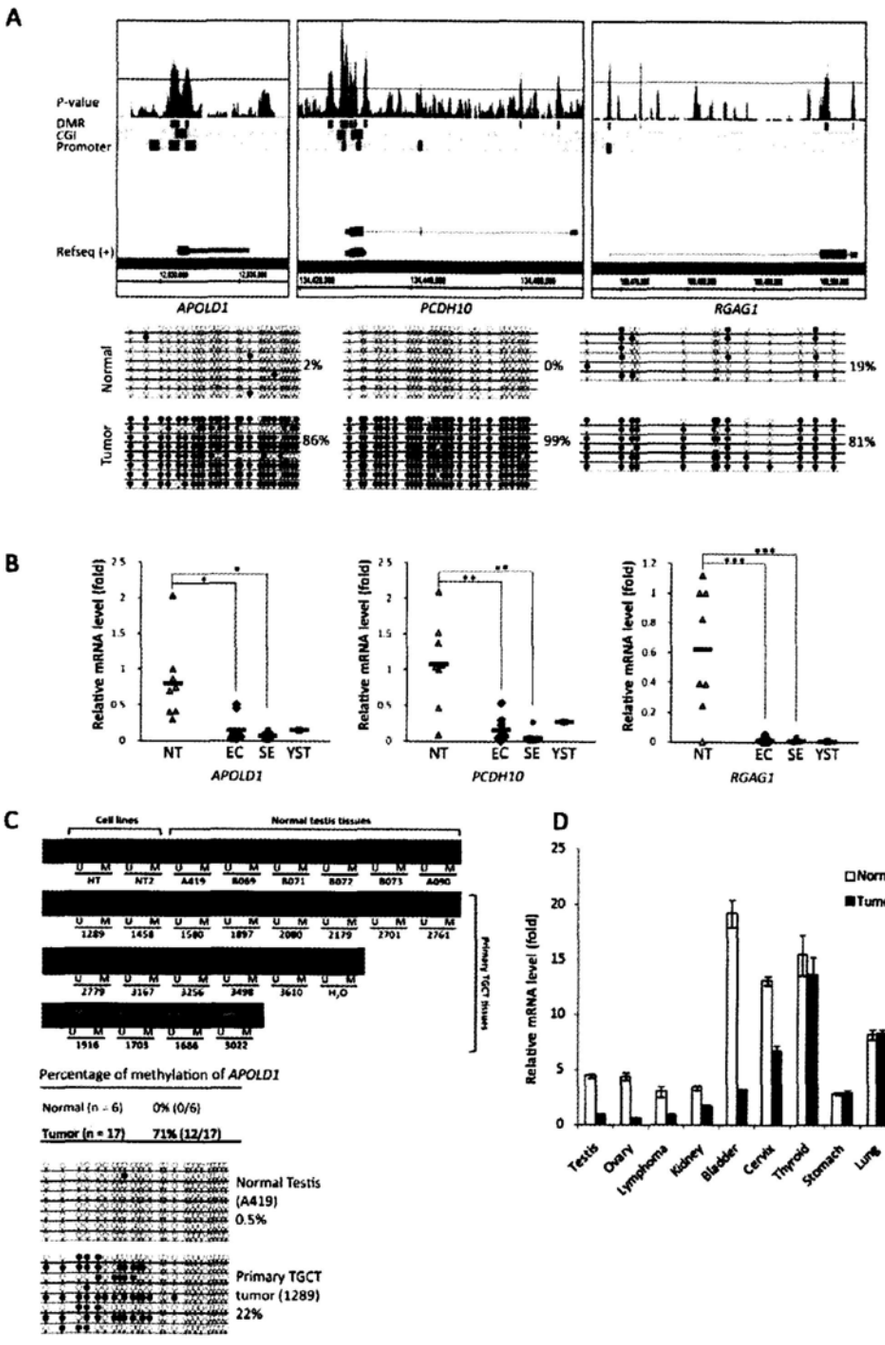
A
P-value
DMR
CGI
Promoter
Refseq (+)
APOLD1
PCDH10
RGAG1
Normal
Tumor
2%
0%
19%
86%
99%
81%
B
Relative mRNA level (fold)
NT
EC
SE
YST
APOLD1
PCDH10
RGAG1
C
Cell lines
Normal testis tissues
Primary TGCT tissues
Percentage of methylation of APOLD1
Normal (n = 6) 0% (0/6)
Tumor (n = 17) 71% (12/17)
Normal Testis (A419) 0.5%
Primary TGCT tumor (1289) 22%
D
Normal
Tumor
Relative mRNA level (fold)
Testis
Ovary
Lymphoma
Kidney
Bladder
Cervix
Thyroid
Stomach
Lung
Liver

Figure 2.5 Validation of three hypermethylated candidate genes in primary TGCT samples. (**A**) Hypermethylation of the promoters of *APOLD1*, *PCDH10* and *RGAG1* in NT2 cells. Hypermethylation of these genes is confirmed by genomic bisulfite sequencing. (**B**) Downregulated expression of *APOLD*, *PCDH10* and *RGAG1* in primary TGCT. NT: normal testis (n = 8); EC: embryonal carcinoma (n = 9); SE: seminoma (n = 8); YST (n = 1): yolk sac tumor. Mean value of each group is represented by the horizontal bar. **P*<0.005; ***P*<0.05; ****P*<0.001 by 2-tailed Student's *t*-test. (**C**) Promoter hypermethylation of *APOLD1* in primary TGCT. MSP is performed to compare the relative methylation of each patient. 71% of TGCT patients are partially methylated (n = 17) while none of normal testis (n = 6) is methylated. One case from the tumor group (1289) and normal group (A419) are selected and confirmed by bisulfite sequencing. U: unmethylated; M: methylated. (**D**) Expression of *APOLD1* in other tumors. RNA samples of each tumor and normal adjacent tissue were isolated from a single individual. Error bars indicate s.e.m. of triplicate experiments.

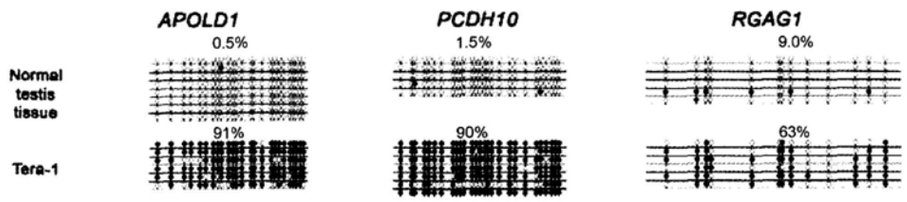

Figure 2.6 Bisulfite sequencing of the 3 candidate genes (*APOLD1*, *PCDH10* and *RGAG1*) in normal testis tissue and another human testicular embryonal carcinoma cell line Tera-1.

2.3.6 Differentially methylated non-coding RNAs and their dysregulation in cancer

The fact that the majority of DMRs occur in non-repetitive intergenic and intronic regions raises the question of their potential regulatory function. I proposed that intergenic and intronic methylation may play a role in regulating ncRNAs. There are several groups of ncRNAs regulating diverse cellular processes. miRNA is a class of short ncRNA that has been known to destabilize or repress translation of mRNA at a post-transcriptional level. To explore the role of intergenic or intronic DMRs, I mapped the identified DMRs to the miRBase Registry, a database for miRNA. The loci of three miRNAs, namely, hsa-mir-199a-2, hsa-mir-124a-2, and hsa-mir-184, were found overlapping with the hypermethylated DMRs (**Figure 2.7A**). Hypermethylation of these 3 miRNAs in NT2 cells was confirmed by genomic bisulfite sequencing. To examine the effect of hypermethylation on the expression of the miRNA, the level of the mature miRNAs in cancer and normal cells was measured by real-time qPCR. Among the three miRNAs, only hsa-mir-199a-2 was downregulated in cancer cells (741-fold downregulation), while hsa-mir-124a-2 and hsa-mir-184 showed 19 562 and 37 fold upregulation, respectively (**Figure 2.7B**). Treatment of NT2 cancer cells with 5-aza up-regulated the expression of hsa-mir-199a-2 by 42 fold, indicating that the expression of this miRNA was suppressed by methylation (**Figure 2.7C**). 5-aza treatment also up-regulated the ex-

pression of hsa-mir-184 by 25 fold but had no effect on the expression of hsa-mir-124a-2.

hsa-mir-199a-2 may be a candidate gene that is epigenetically regulated in TGCT. I thus studied its expression in primary TGCT tissue. By real-time qPCR, the expression level of hsa-mir-199a-2, after normalized with that of hsa-mir-191, was down-regulated in embryonal carcinomas (n = 9; $P<0.05$ by 2-tailed Student's *t*-test) and more significantly in seminomas (n = 8; $P<0.00005$ by 2-tailed Student's *t*-test) (**Figure 2.7D**). In Chapter 3, I surveyed the expression profile of hsa-mir-199a-2 with a larger sample size, which would give a more statistically significant value.

In addition to miRNA, we also mapped the DMRs to snoRNA-LBME-db, a database of for small nucleolar RNAs (snoRNA). Three snoRNAs, namely, HBII-240, ACA33, and ACA8 were hypomethylated (**Figure 2.8A**). Quantitation of expression by real-time qPCR analysis of these snoRNAs in cancer and normal cell lines revealed HBII-240 and ACA33 were upregulated by approximately 3-fold (**Figure 2.8B**). In a proportion of primary TGCT tumors, we found these 3 snoRNAs were also upregulated as compared with normal testis tissue (**Figure 2.9**). However, the upregulation varied case by case, resulting in stastically insignificant (ACA33: $P = 0.322$; ACA8: $P = 0.204$; HBII-240: $P =$

0.947; 2-tailed Student's *t*-test). The specific role of these snoRNAs in testicular germ cell tumorigenesis remains to be elucidated.

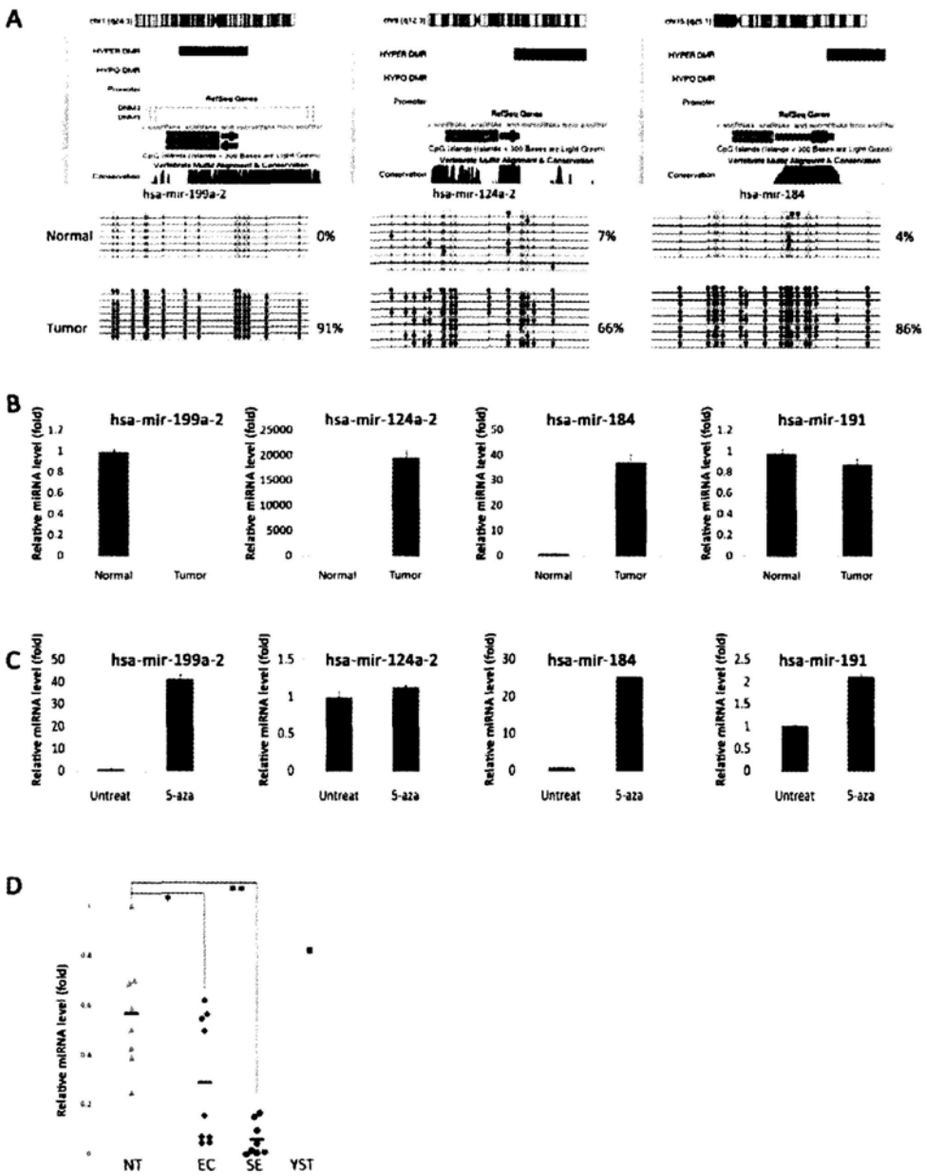

Figure 2.7 Hypermethylation and differential expression of miRNAs. (**A**) Hypermethylated DMRs at the loci of hsa-mir-199a-2 (Chr.1q4.3), hsa-mir-124a-2 (Chr.12q12.3) and hsa-mir-184 (Chr.15q25.1). hsa-mir-199a-2 embeds in the intron of *DNM3* while hsa-mir-124a-2 and hsa-mir-184 reside in intergenic regions. Hypermethylation of these DMRs in NT2 cells is confirmed by bisulfite sequencing. (**B**) Expression of the 3 hyper-

methylated miRNAs as determined by real-time qPCR. hsa-mir-191 is included as an internal control. Error bars indicate s.e.m. of triplicate experiments. (**C**) Effect of 5-aza treatment on expression of the 3 hypermethylated miRNAs. (**D**) Dysregulation of hsa-mir-199a-2 in primary TGCT. Mean value of each group is represented by the horizontal bar. NT: normal testis (n = 8); EC: embryonal carcinoma (n = 9); SE: seminoma (n = 8); YST (n = 1). *P<0.05; **P<0.00005 by 2-tailed Student's t-test.

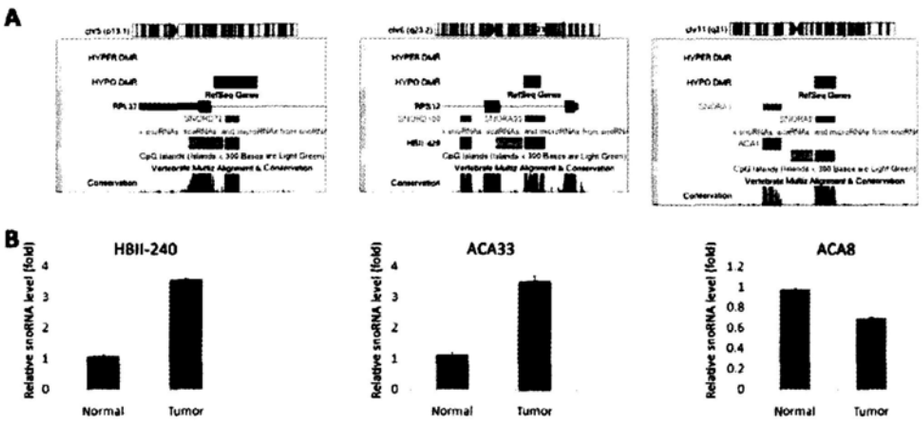

Figure 2.8 Hypomethylation of snoRNAs. (**A**) Three conserved snoRNAs, HBII-240, ACA33 and ACA8, are hypomethylated. HBII-240 and ACA33 reside in the introns of *RPL37* and *RPS12* respectively, while ACA8 is found in intergenic region. (**B**) Real time qPCR analysis on the expression of the 3 snoRNAs. Both ACA33 and HBII-240 are upregulated by 3-fold in NT2 cancer cells. Error bars indicate s.e.m. of triplicate experiments.

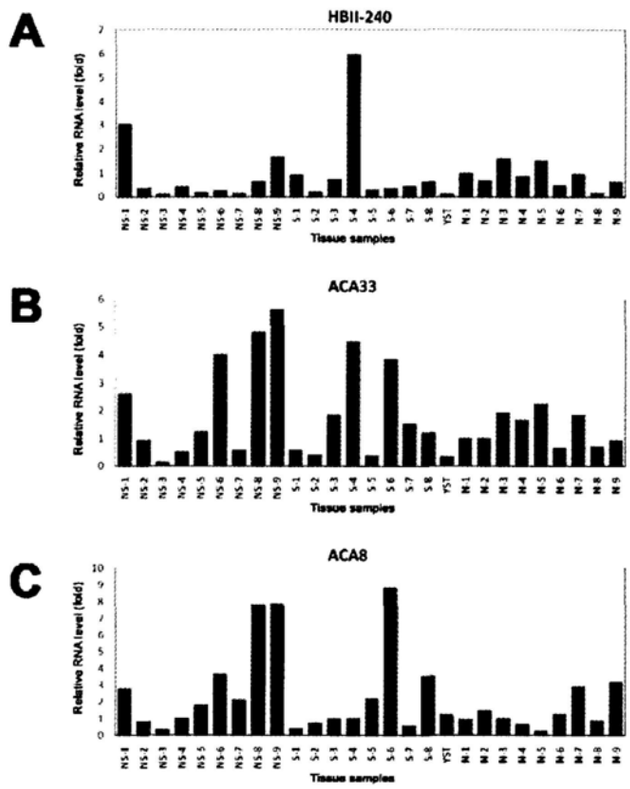

Figure 2.9 Real time qPCR analysis on the expression of the 3 snoRNAs in primary TGCT. (**A**) HBII-240. (**B**) ACA33. (**C**) ACA8. NS: non-seminoma; S: seminoma; YST: yolk sac tumor; N: normal testis. ACA33: $P = 0.322$; ACA8: $P = 0.204$; HBII-240: $P = 0.947$; 2-tailed Student's *t*-test.

2.4 Discussion

Aberrant DNA methylation is common in cancer cells. This Chapter demonstrates a genome-wide approach for identification of differentially methylated genes and ncRNAs using MeDIP-Chip for methylation analysis and expression microarray array for expression analysis.

CpG island hypermethylation results in changes of chromatin structure and appears to repress gene transcription. In this study although many genes were differentially methylated, only ~20% of genes showed an association between hypermethylation and gene repression. The role of DNA methylation on repression of these genes was validated by treatment with 5-aza, which inhibited DNA methylation and restored expression of the genes. I also demonstrated another group of genes that, although hypermethylated in their promoters, were insensitive to demethylation. The existence of methylation insensitive genes highlights the need to experimentally link epigenetic changes to altered transcriptional activity. Moreover, a group of genes (Group C) showed differential promoter methylation but the change of expression was marginal (fold change ranges from -2 to 2). The expression of this group of genes appears to be methylation independent. However, there is a possibility that false positive results

were generated from microarray experiments. Thus, only significantly differentially expressed genes (fold change > 2 or < -2) were selected for further analysis.

Whole genome tiling hybridization allowed us to observe widespread methylation changes. Only a small proportion of DMRs were found in promoters of known genes. A substantial number of DMRs were located in intronic or intergenic regions. Methylation changes in intronic or intergenic regions previously reported have largely been ignored because of a failure to investigate transcriptional consequences. The role of intergenic DMRs remains an enigma. They may be a consequence of inappropriate epigenetic change during transformation. They may play a role in maintenance of genomic stability or chromatin condensation (Ahuja *et al*, 1997; Ballestar & Esteller, 2002). Another possible function of intronic and intergenic DMRs is the regulation of genetic elements not identified by conventional algorithms. Many non-coding RNAs are located in intronic and intergenic regions and their regulation is unknown. In this study DMRs were mapped to miRNA and snoRNA databases to explore whether methylation changes occur in regions of ncRNAs. This allowed identification of 3 hypermethylated miRNAs and 3 hypomethylated snoRNAs.

Though the three miRNAs were hypermethylated, expression and 5-aza treatment experiments indicated that only hsa-mir-199a-2 was suppressed by hypermethy-

lation. The unexpected behavior of hsa-mir124a-2 and hsa-mir-184 could probably be explained by the location of the partially methylated regions near the 3'-end of the transcribed locus while the DMR of hsa-mir-199a-2 covers the 5' upstream and transcribed locus. Studies of cancers report that miRNA dysregulation is often associated with tumor progression or metastasis, probably a consequence of post-transcriptional silencing of target oncogenes or tumor suppressor genes (Mendell, 2005; Zhang *et al*, 2007). The present study implicates methylation as one of the causes.

snoRNAs are another group of ncRNAs that guide modification of rRNAs or spliceosomal RNAs. These conserved small RNA regulators modify alternative splicing of many transcripts (Bachellerie *et al*, 2002). The identification in the present study of hypomethylation and enhanced expression of the three snoRNAs suggests a potential relationship of cancer and dysregulation of snoRNAs.

An *in vitro* cell culture system was exploited in this study because of the ease of its manipulation. It is recognized that methylation changes in cultured cells may not reflect *in vivo* changes. Despite this I found a number of differentially methylated genes in the culture system that were concordant with those of primary tissue samples. Three hypermethylated genes, *PCDH10*, *APOLD1*, and *RGAG1* were investigated as examples. These genes were silenced in primary TGCTs and their expression was restored upon

demethylation. *PCDH10* encodes a membrane protein for cell adhesion. It has been implicated to be a tumor suppressor gene in studies of nasopharyngeal, esophageal, breast, colorectal, cervical, lung and hepatocellular carcinoma cell lines. Expression of *PCDH10* in these cell lines was suppressed by DNA hypermethylation (Ying *et al*, 2006). Interestingly it has also been identified as one of the deleted loci in patients with autism (Morrow *et al*, 2008). *RGAG1* and *APOLD1,* prior to this study, were not known to be epigenetically silenced in cancers. *RGAG1* (also known as *MART9*) is an X-linked retrotransposon-derived neogene of unknown function (Brandt *et al*, 2005). Expressed sequence tags (EST) of *RGAG1* were found predominantly in testis, suggesting that this retrogene might be important in germ cell development. *APOLD1* is another uncharacterized gene identified in this study. Its open reading frame encodes an apolipoprotein-L domain-containing protein whose function is unknown. Remarkably, *APOLD1* is located in 12p13.1, a TGCT susceptibility locus previously identified by genetic linkage analysis (Crockford *et al*, 2006). While genetic susceptibility loci in this gene have not been identified, the coincidence of an epigenetically silenced gene in this locus may provide new insight into interactions between genetic and epigenetic factors. The functions of these three candidate genes need to be further investigated.

In summary, this study provides comprehensive data for identification of both protein-coding genes and non-coding RNAs that are epigentically regulated by DNA methylation. Methylation occurs in promoters and CpG islands, as well as in intragenic and intergenic regions. Only a subset of hypermethylated genes are directly regulated by DNA methylation. I also demonstrated dysregulation of the selected candidate genes and ncRNAs in primary TGCT. Two of the genes, *APOLD1* and *RGAG1* are novel genes whose biological function needs further investigation. hsa-mir-199a-2 is another developmentally regulated miRNA that is implicated in cancer invasion (Migliore *et al*, 2008). The function of hsa-mir-199a-2 is discussed in Chapter 3. For simplicity, hsa-mir-199a-2 is denoted as conventional symbol "miR-199a" in the following chapters.

Chapter 3

Methylation of an Intronic Region Regulates Testicular Cancer Invasiveness via miR-199a

3.1 Introduction

DNA methylation is a fundamental epigenetic modification that regulates many different biological processes. It has a functional role in cellular differentiation, genomic imprinting, gene silencing, and probably aging, allowing cells of different tissues to stably maintain diverse characteristics despite the same genetic makeup (Jones & Takai, 2001; Liu *et al*, 2009). In cancer cells, hypermethylation of tumor suppressor genes, and/or hypomethylation of oncogenes or heterochromatin results in aberrant expression of genes leading to suppression of tumorigenesis or promotion of cell proliferation (Cheung *et al*, 2009). Recent reports have suggested methylation may play a role in the regulation of cancer progression (Aleman *et al*, 2008; Li *et al*, 2001; Watts *et al*, 2008).

Testicular cancer is one of several aggressive tumors in young males. Testicular cancer invasiveness is defined by the extent to which the primary tumor has spread to tissues adjacent to the testes, regional lymph nodes, distant organs and demonstration of increased serum levels of tumor marker proteins (Krege *et al*, 2008). Metastasis of testicular cancer includes several steps. Initially, noninvasive neoplastic cells (carcinoma *in situ*) are formed in the tubules, probably derived from primordial germ cells (Tis). Subsequently, the tumor may invade and grow through the inner layer surrounding the testis (tunica albuginea), but not involving the outer layer covering the testicle (tunica

vaginalis) (T1). Further invasion is defined by hematogenous or lymphatic spread near the tumor or tunica vaginalis (T2). It may invade the spermatic cord (T3), and eventually the skin surrounding the testicles (scrotum) (T4) (Albers *et al*, 2005). Cancer invasion is a critical step in the initiation of metastasis; however, the basis for this phenomenon is not well understood.

I hypothesize that aberrant DNA methylation is a factor that contributes to testicular cancer progression. In Chapter 2, I reported the use of a malignant testicular cancer cell line NT2 for the identification of differential methylation in this cancer. Using methylated DNA immunoprecipitation (MeDIP) and tiling array hybridization, I identified 35208 differentially methylated regions (DMR). The majority of DMRs did not associate with promoters of protein-coding genes. Instead, they were largely found in introns or intergenic regions. Mapping of these DMRs pinpointed 3 microRNAs (miRNA) and 3 small nucleolar RNAs (snoRNA) that were differentially methylated. One miRNA, miR-199a, was previously implicated in the progression and prognosis of gastric and ovarian cancers (Nam *et al*, 2008; Ueda *et al*, 2009). In this Chapter I document that miR-199a was generally hypermethylated in malignant testicular cancer; this hypermethylation correlated with its downregulation during cancer progression. Expression of miR-199a in these cancer cells suppressed their cancer invasive phenotype. I identified

podocalyxin-like protein 1 (PODXL) as a target of miR-199a-5p. PODXL is an anti-adhesive protein which was aberrantly upregulated in malignant testicular cancer, and negatively correlated with miR-199a-5p expression. Its expression correlated with cancer progression. Knockdown of this protein suppressed cancer invasion. The data suggest a mechanism for this phenomenon; acquired methylation in an intronic region is one of the factors linked to testicular cancer progression. Altered methylation suppresses miR-199a expression, leading to increased levels of PODXL and progression of testicular cancer.

3.2 Materials and methods

Normal and tumor tissues

Testicular normal and tumor tissues were purchased from Oncomatrix (San Marcos, CA, USA). The testis disease spectrum tissue arrays (T231 & TE2081) for testicular cancer progression were purchased from US Biomax (Rockville, MD, USA). Clinical stage of all tumor tissues was TNM graded and the pathology of patients were available from the vendors' websites (http://www.oncomatrix.com/products/Cancer/default.asp and http://www.biomax.us/tissue-arrays/Testis/).

Cell lines and cell culture

NT2, Tera-1, Tera-2, NCCIT and HT cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). 833K was kindly provided by Dr. Y.F. Lau. HT, NT2 and its sublines (NT2-GFP, NT2-199a, NT2-VC and NT2-PODXLi) were cultured in DMEM medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS. NCCIT and 833K were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS. Tera-1 and Tera-2 cells were cultured in McCoy's 5a Medium Modified (ATCC, Manassas, VA, USA) supplemented with 15% FBS. All cells were maintained in a humidified incubator at 37°C with 5% CO_2.

Isolation of RNA and DNA from archived tissues and cultured cells

RNA was isolated from formalin-fixed, paraffin-embedded (FFPE) tissues using the RecoverAll™ Total Nucleic Acid Isolation Kit (Ambion, Austin, TX, USA). RNA was isolated from cultured cells using mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA) for miRNA expression analysis, or using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) for mRNA expression analysis. Genomic DNA was isolated from FFPE tissues using the EZ DNA Methylation-Direct™ Kit (Zymo Research, Orange, CA, USA), followed directly by bisulfite treatment. For cultured cells, genomic DNA was isolated using the Gentra Puregene Kit (Qiagen, Valencia, CA, USA). All procedures were performed according to the manufacturers' instruction.

Genomic bisulfite sequencing and Methylight qPCR

Genomic DNA was treated with sodium bisulfite using the EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA, USA). Bisulfite-treated DNA was purified and used for PCR amplification. For bisulfite sequencing, the PCR product was TOPO-cloned into pCR4 vector (Invitrogen, Carlsbad, CA, USA) and 6 positive clones were sequenced. For Methylight qPCR, bisulfite-converted DNA was used for real-time PCR using a pair of custom-made TaqMan probes (Applied Biosystems, Foster City, CA, USA) specific for either

methylated (M) or unmethylated (U) region of the promoter of miR-199a. Sequences of the probes are: M: 6FAM-TGC GTT GTG TCG TTG GAG AGA TCG-MGBNFQ; U: VIC-TGT GTT GTG TTG TTG GAG AGA TTG TTA G-MGBNFQ. Methylation of miR-199a was calculated by: $C_{meth} = 100/[1+2^{(Ct_{CG}-CT_{TG})}]\%$, where Ct_{CG} and Ct_{TG} are the threshold cycles of M (FAM channel) and U (VIC channel) detectors respectively (Eads *et al*, 2000).

Reverse transcription and real-time PCR of miRNA and mRNA

Reverse transcription and real-time PCR of mRNA was performed as previously described (Cheung *et al*). For miRNA expression analysis, total RNA was converted to cDNA using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed using the TaqMan MicroRNA Assays, according to the manufacturer's instruction (Applied Biosystems, Foster City, CA, USA). miR-191 was used as a normalization control (Peltier & Latham, 2008).

miRNA transfection and establishment of stable cell lines

miR-199a-5p mimics, miRNA scramble control and miR-199a inhibitors were purchased from Ambion. Cells were transfected with indicated amount of miRNA molecules using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Cells were harvested for RNA or protein extraction 72 hours after transfection. For establishment of stable cell lines

NT2-GFP and NT2-199a, NT2 parent cells were infected with lentiviral particles carrying an expression vector of miR-199a (NT2-199a) or vector alone (NT2-GFP) (System Biosciences, Mountain View, CA, USA). Seventy two hours after infection, positive infected cells, as indicated by co-expression of GFP protein, were sorted by FACSAria Flow Cytometer (BD Biosciences, San Jose, California, USA). For establishment of stable PODXL knockdown cell lines NT2-VC and NT2-PODXLi, NT2 parent cells were transfected with vectors expressing shRNAs against PODXL or GFP (vector control) (Origene, Rockville, MD, USA). Stable RNAi sublines were selected by Puromycin. Four different shRNA sequences were tested and the vector with highest RNAi efficiency was employed in subsequent experiments.

Wound healing migration assay

Cells were grown to confluence on 12-well plates. Monolayer was scratched to generate the "wounds" using a P10 pipette tip. Wells were gently washed with PBS to remove cell debris and then replaced with fresh complete medium. Cells were incubated at 37°C for 17-24 hours. Images were captured with a microscope at 10X (Carl Zeiss, Thornwood, NY, USA). Distance between the edges was measured by software AxioVi-

son (Carl Zeiss, Thornwood, NY, USA). Three independent experiments, each with 6 replicates, were performed.

Cell invasion assay

In vitro cell invasion assay was performed using Growth Factor Reduced Matrigel Invasion Chambers with 8 μm pore size (BD Biosciences, San Jose, California, USA). Subconfluent cells (70-80%) were resuspended in serum-free DMEM medium. 5 x 10^4 cells (0.5 ml) and were added to matrigel-coated inserts and placed in the wells, containing 0.6 ml of complete medium supplemented with 10% FBS as chemoattractant. After 6 and 18 hours of incubation at 37°C, cells that had not invaded the matrigel were removed from the interior sides of the inserts by cotton swabs. Invaded cells on the exterior sides were stained with crystal violet and counted with a microscope (Carl Zeiss, Thornwood, NY, USA). Three independent experiments, each with 3 replicates, were performed.

Soft agar colony formation assay

Soft agar assay was performed as previously described (Tsang *et al*, 2007). Each well of 6-well plates contained a bottom layer of 0.6% Noble agar (USB, Cleveland, Ohio, USA) in serum-free DMEM. 2 x 10^3 NT2-GFP or NT2-199a cells were resuspended in 0.3%

Noble agar in DMEM supplemented with 10% FBS. The plates were incubated at 37°C and the medium was changed every 3 days. After 8 weeks, cells were stained with 0.05% crystal violet and visualized with a microscope.

Cloning of 3'-UTR and luciferase reporter assay

The flanking sequences containing the predicted miRNA binding sites were amplified by PCR and TOPO-cloned to pCR4 vectors (Invitrogen, Carlsbad, CA, USA). The fragments were restricted by XbaI and sub-cloned to the *Firefly* luciferase reporter vector pGL4.13 (Promega, Madison, WI, USA). The mutant plasmids were generated by PCR method using the Phusion Site-directed Mutagenesis Kit (Finnzymes, Woburn, Massachusetts, USA). The seed sequence in the mutant constructs was mutated to its complementary base. Luciferase reporter assay was performed as previously described (Pang *et al*, 2009). 100 ng of pGL4.13-UTR were co-transfected with 33 nM miR-199a-5p mimics or scramble control, and 2 ng *Renilla* luciferase vector pGL4.73 (normalization control), into NT2 cells (12-well format in triplicate) with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). 48 hours after transfection, luciferase activity was measured by Luminometer (Turner Biosystems, Madison, WI, USA) using the Dual-Luciferase Reporter Assay System Kit (Promega, Madison, WI, USA).

Western blot analysis

Western blot analysis was performed as previously described (Pang *et al*, 2009). Primary antibodies used were: PODXL (clone 3D3, 3 μg/ml, Santa Cruz, Santa Cruz, CA, USA); GAPDH (1:8000, Genway, San Diego, CA, USA). Secondary antibodies (1:10000) were purchased from Bio-Rad (Hercules, CA, USA).

Immunohistochemistry

Immunohistochemistry was performed as previously described (Li *et al*, 2007). Briefly, FFPE tissue arrays were deparafinized in xylenes and hydrated in a gradual series of ethanol. Antigen retrieval was done by heating the slides in citrate buffer at 100°C. The slides were probed with anti-PODXL antibody (1:200, Atlas Antibodies, Stockholm, Sweden) overnight at room temperature. Signal was developed using DAB Histochemistry Kit (Invitrogen, Carlsbad, CA, USA). Cells were counter-stained with hematoxylin. Expression of PODXL was scored as 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). Triplicate experiments were performed.

Animal studies of tumor growth and metastasis

For *in vivo* tumor growth study, 5-week-old male athymic nude mice (Charles River, Boston, MA USA) were injected subcutaneously with 1 x 10^7 NT2-GFP or NT2-199a (n = 10 for each group) in each flank of each mouse. Mouse weight and tumor size were measured every Monday and Thursday. Tumor volume was calculated as: length x width^2 x 1/2. All mice were killed 60 days after implantation. The mean tumor volume $\pm$ s.e.m. of each group was calculated. For metastasis study, 5-week-old male athymic nude mice were injected intravenously with 1 x 10^6 of NT2-GFP or NT2-199a cells (n = 11 for each group) via tail vein. Three mice from each group were sacrificed 49 and 64 days after implantation. The remaining mice were sacrificed 82 days after implantation. Metastasis was examined in major organs including brain, liver, kidney, lung and testis by necropsy and histochemistry.

Cell adhesion assay

Primary human umbilical vein endothelial cells (HUVEC) (Invitrogen, Carlsbad, CA, USA) were seeded on 12-well plates for 48 hours to form monolayer. 3 x 10^5 resuspended NT2-GFP or NT2-199a cells were added to the HUVEC cells and incubated at 37°C for 45, 90 and 150 minutes. Non-adherent cells were washed by PBS 4 times. Adherent cells

were visualized with a fluorescent microscrope at 10X (Carl Zeiss, Thornwood, NY, USA). Triplicate experiments were performed.

Statistical analysis

The differences in miR-199a-5p and miR-199a-3p expressions between normal, benign and malignant groups were analyzed by Wilcoxon Two Sample Test. The differences for wound healing assay, invasion assay, tumor growth and luciferase assay were analyzed by two-tailed Student's *t*-test, assuming equal variance. Results were represented as mean ± s.e.m. The correlations between miR-199a expression and methylation, miR-199a expression and PODXL level, tumor progression and PODXL level, were analyzed by Spearman's rank correlation coefficient. $P < 0.05$ is considered statistically significant.

3.3 Results

3.3.1 Identification of a hypermethylated intronic region in testicular cancer

To understand methylation changes in testicular cancer, I previously profiled the DNA methylome of a malignant cancer cell line NT2. The majority of identified DMRs were mapped to intronic or intergenic regions (Chapter 2). I postulated these DMRs might link to non-coding RNAs which could act as riboregulators. Conseqeuntly I identified hypermethylation of 3 miRNAs. miR-199a was one of the downregulated miRNAs. It is embedded in intron-14 of *dynamin 3* (*DNM3*) at 1q24.3. A conserved hypermethylated region of ~700 bp spanning miR-199a and its upstream promoter was identified (**Figure 3.1A**). I examined several testicular cancer cell lines (NT2, Tera-1, Tera-2, NCCIT and 833K) and a non-cancerous fetal testicular cell line (HT) with genomic bisulfite sequencing. The miRNA-199a loci in all cancer lines were highly or partially methylated, whereas in the non-cancerous testis cell line it was unmethylated (**Figure 3.1B**).

3.3.2 Aberrant methylation of miR-199a is associated with testicular cancer progression

To investigate whether aberrant methylation of miR-199a is related to cancer progression, I obtained biopsies from testicular cancer patients with different stages of

metastasis, together with 3 normal individuals as controls. Bisulfite sequencing analysis revealed an acquired methylation pattern as cancer cells start to invade the surrounding tissues (T1) and metastasize blood or lymph vessels near the tumor (T2) (**Figure 3.1C**). To confirm the methylation change with cancer progression, I used a high-throughput methylation assay (Methylight) to analyze genomic DNAs extracted from tissue arrays (n = 105). The results indicated that the neoplastic invasiveness increased with methylation. In contrast, the 'non-invasive' (normal or benign) case was inversely related to methylation (**Figure 3.1D**). These data suggest that methylation of miR-199a is associated with testicular cancer progression.

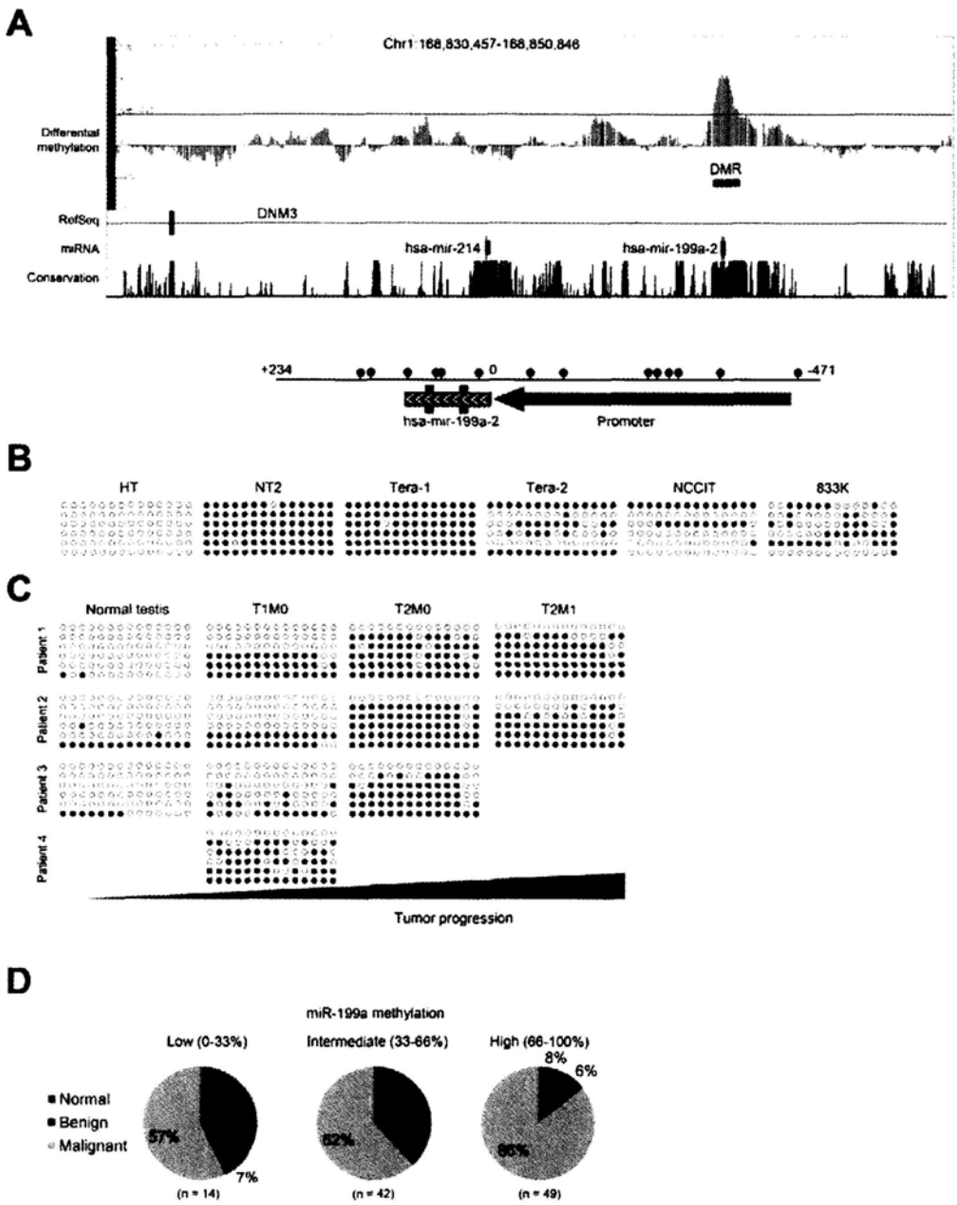

Figure 3.1 Methylation of miR-199a is associated with testicular cancer progression. (**A**) Genomic representation of differential methylation from chr1:168,830,456 - 168,850,846 (hg 17). A hypermethylated DMR embedded in intron of DNM3 was identified by MeDIP-chip and mapped to miR-199a and its upstreaming promoter. (**B**) Genomic bisulfite sequencing of miR-199a in different cultured testicular cancer cell lines (NT2,

Tera-1, Tera-2, NCCIT & 833K) and a non-cancerous fetal testicular cell line (HT). (**C**) Genomic bisulfite sequencing of miR-199a in patients with testicular cancer at different stages. Normal testicular tissues were included as normal control. T1: the tumor has not spread beyond the testicle and epididymis; T2: the tumor has spread to blood or lymph vessels near the tumor or tunica vaginalis; M0: no distant metastasis; M1: distant metastasis is present. (**D**) Proportion of cancer samples in different extent of miR-199a methylation. Methylation of miR-199a was divided into 3 groups: low, intermediate and high. Different tumor grade (normal, benign and malignant) was represented as percentage in each group.

3.3.3 Expression of miR-199a-5p is associated with testicular cancer progression

miR-199a refers to two mature miRNA species, namely miR-199a-5p and miR-199a-3p, both are processed from the same precursor RNA (**Figure 3.2A**). However, they have different seed sequences that regulate different targets. To determine whether the expression of these miRNAs is related to testicular cancer progression, I employed quantitative real-time RT-PCR. Comparison of the normal and malignant groups showed that miR-199a-5p was significantly downregulated in malignant cancers ($P = 0.00017$). The difference between normal and benign tumors, however, was insignificant ($P = 0.463$). Although processed from the same precursor RNA, miR-199a-3p was not significantly changed as contrasted to miR-199a-5p in malignancy ($P = 0.0233$). I also observed a significant upregulation of miR-199a-3p in benign tumors ($P = 0.0044$). These results indicate that miR-199a-5p, but not miR-199a-3p, is involved in testicular cancer progression (**Figure 3.2B**).

3.3.4 Reciprocal relationship between methylation and expression

Increased methylation in promoters is one mechanism for transcriptional silencing. The relationship between methylation and expression was demonstrated by correlation analysis of the genomic DNA and RNA isolated from the same individuals. We uti-

lized Spearman's rank correlation coefficient to assess the trend. Negative correlations were observed for both miR-199a-5p (correlation = -0.370, $P = 0.0001$) and miR-199a-3p (correlation = -0.298, $P = 0.0024$), suggesting that methylation is a negative regulator of miR-199a (**Figure 3.2C**). The role of methylation as a transcription inhibitor was investigated by treating cultured NT2 cells with the demethylation agent 5-aza-2'-deoxycytidine (5-aza). 5-aza inhibits *de novo* methyltransferase to reverse the acquired methylation lesion. As anticipated, 5-aza treatment restored miR-199a expression by more than 40 fold (**Figure 3.7B**). Taken together, these data show that methylation plays a critical role in the regulation of miR-199a expression.

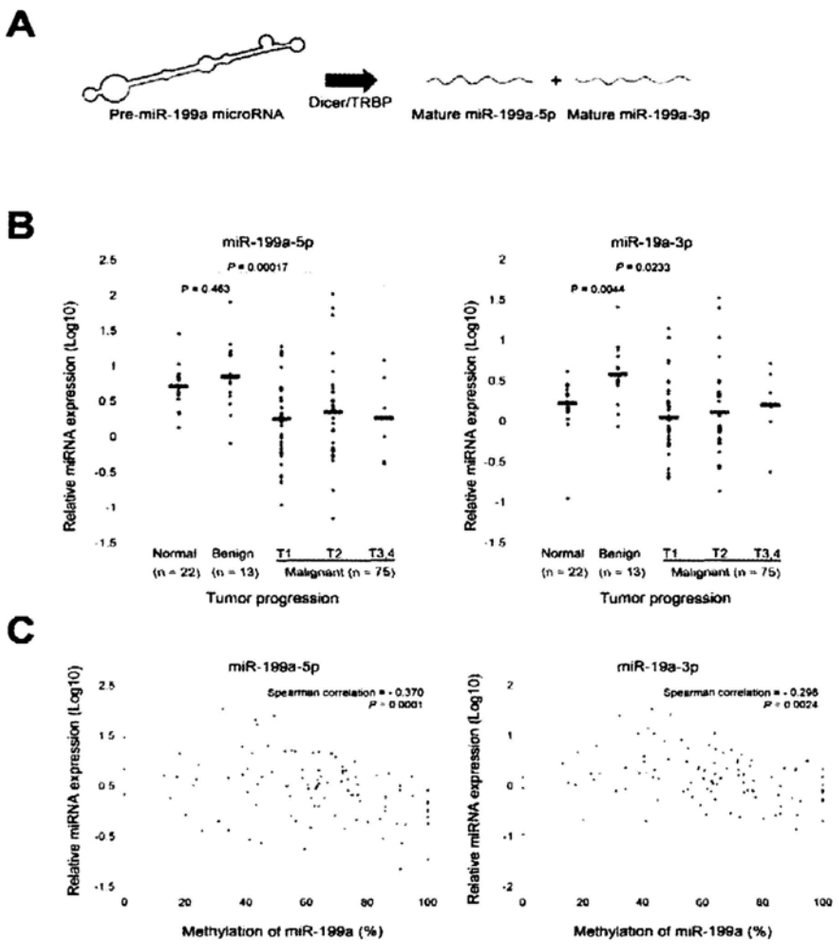

Figure 3.2 Expression of miR-199a is associated with testicular cancer progression and negatively correlated with methylation. (**A**) Pathway of miR-199a biogenesis. The precursor RNA of miR-199a is processed to 2 different mature miRNA species: miR-199a-5p and miR-199a-3p. (**B**) Expression of miR-199a-5p and miR-199a-3p in normal, benign and malignant testicular tumors. The difference between normal and malignant groups is significant (miR-199a-5p: $P = 0.00017$; miR-199a-3p: $P = 0.0233$; Wilcoxon two sample test). T3: the tumor invades the spermatic cord; T4: the tumor invades the scrotum. Red bar: mean (**C**) Scatter plots of miR-199a-5p and miR-199a-3p (y-axis) expression against methylation (x-axis). Expression of both miR-199a-5p and miR-199a-3p correlates negatively with methylation (miR-199a-5p: Spearman correlation = -0.370, $P = 0.0001$; miR-199a-3p: Spearman correlation = -0.298, $P = 0.0024$).

3.3.5 Expression of miR-199a suppresses cancer migration, invasion and cell growth

To study the function of miR-199a, miR-199a was constitutively expressed in cancer cells by integrating the genomic sequence of miR-199a linked to a CMV promoter. Lentivirus carrying a vector containing miR-199a and a GFP reporter was used to infect NT2 cells. Positive cells were sorted by flow cytometry. These cells (NT2-199a) expressed more than 500 fold of miR-199a-5p and 200-fold of miR-199a-3p when compared to the vector infected control cells (NT2-GFP) (**Figure 3.3A**). Change of cell motility is one of the characteristics of metastasis (Sahai, 2005). Using the wound-healing assay, I found that NT2-199a migrated more slowly than NT2-GFP ($P < 0.005$) (**Figure 3.3B**). Another feature of metastasis is its ability to invade extracellular matrix (Sahai, 2005). I used the Matrigel invasion assay to measure cancer cell invasion. Expression of miR-199a significantly suppressed the ability of NT2 cells to invade the Matrigel basement ($P < 0.005$) (**Figure 3.3C**). Moreover, I assessed the ability of cells to form colonies in an anchor-independent condition using a soft agar assay. I did not observe any difference in colony formation (**Figure 3.4**). However, a difference was observed when these cells were grown *in vivo*. Two months after subcutaneous implantation of transfected cells in athymic nude mice, the average size of the tumors in the NT2-199a group was ~33% smaller than that in the control group ($P = 0.145$) (**Figure 3.3D**). In addition, reduced cell

growth was confirmed by direct counting of *in vitro* cultured cells grown on fibronectin-coated plates (**Figure 3.5**). These results suggest that miR-199a suppresses cancer cell migration, invasion and growth, and probably has an anti-metastatic function.

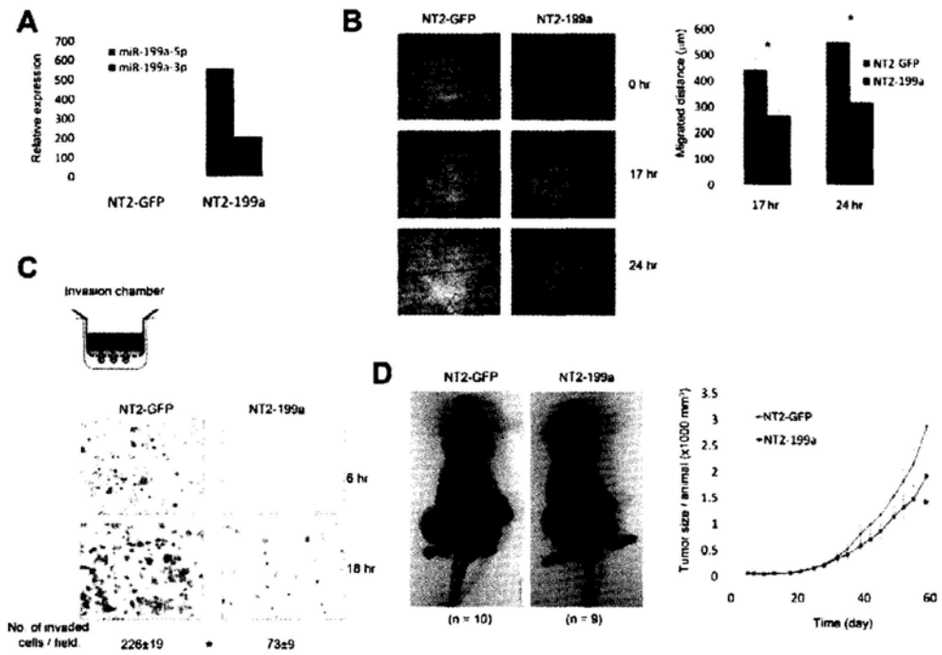

Figure 3.3 miR-199a suppresses cancer cell migration and invasion, and decreases cancer growth. (**A**) Ectopic expression of miR-199a in a metastatic testicular cancer cell line NT2. NT2 cells were infected by pseudo lentiviruses carrying either miR-199a (NT2-199a) or an empty vector (NT2-GFP). Stable cells which express GFP were sorted by flow cytometry and the level of miR-199a-5p and miR-199a-3p was determined by real-time PCR. (**B**) Wound healing assay for assessment of cancer cell migration. Same number of NT2-GFP & -199a cells was seeded on plates overnight to form monolayer and the width of the gap was measured after 17 and 24 hrs. The difference between two groups is significant (*P < 0.005, 2-tailed Student's t-test). Error bar: s.e.m. of triplicates. (**C**) Matrigel invasion assay for assessment of cancer invasion. Same number of NT2-GFP and NT2-199a cells was suspended in serum-free medium and allowed to invade through the matrigel coated on the membrane of the insert. Invaded cells were stained with crystal violet and counted. The difference is significant (*P < 0.005, 2-tailed Student's t-test) (**D**) Growth of NT2-GFP and NT2-199a cells in athymic nude mice. Cancer cells were injected subcutaneously into 2 groups of nude mice (n = 10 for NT2-GFP; n = 9 for NT2-199a) and the tumor size was monitored at different time points. Mean size of the tumors per animal was plotted (*P = 0.145, 2-tailed Student's t-test). Error bar: s.e.m.

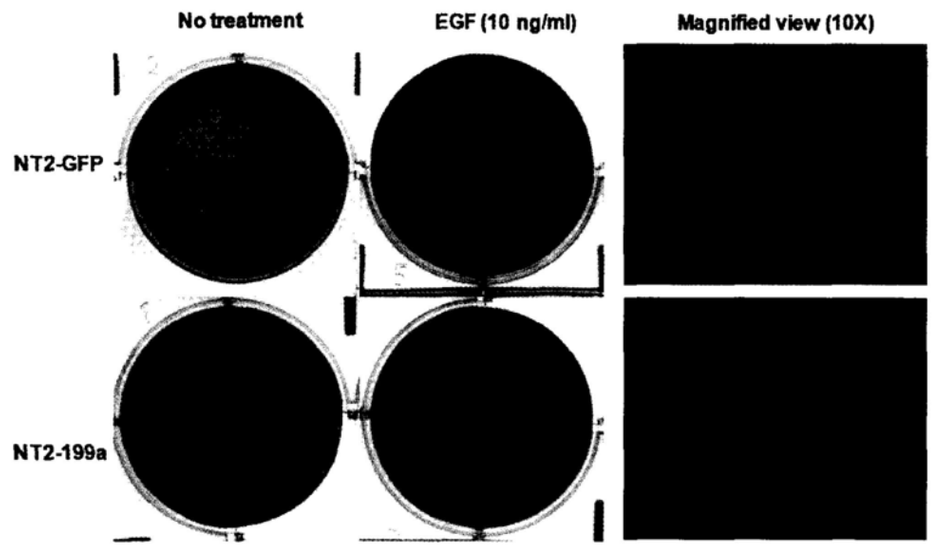

Figure 3.4 Growth of NT2-GFP and NT2-199a cells by soft-agar colony formation assay

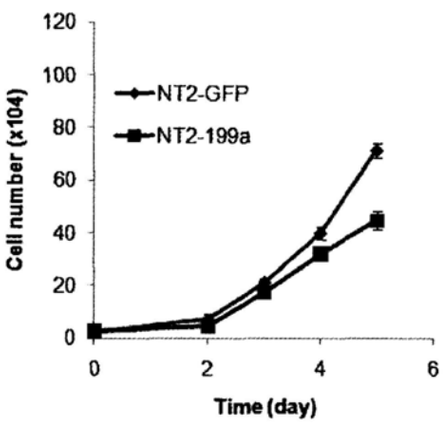

Figure 3.5 Growth of NT2-GFP and NT2-199a cells *in vitro*. Same number of cells was plated on fibronectin-coated wells. The number of cells was counted at different time points. Result represents mean ± s.e.m. of triplicate experiments.

3.3.6 miR-199a suppresses cancer metastasis in mouse xenograft model

To further confirm the anti-metastasis property of miR-199a, I used a xenograft animal model to study its function *in vivo*. To do this, equal numbers of NT2-GFP and NT2-199a cells were injected intravenously in athymic nude mice via tail vein (n = 11 for each group). Mice were sacrificed at day-49, -64 and -82 after injection. At day-49 and -64, 3 mice out of 6 from the control group (NT2-GFP) developed pulmonary metastasis. No metastases were found in the NT2-199a group. At day-82, all the remaining mice (5 from each group) were sacrificed. Four mice (80%) from the control group developed metastasis, compared to three mice (60%) from the NT2-199a group (**Figure 3.6A**). Metastasis developed in organs such as lung and liver, which are the common metastatic organs of human testicular cancer (**Figure 3.6B**). Histologic analysis indicated invasion of xenografted cancer cells (NT2-GFP) surrounding liver and lung, but none of the cancer cells expressed miR-199a (NT2-199a) (**Figure 3.6C**). Although at later stage (day-82) miR-199a appeared to be less effective in suppressing metastasis, it inhibited metastasis at day-49 and -64. These data suggest that miR-199a is an early suppressor of metastasis.

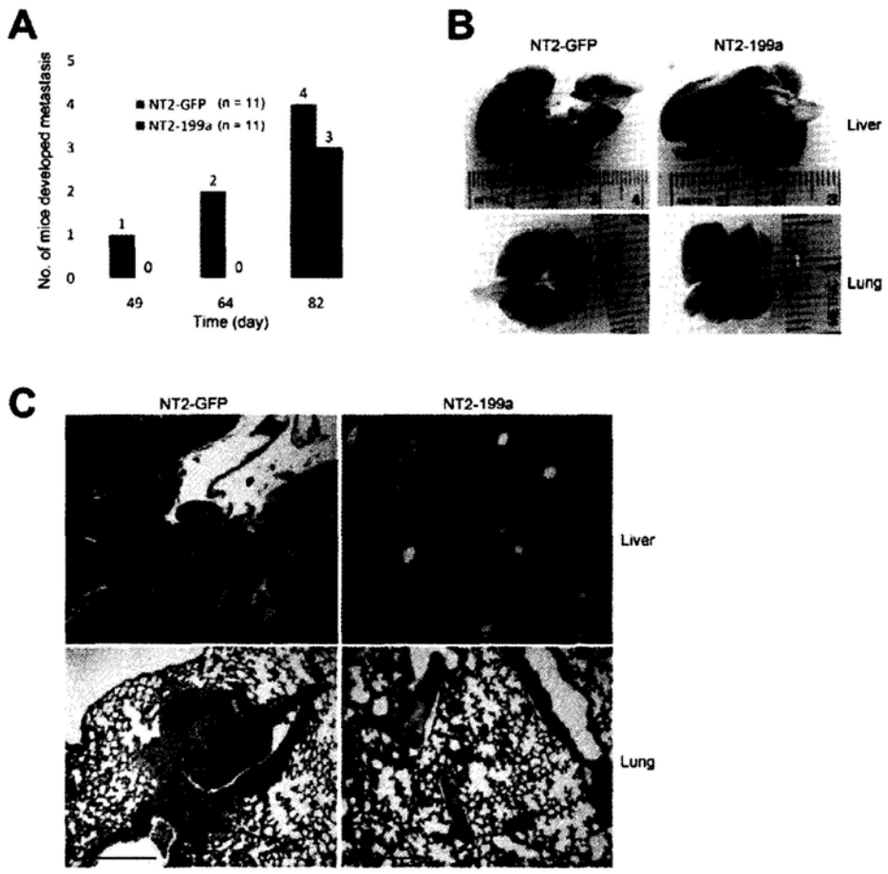

Figure 3.6 miR-199a suppresses cancer metatstasis in mouse xenograft model. (**A**) Number of mice developed metastasis. Same number of NT2-GFP & -199a cells were injected intravenously in athymic nude mice tail vein (n = 11). Animals were sacrificed and necropsied for metastasis at days 49, 64 and 82 post-injection. (**B**) Gross view of cancer metastasis in lungs and livers. No metastasis was observed in NT2-199a group at days 49 and 64. (**C**) H&E stained sections of lungs and livers in NT2-GFP and NT2-199a groups. Metastasis is indicated. Magnification: 10X. Bar: 100 um.

3.3.7 Identification of PODXL as the target of miR-199a-5p

miRNAs are non-coding riboregulators that regulate mRNA stability or translation (He & Hannon, 2004). Since only miR-199a-5p was related to tumor progression I sought to identify the targets of miR-199a-5p to account for its activity (**Figure 3.2B**). I presumed that the targets would be significantly downregulated in the malignant NT2 cells. Therefore, coupling the previous microarray expression data of this line with multiple miRNA target prediction algorithms (TargetScan and PicTar), I generated a list of downregulated predicted target genes (**Table 3.1**). Notably, PODXL was one of the significantly downregulated target genes. It is an anti-adhesive transmembrane sialoglycoprotein normally expressed in kidney podocytes (Kerjaschki *et al*, 1984). However, it is also implicated in the development of aggressive forms of cancers such as malignant astrocytic tumor, breast cancer, prostate cancer, small cell lung carcinoma as well as malignant testicular embryonal carcinoma (Casey *et al*, 2006; Hayatsu *et al*, 2008; Koch *et al*, 2008; Schopperle *et al*, 2003). Western blot analysis confirmed overexpression of this protein in NT2 cells, and a reciprocal relationship with the miR-199a-5p level (**Figure 3.7A**). Furthermore, demethylation of NT2 cells by 5-aza restored the miR-199a-5p level and suppressed PODXL expression, suggesting a link between methylation, miR-199a-5p expression and PODXL level (**Figure 3.7B**). To demonstrate the effect of the

miRNA on PODXL level, I transfected NT2 cells with different concentrations of miR-199a-5p mimics. Seventy-two hours after transfection, PODXL protein was significantly decreased. The same effect was observed when NT2 cells stably expressed miR-199a (NT2-199a) (**Figure 3.7C**). As the NT2-199a cells were transfected with miR-199a-5p inhibitor (5pi), the PODXL level was restored. Surprisingly, miR-199a-3p inhibitors (3pi) also restored PODXL, probably because both inhibitors target the same primary miRNA precursor molecules (**Figure 3.7D**). Regulation of PODXL by miR-199a-5p is most likely through binding of miRNA at its 3'-UTR. To validate this speculation, I cloned the two predicted binding sites in PODXL 3'-UTR linked to *firefly* luciferase vectors. When these luciferase vectors were co-transfected with miR-199a-5p mimics in NT2 cells, luciferase activity of the vector carrying the conserved binding site was significantly suppressed. However, miR-199a-5p did not suppress the vector carrying a poorly conserved binding site. To show that the suppression of luciferase activity is due to binding of the miRNA to the seed sequence, I generated the mutant constructs by mutating the seed sequence. As expected, miR-199a-5p had little effect on the mutant constructs (**Figure 3.7E**). These data show that miR-199a-5p regulates PODXL through a conserved binding site in its 3'-UTR.

Table 3.1 Predicted targets of miR-199a-5p

Gene symbol	Gene name	Probeset ID	P-value	Fold change (Tumor vs Normal)
DEPDC1B	DEP domain containing 1B	226980_at	8.07E-07	37.5518
WNK3	WNK lysine deficient protein kinase 3	232282_at	1.27E-05	19.3676
SEMA6A	sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6A	223449_at	8.21E-07	19.3104
PPP1R9A	protein phosphatase 1, regulatory (inhibitor) sub-unit 9A	228494_at	1.60E-06	18.8923
ASRGL1	asparaginase like 1	218857_s_at	3.56E-05	14.707
JPH3	junctophilin 3	229294_at	5.86E-05	14.6071
GCNT2	glucosaminyl (N-acetyl) transferase 2, I-branching enzyme (I blood group)	230788_at	3.13E-06	14.5888
TAF9B	TAF9B RNA polymerase II, TATA box binding protein (TBP)-associated factor, 31kDa	221618_s_at	5.15E-06	12.1248
AUTS2	autism susceptibility candidate 2	212599_at	4.55E-05	11.9623
EIF2C1	eukaryotic translation initiation factor 2C, 1	228120_at	2.14E-07	11.3943
PAQR9	progestin and adipoQ receptor family member IX	1558322_a_at	1.31E-05	10.9816
ATXN7L1	ataxin 7-like 1	227732_at	7.65E-06	9.97617
PODXL	podocalyxin-like	201578_at	1.14E-05	8.67335
ACVR2B	activin A receptor, type IIB	220028_at	4.23E-06	8.34039
C21orf66	chromosome 21 open reading frame 66	221158_at	1.14E-07	7.99864
CDCA7L	cell division cycle associated 7-like	225081_s_at	2.32E-07	7.43984
PLEKHH1	pleckstrin homology domain containing, family H (with MyTH4 domain) member 1	225727_at	9.83E-05	7.20608
KIAA1553	KIAA1553	227920_at	1.53E-05	6.79427
LRP4	low density lipoprotein receptor-related protein 4	212850_s_at	0.00011295	6.70848
ARHGAP19	Rho GTPase activating protein 19	212738_at	1.65E-05	6.06277
RBBP4	retinoblastoma binding protein 4	217301_x_at	2.79E-07	5.63811
SNN	stannin	218032_at	1.76E-05	5.63048
CDKN1C	cyclin-dependent kinase inhibitor 1C (p57, Kip2)	213182_x_at	1.13E-05	5.54601
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral onco-gene homolog	205051_s_at	0.00015213	5.44202
NUP210	nucleoporin 210kDa	212316_at	1.04E-05	4.90807
FLRT3	fibronectin leucine rich transmembrane protein 3	222853_at	4.46E-06	4.76393
RUNX3	runt-related transcription factor 3	204197_s_at	9.07E-07	4.57197
TTC9	tetratricopeptide repeat domain 9	213172_at	5.36E-05	4.56473
RGMA	RGM domain family, member A	223468_s_at	0.00167113	4.04001
PLXND1	plexin D1	222369_at	0.0001831	3.9669
ARL6IP6	ADP-ribosylation-like factor 6 interacting protein 6	225707_at	5.85E-05	3.85206
SMARCD1	SWI/SNF related, matrix associated, actin depen-dent regulator of chromatin, subfamily d, member	209518_at	1.03E-05	3.68161

	1			
D4S234E	DNA segment on chromosome 4 (unique) 234 expressed sequence	209570_s_at	1.82E-06	3.65204
CCNJ	cyclin J	219470_x_at	6.56E-05	3.5275
SMARCA4	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4	213720_s_at	3.92E-06	3.50942
RANBP2	RAN binding protein 2	201712_s_at	9.23E-06	3.40035

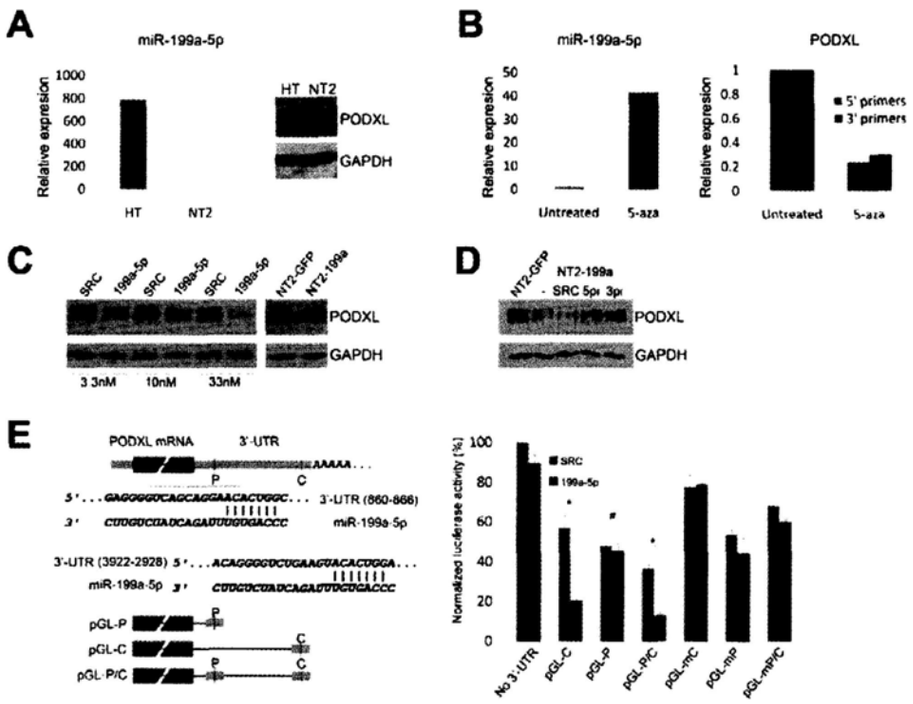

Figure 3.7 Identification of PODXL as the target of miR-199a-5p. (**A**) Reciprocal expression of miR-199a-5p and PODXL in normal (HT) and cancerous (NT2) cell lines. The miRNA level of miR-199a was determined by real-time PCR while PODXL protein level was determined by Western blot analysis. (**B**) Expression of miR-199a-5p and PODXL of NT2 cells treated with or without 5-aza-2'-deoxycytidine (5-aza). Both 5′ and 3′ end primers for PODXL mRNA were shown. (**C**) PODXL level of NT2 cells transiently transfected with miR-199a-5p mimics (199a-5p) or stably express miR-199a-5p (NT2-199a). GAPDH was used as a loading control. SRC: scramble miRNA control. (**D**) PODXL level of NT2-199a cells transiently transfected with miR-199a-5p (5pi) or miR-199a-3p (3pi) inhibitors. (**E**) Luciferase report assay of PODXL 3'-UTR. Two miR-199a-5p targeting sites (P: poorly conserved site; C: conserved site) were cloned to the 3'-end of Firefly luciferase (pGL-P and pGL-C). The mutant constructs (pGL-mC and pGL-mP) were generated by changing the binding sites to complementary sequences. The plasmids were co-transfected with miR-199a-5p mimics (199a-5p) or scramble miRNA control (SRC), together with a *Renilla* luciferase plasmid as a normalization control. Luciferase activity were measured 48 hrs post-transfection (*$P < 0.001$; #$P = 0.806$, 2-tailed Student's *t*-test). Error bar: s.e.m. of triplicates.

3.3.8 PODXL is highly expressed in malignant testicular cancer

Although PODXL was identified to be a target of miR-199a-5p, its role in testicular cancer progression remains unclear. Using the same tissue arrays, I analyzed the level of PODXL protein in tumors of different grades by immunohistochemistry (IHC). I found high levels of PODXL in malignant cancers including seminoma, non-seminomatous embryonal carcinoma and yolk sac tumor, but not in normal or benign tissues (**Figure 3.8A**). Although not all cases of aggressive testicular cancer expressed PODXL, I observed a trend of an increased proportion of malignant tumors with the level of PODXL (**Figure 3.8B**). Spearman's rank correlation test showed a positive correlation between testicular cancer progression and PODXL level (correlation = 0.261, P = 0.0049). PODXL was previously reported to be a predictor of other cancer types (Casey *et al*, 2006; Hayatsu *et al*, 2008; Somasiri *et al*, 2004). Here, my finding supports the role of PODXL in testicular cancer progression.

3.3.9 Reciprocal relationship between miR-199a-5p and PODXL

A reciprocal relationship between miR-199a-5p and PODXL was observed in cultured cells (**Figure 3.7A-D**). This relationship was further confirmed in tissues (n = 110). PODXL level was divided into 4 groups, based on the IHC staining intensity. A scatter

plot of miR-199a-5p or miR-199a-3p against PODXL level was created. The mean value of both miRNA species decreased with increasing level of PODXL. Spearman's rank correlation test indicated a negative correlation for miR-199a-5p only (correlation = -0.187, $P = 0.05$). Correlation of miR-199a-3p with PODXL was not strong or significant (correlation = -0.058, $P = 0.55$) (**Figure 3.8D**). The difference of the correlation coefficient agrees with the finding that PODXL is a target of miR-199a-5p, but not miR-199a-3p.

3.3.10 PODXL knockdown suppresses cancer invasion *in vitro*

As a target of miR-199a-5p, PODXL might participate in the anti-metastatic function of this miRNA. To validate this hypothesis, we stably knocked down PODXL in NT2 cells with RNAi. The stable knockdown cells (NT2-PODXLi) displayed slower migration as revealed by the wound healing assay (**Figure 3.9**). Moreover, the Matrigel invasion assay showed that NT2-PODXLi was less invasive than the vector control cells (NT2-VC) (**Figure 3.8C**). The invasion property of NT2-PODXLi cells was similar to that of NT2-199a cells. However, in NT2-PODXLi cells the level of miR-199a was relatively invariable (**Figure 3.10**). Thus, we demonstrated that knockdown of PODXL alone without changing the level of its riboregulator miR-199a-5p would suppress cancer invasion similar to the effect of over-expression of miR-199a, implying that PODXL is a downstream target

of miR-199a-5p.

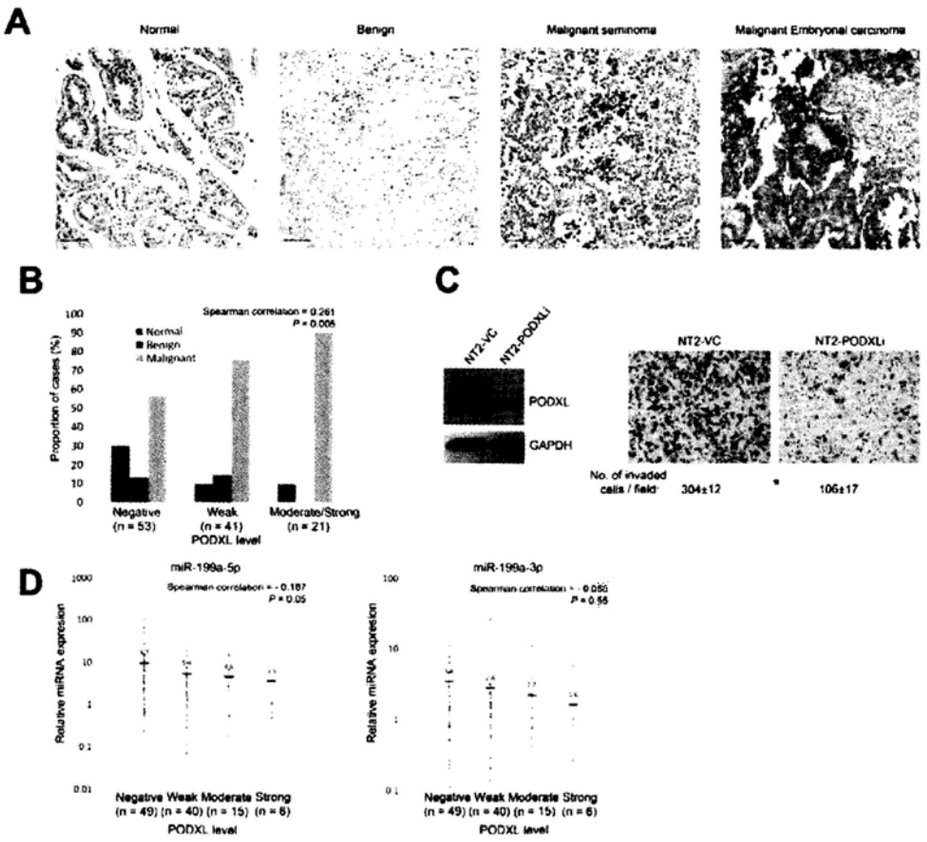

Figure 3.8 PODXL protein is highly expressed in malignant testicular cancers and negatively correlated with miR-199a-5p level. (**A**) Immunohistochemistry of PODXL in normal, benign and malignant testicular cancer sections. Images were captured at 10X magnification. Bar: 100 μm. (**B**) Proportion of patients expressing different levels of PODXL protein in testicular specimens. Samples were divided into 3 groups based on the PODXL level (negative, weak, moderate or strong) and proportion of different grades of tumors were counted. (**C**) Knockdown of PODXL suppresses cell invasion *in vitro*. PODXL was stably knocked down by RNAi in NT2 cells (NT2-PODXLi). The property of cancer cell invasion was assessed by Matrigel invasion assay as contrasted to the vector control cells (NT2-VC). Number of invaded cells per field was counted (mean ± SD). (**D**) Scatter plots of miR-199a-5p and miR-199a-3p expression against PODXL level. Expression of miR-199a-5p, but not 3p, correlates negatively with PODXL level (miR-199a-5p: Spearman correlation = -0.187, $P = 0.05$; miR-199a-3p: Spearman correlation = -0.058, P = 0.55).

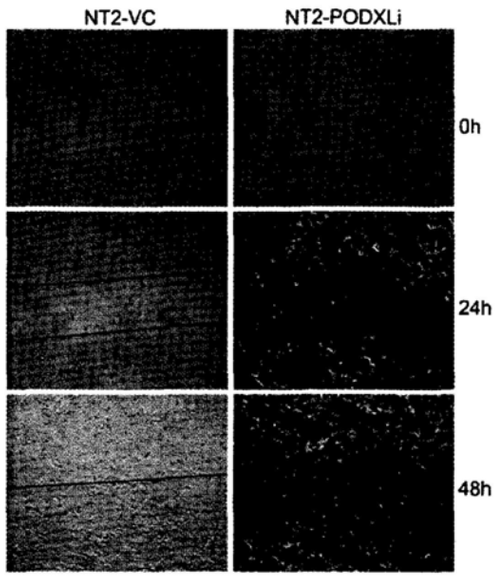

Figure 3.9 Wound healing assay of NT2 cells after PODXL knockdown. NT2-VC: NT2 cells transfected with vector control plasmid; NT2-PODXLi: NT2 cells transfected with PODXL shRNA plasmid.

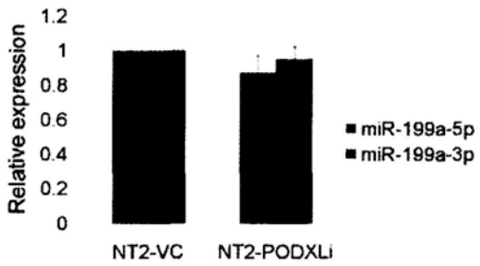

Figure 3.10 Expression of miR-199a in NT2-VC and NT2-PODXLi cells. The relative expression level of miR-199a-5p and miR-199a-3p was determined by real-time RT-qPCR. Error bar: s.e.m. of triplicates.

3.4 Discussion

Primary tumors must be able to invade their surrounding tissues to develop metastasis (Hirohashi & Kanai, 2003). Therefore, cancer invasion is a critical step in metastasis. The molecular basis of invasion could be dysregulation of cell-cell adhesion molecules such as cadherins, integrins and selectins (Hirohashi & Kanai, 2003; Makrilia *et al*, 2009). Genetic (germline mutation), epigenetic (DNA methylation or histone modification) or genomic (loss of heterozygosity or copy number variation) alterations can all contribute to gene dysregulation. Despite the efforts invested in tracking the genes for the disease by linkage analysis, no specific testicular cancer genes have been identified (Krausz & Looijenga, 2008; Rapley, 2007). Testicular germ cell tumor initially develops in the seminiferous tubules where germ cells differentiate. Aggressive tumor invades the tunica albuginea, a thin layer of tissue surrounding the tubules. Further invasion occurs when tumor cells invade the tunica vaginalis, lymph or blood vessels next to the tumor. The mechanism of testicular cancer invasion is not clear. It might share features common to other cancer types, for instance, expression of matrix metalloproteinase (MMP) for digestion of extracellular matrix (Nabeshima *et al*, 2000). In this Chapter, I described an epigenetically linked dysregulation of a conserved miRNA 199a. This is caused by aberrant methylation in an intronic region of *DNM3* at 1q24.3. Intronic methylation has

been largely ignored in previous genome-wide profiling, due to its unclear role in gene regulation. Here I found that hypermethylation in the *DNM3* intron leads to miR-199a silencing. Both miR-199a methylation and expression are correlated with tumor progression. I demonstrated the anti-invasiveness and anti-metastasis properties of miR-199a. Subsequently I identified an embryonal carcinoma tumor antigen, PODXL, as the target of miR-199a-5p. PODXL is an anti-adhesive protein that is upregulated in many aggressive tumors (Casey *et al*, 2006; Hayatsu *et al*, 2008; Somasiri *et al*, 2004), but the mechanism of this event is unknown. We showed that miR-199a-5p is a negative regulator of PODXL. Based on the data I propose that epigenetic alteration in an intron of DNM3 leading to dysregulation of miR-199a and PODXL is one of the causes for testicular cancer invasion.

miRNA is recognized as an important class of riboregulator. They regulate a variety of processes such as cell differentiation, development, tumorigenesis and cancer progression (Bartel, 2004). miRNA can be oncogenic or tumor suppressive (Esquela-Kerscher & Slack, 2006). Specifically, some miRNA such as miR-122, miR-148a, miR-34b/c, miR-21, miR-373 and miR-520 (Huang *et al*, 2008; Lujambio *et al*, 2008; Tsai *et al*, 2009; Zhu *et al*, 2008) have been shown to be important in cancer metastasis. However, few miRNAs for testicular cancer metastasis/invasion are known. miR-199a was initially

identified to be an evolutionarily conserved small RNA essential for development (Chakrabarty *et al*, 2007; Friedman *et al*, 2009; Lee *et al*, 2009; Lin *et al*, 2009). Recently it is also reported to be linked to other aggressive tumor types, such as gastric cancer (Ueda *et al*, 2009), bladder cancer (Ichimi *et al*, 2009), uveal melanoma (Worley *et al*, 2008) and ovarian cancer (Chen *et al*, 2008; Iorio *et al*, 2007; Nam *et al*, 2008). The anti-invasion/metastasis property of miR-199a demonstrated in this study further supports the tumor suppressor role of this miRNA.

PODXL is another frequently upregulated protein in malignant tumors (Casey *et al*, 2006; Hayatsu *et al*, 2008; Somasiri *et al*, 2004). It is an anti-adhesion transmembrane protein that inhibits cell-cell interaction through the charge-repulsive effects of its extensively sialoglycosylated extracellular domain (Takeda *et al*, 2000). Disruption of cell-cell interaction at primary sites is a crucial step in developing an invasive phenotype. For the first time, in my study, the link between PODXL and miR-199a-5p was established. In another independent study, forced expression of PODXL in MCF-7 breast carcinoma cells perturbed cell-cell interaction (Somasiri *et al*, 2004). In my study, forced expression of miR-199a (as associated with suppression of PODXL) in testicular cancer cells enhanced cell-cell interaction with endothelial cells (**Figure 3.11**). These data indicate that miR-199a regulates cell-cell interaction, consistent with that of PODXL. How-

ever, I cannot rule out targets other than PODXL that modulate cell-cell interactions. The increase in adhesion to HUVEC cells might also promote metastasis in blood vessels. Animal study is needed to prove this potential.

In summary, I reported an epigenomic approach for screening disease-related "hotspots" in testicular cancer; it revealed that miR-199a was regulated by DNA methylation. My data support the role of miR-199a as an anti-invasive/metastatic miRNA, in part through its target protein PODXL.

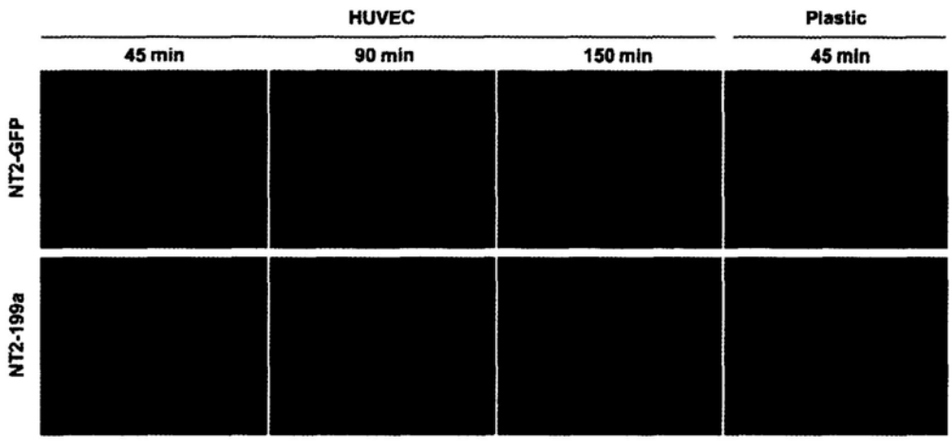

Figure 3.11 Adhesion of NT2-GFP and NT2-199a cells to HUVEC cells. Cells adhered to HUVEC cells were observed at 45, 90 and 150 minutes. The number of cells was indicated by the green fluorescent signal.

Chapter 4

General Discussion and Conclusion

4.1 Overview of the project

The present research is focused on the epigenetic changes that are related to human diseases. Epigenetic change, in contrast to genetic mutation, refers to the alteration of several types of chromatin modifications other than the sequence of DNA. In this regard, DNA methylation plays a critical role in various molecular events such as X inactivation, genomic imprinting, reprogramming. Defect in these processes accounts for a number of inherited diseases. The aberrant methylation changes that occur in cancer raise particular concerns.

Testicular cancer is a common reproductive malignancy in young men. Since most testicular cancers originate from germ cells, they represent a special class of cancers that share some properties of cancer stem cells, such as expression of embryonic stem cell markers. Embryonal carcinoma, one of the testicular cancers, is undifferentiated cancer cell that can form teratoma in nude mice. It is also capable of differentiating into neural cells upon treatment of retinoic acid.

My research began with the genome-wide profiling of DNA methylation in embryonal carcinoma. Interesting features unique in embryonal carcinoma were found. In particular, genes that are affected by DNA methylation only represent a small propor-

tion (~20%) of all differentially methylated genes. For those genes controlled by methylation, the presence of CpG island is irrelevant. Some genes, such as *RGAG1* and miR-199a, lack CpG island but the presence of less dense CpG dinucleotides is able to suppress gene transcription.

In subsequent analysis to link methylation changes to testicular cancer, I found some previously uncharacterized genes and ncRNAs that seem to be important in testicular tumorigenesis. It is known that the expression profiles during testicular germ cell tumorigenesis resemble early embryogenesis (Skotheim *et al*, 2005). Some genes, in addition to their presumable role in cancer biology, may also play some unknown functions in spermatogenesis, an important developmental process in human reproduction. This is perhaps demonstrated by the finding of a developmentally regulated transcript that is silenced in testicular cancer by DNA methylation. The gene, named *ZSWIM2*, is a male germ cell specific gene whose function is currently unclear. It might participate in the ubiquintination pathway by act as an E3 ubiquitin ligase. Interestingly, I found a developmentally regulated expression pattern of murine *Zswim2* in spermatogenesis. Its prominent expression in round spermatids suggests a role in late spermatogenesis. The generation of transgenic knockout mice would help us understand the role of this gene on spermatogenesis and perhaps germ cell tumorigenesis.

My ultimate goal is to explain the observed epigenetic changes in testicular cancers that are relevant to tumorigenesis or spermatogenesis. The present study reveals the complexity of gene regulation that involves different layers of regulation. As demonstrated in this thesis, methylation of DNA directs silencing of both protein-coding genes and ncRNAs. DNA methylation suppresses miRNAs that in turn disturb the equilibrium of oncogenes and tumor suppressor genes by post-transcriptional regulation. For example, I demonstrated that miR-199a appeared to regulate testicular cancers progression by targeting PODXL to mediate cancer metastasis and invasiveness. These observations suggest that DNA methylation can contribute to tumorigenesis by different mechanisms, other than our previous knowledge on germline mutation.

4.2 Summary and conclusion

To summarize, DNA methylation profiling revealed a large number of DMRs in testicular cancer. Some of the DMRs were coupled with transcriptional regulation of genes and ncRNAs. Three genes were validated and confirmed in primary tissue; these include *APOLD1*, *PCDH10* and *RGAG1*. Moreover, 3 snoRNAs (HBII-240, ACA33 and ACA8) and 3 miRNAs (hsa-mir-199a-2, hsa-mir-124a-2, and hsa-mir-184) were also differentially methylated. miR-199a was selected for in-depth studies. Analysis of 105 primary testicular tissue samples revealed a correlation of miR-199a methylation with tumor progression. An inverse relationship between methylation and expression was also observed. Expression of miR-199a in cancer cell line suppressed cancer invasive and metastatic phenotypes, as well as changed tumor growth and cell adhesion properties. Genome-wide screening and bioinformatic prediction identified PODXL to be a target of miR-199a-5p. PODXL was confirmed as a target by various assays in *in vitro* cultured cell system. The reciprocal relationship between PODXL and miR-199a level was also demonstrated in primary tissue *in vivo*. Significantly, PODXL level was also correlated with testicular cancer progression. Knockdown of PODXL resulted in anti-invasive property similar to overexpression of miR-199a, suggesting PODXL is a downstream target of miR-199a for cancer invasiveness or metastasis.

To conclude, MeDIP-Chip is a useful genome-wide approach for identification of epigenetic "hotspot" in cancers. Coupling with expression microarray array and pharmacological approcah, those differentially expressed genes that are governed by DNA methylation can be surveyed. In addition, miR-199a is related to testicular cancer progression through targeting PODXL, both of which are important in testicular cancer invasion and metastasis.

4.3 Future work

Many genes and ncRNAs were identified in this project. Importantly, several of them have not been characterized and the function in tumorigenesis remains elusive. It is worthwhile to study the biological function of every dysregulated genes. For instance, *APOLD1* is frequently methylated in testicular cancer patients. It is downregulated in different cancer types in addition to testicular cancer. The function of *APOLD1* is unknown. It might act as a tumor suppressor gene. Future study can focus on the role of this protein in tumorigenesis.

Genes that are silenced in testicular tumorigenesis might play an important role in normal development, as discussed above (Chapter 4.1 Overview of the project). *ZSWIM2* is one of the genes discovered that may regulate spermatogenesis. Currently, a transgenic animal model of *Zswim2* knockout is being produced at the Laboratory of Clinical Genomics, *Eunice Kennedy Shriver* National Institute of Child Health and Human Development. With this animal model available, I hope we can begin to understand the physiological function of this gene in both normal germ cell development and testicular cancer tumorigenesis.

Supplementary Materials

Supplementary Table 2.1 Differentially methylated promoters with expression data of the corresponding genes.

Hypermethylated promoters

Chr.#	Promoter Start	Promoter End	Gene Symbol	Affy Probeset ID	P-value (Annova)	Fold Change
chr7	93667640	93669030	COL1A2	202403_s_at	4.39E-09	-248.109
chr15	46957082	46957725	EID1	211698_at	6.76E-07	-244.542
chr7	23058561	23059818	GPNMB	201141_at	3.93E-07	-137.799
chrY	2752457	2753103	RPS4Y1	201909_at	1.27E-06	-133.937
chr1	159332214	159332814	DDR2	225442_at	4.76E-09	-80.8287
chr12	13240401	13241090	EMP1	201324_at	3.31E-06	-74.929
chrX	102390793	102391410	TCEAL7	227705_at	5.11E-07	-60.2281
chr4	134427575	134428175	PCDH10	228635_at	8.25E-07	-53.0055
chr1	183381661	183382261	PTGS2	204748_at	1.88E-06	-51.9979
chr5	38881218	38882028	OSMR	205729_at	2.38E-05	-46.8265
chr1	159769520	159770487	RGS4	204338_s_at	1.14E-08	-34.565
chr2	110012917	110013781	LIMS3	223800_s_at	2.95E-05	-30.7439
chr12	45758445	45759045	AMIGO2	222108_at	1.54E-05	-29.733
chr8	104453338	104453938	CTHRC1	225681_at	1.20E-06	-25.8796
chr10	14090438	14091038	FRMD4A	225163_at	1.68E-08	-20.5507
chr3	147361880	147362665	PLOD2	202619_s_at	3.70E-08	-17.4504
chr14	22520734	22522192	JUB	225806_at	1.65E-06	-14.5991
chr1	147593709	147594385	CTSK	202450_s_at	7.20E-07	-13.9574
chr3	115825642	115826652	ZBTB20	235308_at	9.22E-06	-13.7445
chr6	134540551	134541151	SGK	201739_at	3.22E-08	-13.1976
chr20	19817535	19818135	RIN2	209684_at	1.09E-06	-12.0817
chr3	153499240	153499992	MBNL1	201152_s_at	7.55E-07	-11.5347
chr1	78797129	78797729	IFI44L	204439_at	6.50E-05	-9.39665
chr10	124218232	124218832	HTRA1	201185_at	7.37E-07	-9.03052
chr3	29296856	29298293	RBMS3	243041_s_at	2.71E-07	-8.61761
chr1	233012280	233012881	LGALS8	208934_s_at	3.80E-07	-7.97623
chr1	85497857	85498663	DDAH1	229456_s_at	7.38E-07	-7.42616
chr1	194620263	194620863	LHX9	1562736_at	4.32E-05	-7.37534
chr2	33246801	33247401	LTBP1	202729_s_at	8.69E-06	-7.25582
chr11	58668061	58668661	FAM111A	218248_at	4.84E-06	-6.25441
chr2	28529247	28530338	FOSL2	218880_at	2.13E-05	-6.12287
chr6	101952678	101953976	GRIK2	213845_at	0.00040358	-5.10917
chr4	6685176	6685776	MAN2B2	214703_s_at	1.22E-05	-4.908
chr1	146721090	146721816	MTMR11	205076_s_at	2.82E-06	-4.73889
chr20	1843221	1843821	SIRPA	202896_s_at	1.38E-06	-4.62705

chr12	107723693	107724293	SSH1	221753_at	6.55E-06	-4.50284
chr2	159817717	159818317	TANC1	225308_s_at	1.38E-05	-4.44992
chr3	187562398	187563254	DGKG	235843_at	6.89E-05	-4.29688
chr8	108578861	108579979	ANGPT1	205609_at	0.00041234	-4.06368
chr2	19478201	19478801	OSR1	228399_at	0.0010305	-3.68572
chr3	55489985	55490961	WNT5A	213425_at	1.18E-07	-3.58959
chr12	12951694	12952933	GPRC5A	203108_at	0.00015837	-3.36443
chr4	138811134	138811734	PCDH18	225975_at	0.00030213	-3.26266
chr12	9913625	9914225	CLEC2B	209732_at	0.00012359	-3.20578
chr5	141328306	141328993	RNF14	201823_s_at	1.39E-05	-3.09801
chr7	137065333	137065933	CREB3L2	212345_s_at	7.17E-06	-3.03128
chr6	29902997	29903597	HLA-G	210514_x_at	2.67E-07	-3.00268
chr12	14818062	14818706	H2AFJ	224301_x_at	2.26E-05	-2.99456
chr8	23454428	23455029	SLC25A37	226179_at	5.49E-06	-2.98191
chr1	164266729	164267329	CREG1	201200_at	2.74E-05	-2.88774
chr3	169224982	169225582	GOLPH4	204324_s_at	1.05E-05	-2.88338
chr1	93786058	93786658	BCAR3	239908_at	0.00061847	-2.85607
chr1	183529189	183529889	PLA2G4A	210145_at	0.00130673	-2.82478
chr1	146684133	146684733	BOLA1	219345_at	3.34E-05	-2.74018
chr14	20340278	20340898	RNASE1	201785_at	0.00076033	-2.72543
chr4	160506472	160507119	RAPGEF2	1569238_a_at	1.24E-05	-2.69554
chr7	89982863	89983934	PFTK1	204604_at	0.00015037	-2.63763
chr11	110991672	110992272	SNF1LK2	233648_at	0.00663379	-2.63301
chr1	232372881	232373514	LYST	210943_s_at	9.35E-06	-2.63211
chr17	38220797	38221397	BECN1	208946_s_at	6.50E-06	-2.5981
chr7	110324218	110325423	LRRN3	209841_s_at	0.00020172	-2.56467
chr9	112931243	112931843	SLC31A2	204204_at	0.00072585	-2.55756
chr1	242134935	242135535	KIF26B	1561689_at	0.00024541	-2.37907
chr1	86881687	86882705	SH3GLB1	209091_s_at	2.77E-05	-2.34655
chr5	140531925	140532541	PCDHB7	231738_at	0.00193953	-2.30601
chr10	103549092	103549692	MGEA5	223494_at	0.00147797	-2.28782
chr3	36924847	36925512	LBA1	213261_at	0.00105052	-2.24716
chr16	85169116	85169716	FOXL1	243409_at	0.00968824	-2.20589
chr4	170564992	170565689	SH3RF1	225590_at	3.99E-05	-2.17185
chr3	20055814	20056414	PCAF	203845_at	0.00476313	-2.10626
chr17	4682804	4683477	MINK1	214246_x_at	5.38E-06	-2.06356
chr1	177734187	177734897	MR1	210224_at	0.00387801	-2.0623
chr20	1507265	1507865	SIRPB1	206934_at	0.00065956	-2.01731
chr16	31014666	31015266	VKORC1	217949_s_at	2.14E-06	-1.87079
chrX	76971840	76972483	ATP7A	205197_s_at	0.00439531	-1.80436
chr16	83241454	83242079	C16orf44	219453_at	0.000461	-1.78027

chr3	135006823	135007492	SRPRB	227737_at	3.29E-05	-1.76981
chr1	68228381	68228981	DIRAS3	215506_s_at	0.00131237	-1.73675
chrX	32906102	32906702	DMD	207660_at	0.00226729	-1.72811
chr3	39518059	39518659	MOBP	207659_s_at	0.00126208	-1.71944
chr15	70308017	70308617	PKM2	201251_at	0.00071046	-1.71607
chr8	11571377	11571977	GATA4	1570276_a_at	0.00018933	-1.70111
chr1	37691113	37691790	DNALI1	205186_at	0.00717893	-1.63706
chr15	80896068	80896668	LOC440295	217643_x_at	0.0315694	-1.61397
chr2	158520197	158520846	ACVR1	203935_at	0.00482816	-1.61137
chr3	159771124	159771846	MLF1	204784_s_at	0.00035105	-1.61037
chr19	8918840	8919440	MUC16	220196_at	0.0838416	-1.56636
chr1	153248423	153249023	MEF2D	203003_at	0.0010073	-1.56604
chr18	30512928	30513530	DTNA	208430_s_at	0.00330319	-1.56344
chr1	157348469	157349069	CD84	211189_x_at	0.0107839	-1.55891
chrX	133666244	133667250	FAM122C	241991_at	0.0138248	-1.54298
chr3	25444258	25444858	RARB	231673_at	0.0169592	-1.52402
chr1	189887281	189887940	B3GALT2	210121_at	0.00086452	-1.51176
chr13	77390534	77392025	EDNRB	204271_s_at	0.0018726	-1.50206
chr1	43423792	43424392	TIE1	204468_s_at	0.0324728	-1.49842
chr8	4264896	4265496	CSMD1	243724_at	0.00021673	-1.49581
chr8	1979990	1980755	MYOM2	205826_at	0.00998197	-1.47084
chr12	56159355	56160203	ARHGAP9	232543_x_at	0.0269643	-1.46005
chr16	20494999	20495673	ACSM2	241914_s_at	0.0236942	-1.45638
chrX	101430645	101431245	NXF2	234173_s_at	0.0207138	-1.45309
chrX	53233680	53234280	IQSEC2	229840_at	0.0182576	-1.44157
chr12	1807077	1807677	LRTM2	1558530_at	0.0183913	-1.44048
chr17	3283785	3284385	OR1E2	208587_s_at	0.013833	-1.43448
chr12	12830609	12831209	APOLD1	221031_s_at	0.00469257	-1.42546
chr3	124620646	124621246	ADCY5	242891_at	0.0083467	-1.42065
chr12	9159725	9160325	A2M	1558450_at	0.0147966	-1.42033
chr1	155978773	155979373	CADM3	217442_at	0.00796208	-1.39875
chr4	24657995	24658595	DKFZp761B107	226141_at	0.00319572	-1.38945
chr11	101692892	101693505	BIRC3	210538_s_at	0.0168651	-1.38544
chr4	45966742	45967773	GABRG1	241805_at	0.00661567	-1.38111
chr12	103199484	103200116	EID3	231292_at	0.139438	-1.37579
chr12	27289358	27289958	STK38L	212572_at	0.0008475	-1.368
chr12	99369455	99370321	NR1H4	1554375_a_at	0.013367	-1.36646
chr1	143411711	143412668	PDZK1	243168_at	0.00217799	-1.35927
chrX	100903808	100904408	NXF5	234648_s_at	0.00420831	-1.3548
chr12	8705866	8706466	MFAP5	213765_at	0.0974382	-1.35006

chr2	187539262	187539922	ZSWIM2	1554932_at	0.0109417	-1.34956
chr6	46934675	46935275	GPR116	212951_at	0.0012891	-1.33229
chr7	100267813	100268413	MUC17	232407_at	0.0185937	-1.32556
chr15	72888205	72888805	LMAN1L	220420_at	0.0232909	-1.32276
chrX	52071339	52072096	XAGE1	220057_at	0.104124	-1.31294
chr1	74851343	74852368	C1orf173	229973_at	0.00187845	-1.30397
chr21	44597068	44598113	TRPM2	1564242_at	0.00232856	-1.30195
chr1	243905000	243905616	NLRP3	216015_s_at	0.188484	-1.299
chr1	155136113	155136713	CD1E	208592_s_at	0.00306802	-1.29858
chr21	31175645	31176245	KRTAP11-1	1564803_at	0.109809	-1.29835
chr10	30343359	30343959	KIAA1462	213316_at	0.0540907	-1.29595
chr12	113299137	113299737	TBX5	207155_at	0.039101	-1.2952
chr11	19128354	19128954	ZDHHC13	219296_at	0.00081394	-1.27552
chr12	14024219	14024819	GRIN2B	210412_at	0.0252612	-1.27015
chr1	24214777	24215443	IL22RA1	220056_at	0.0018694	-1.2652
chr7	55904094	55904694	SUMF2	225002_s_at	0.00305955	-1.26266
chr2	167940188	167940788	CMYA3	228794_at	0.0172357	-1.26138
chr8	7308357	7309102	SPAG11	207271_x_at	0.00370898	-1.25628
chr20	32577233	32577833	DYNLRB1	1565612_at	0.00330288	-1.25438
chr11	7393350	7393950	SYT9	232445_at	0.0940229	-1.25125
chr8	7319913	7320514	DEFB104A,DEFB104B	1552411_at	0.0215272	-1.24912
chr2	208845066	208845666	CRYGB	207715_at	0.0677032	-1.24469
chr12	98050840	98051440	ANKS1B	243533_x_at	0.0889346	-1.24357
chr16	20398869	20399469	FLJ20581,LOC123876	89977_at	0.00140856	-1.24036
chr15	80512981	80513581	FLJ40113,LOC440295	232402_at	0.265093	-1.23734
chr13	96444504	96445105	OXGR1	1553319_at	0.157415	-1.22995
chr1	244247649	244248249	OR1C1	1567055_at	0.0012821	-1.22993
chr1	85239503	85240191	WDR63	243087_at	0.0696322	-1.22717
chrX	72449369	72450634	CDX4	221340_at	0.0242089	-1.22667
chr8	39290808	39291439	ADAM32	1552266_at	0.0505779	-1.22578
chr1	47317972	47318572	CYP4A22	217319_x_at	0.08087	-1.22286
chr3	9829487	9830087	ARPC4,TTLL3	1554950_at	0.0543925	-1.21787
chr12	2814228	2815380	NRIP2	215104_at	0.0537463	-1.21528
chr1	197912927	197913527	DKFZp434B1231	229275_at	0.0215488	-1.21262
chr1	155072132	155072747	CD1C	205987_at	0.139793	-1.20726
chr1	155036439	155037600	CD1A	210325_at	0.157869	-1.20719
chr7	2091103	2091703	SNX8	223241_at	0.0359824	-1.20645
chr5	168659358	168659958	SLIT3	1561574_at	0.3719	-1.20245

chr6	50893755	50894665	TFAP2B	215686_x_at	0.0942509	-1.20238
chr1	116365693	116366519	C1orf161	1553333_at	0.0493875	-1.20123
chr6	160982383	160982983	LPA	207584_at	0.032047	-1.20101
chr2	26481162	26481762	GPR113	1553016_at	0.103831	-1.19855
chr3	194755298	194756576	ATP13A4	233535_at	0.0753081	-1.19272
chr3	148606518	148607118	ZIC4	236711_at	0.0868127	-1.18609
chr12	4894418	4895018	KCNA1	208479_at	0.102082	-1.18339
chr3	89610858	89611458	EPHA3	206071_s_at	0.0762291	-1.17713
chr16	3193748	3194348	OR1F1	221402_at	0.0583272	-1.17705
chr7	42924938	42925598	HECW1	215584_at	0.0111289	-1.17246
chr16	24173876	24175257	CACNG3	206384_at	0.17346	-1.16784
chr12	108208745	108209345	FOXN4	1564713_a_at	0.00024563	-1.16783
chr3	127388514	127389114	ALDH1L1	215798_at	0.10218	-1.16763
chr7	142088146	142088746	TRPV6	1559405_a_at	0.00716174	-1.16761
chr12	3652851	3653451	EFCAB4B	223955_at	0.00477607	-1.16601
chrX	56473761	56474361	UBQLN2	215884_s_at	0.079516	-1.1652
chr19	7736143	7736743	CLEC4M	207995_s_at	0.227735	-1.16476
chrX	47810947	47811547	SSX5	208528_x_at	0.271405	-1.16343
chr16	7322252	7322852	A2BP1	1566866_at	0.00207135	-1.16161
chr12	11402017	11402617	PRB1,PRB2	211531_x_at	0.0193021	-1.16075
chr12	8893571	8894171	A2ML1	1564307_a_at	0.0609421	-1.15824
chr5	155685835	155686873	SGCD	230730_at	0.0139655	-1.158
chr3	125181917	125182517	ROPN1	231535_x_at	0.121168	-1.15739
chr4	82748923	82749523	RASGEF1B	1554999_at	0.180739	-1.15573
chr15	72209141	72210576	ISLR2	232208_at	0.0354864	-1.15112
chr11	56949652	56950944	SLC43A3	213113_s_at	0.0301199	-1.14763
chr12	12994484	12995366	GPRC5D	221297_at	0.112942	-1.14378
chr1	158374935	158375535	FCGR2B	210889_s_at	0.246578	-1.14007
chr1	167958221	167958978	FMO1	205666_at	0.440535	-1.13667
chr10	53129249	53129861	CSTF2T	212905_at	0.0783426	-1.1354
chr4	15616113	15616740	FGFBP1	205014_at	0.21066	-1.13308
chr5	180347952	180348607	BTNL3	217207_s_at	0.119826	-1.1185
chr12	242163	242763	SLC6A13	237058_x_at	0.0104643	-1.11739
chr16	20609978	20610579	ACSM1	215432_at	0.348934	-1.11645
chr12	8650776	8651378	AICDA	219841_at	0.196712	-1.1118
chr5	151284254	151285406	GLRA1	207972_at	0.283962	-1.11044
chr5	169611174	169611774	LCP2	244578_at	0.047782	-1.10833
chrX	2977714	2978315	ARSF	214490_at	0.20856	-1.10287
chr7	149611688	149612288	GIMAP8	235306_at	0.278888	-1.09975
chr7	116556893	116557794	WNT2	205648_at	0.26502	-1.0955
chr7	94670204	94670838	PON3	213695_at	0.516715	-1.09542

chr1	198130551	198131151	TNNI1	205177_at	0.0943276	-1.09194
chr12	8215917	8216517	ZNF705A	237711_at	0.115187	-1.09128
chr7	65287279	65287879	LOC285908	235397_at	0.344884	-1.07586
chr21	42696126	42696726	UBASH3A	220418_at	0.151757	-1.0749
chr1	43404880	43405600	TMEM125	225822_at	0.400465	-1.07437
chr12	99252517	99253739	SLC17A8	1553415_at	0.501371	-1.06921
chr7	5341507	5342750	ACTB	200801_x_at	0.00162793	-1.06581
chr2	73781092	73781711	NAT8	206963_s_at	0.708048	-1.06342
chr12	10219828	10220428	C12orf59	236646_at	0.58571	-1.05512
chr12	11354533	11355133	PRB4	243389_at	0.0286813	-1.04185
chr12	10972601	10973201	PRH2	243389_at	0.0286813	-1.04185
chr5	22248290	22248890	CDH12	207149_at	0.691769	-1.0236
chr6	26348133	26348733	HIST1H4F	208026_at	0.788394	-1.02078
chr12	7791285	7791885	CLEC4C	1552552_s_at	0.379152	-1.0113
chr20	56158865	56159528	C20orf85	229542_at	0.903475	-1.01025
chr1	152103094	152103694	C1orf104	1552862_at	0.944968	-1.00489
chr8	33542145	33542745	RNF122	219897_at	0.625711	1.01258
chr3	15538035	15538855	COLQ	206073_at	0.832997	1.01902
chr21	41610031	41610631	FAM3B	227194_at	0.742727	1.02325
chr3	49289172	49289956	C3orf62	241817_at	0.56242	1.03104
chr1	171768151	171768753	TNN	215271_at	0.796337	1.03117
chr12	4358908	4359712	FGF23	221166_at	0.496373	1.04231
chr8	64161004	64161666	TTPA	210614_at	0.693916	1.04492
chr14	60021448	60022048	C14orf39	1561985_at	0.2643	1.05499
chrX	52808896	52811530	TMEM29	223203_at	0.22711	1.07757
chr1	238238488	238239088	WDR64	1553373_at	0.537422	1.09327
chr4	3408193	3408793	RGS12	214361_s_at	0.00160603	1.09967
chr5	140481265	140481865	PCDHB4	240317_at	0.220441	1.13001
chr7	24096523	24097166	NPY	206001_at	0.28104	1.1368
chr7	2575914	2576702	GNA12	224681_at	0.0103603	1.14784
chrX	47972296	47972904	SSX3	207666_x_at	0.374869	1.17477
chr13	114048207	114048807	CDC16	202717_s_at	0.00155917	1.18189
chr15	81093071	81093671	CPEB1	219578_s_at	0.107698	1.18916
chrX	103306228	103306828	ESX1	1552445_a_at	0.0928371	1.19903
chr12	6793123	6793723	CD4	203547_at	0.0843677	1.20014
chr11	64877824	64878424	TIGD3	229789_at	0.135646	1.20501
chr1	46041570	46042892	MAST2	215660_s_at	0.00707749	1.20793
chr1	202057562	202058284	KLHDC8A	219331_s_at	0.0565727	1.20896
chr7	5504050	5504650	TRIAD3	227065_at	0.0120187	1.21203
chr2	137581694	137582294	THSD7B	232327_at	0.146576	1.21801
chr5	140546577	140547177	PCDHB9	223927_at	0.0925893	1.23188

chr1	85885409	85886666	C1orf181	218932_at	0.0124928	1.26001
chr8	42130840	42131440	AP3M2	203410_at	0.0001554	1.29418
chr11	63204987	63205902	RTN3	219549_s_at	0.00200115	1.29733
chr7	112319802	112320402	GPR85	234303_s_at	0.0948381	1.3016
chr12	46643758	46644359	TMEM106C	201764_at	0.0303604	1.3042
chr1	225785366	225785966	C1orf96	225904_at	0.00099866	1.32085
chr2	201551112	201551712	CLK1	214683_s_at	0.119179	1.34423
chr5	176218905	176219505	UNC5A	236448_at	0.00361638	1.35215
chr1	233206698	233207300	ACTN2	203862_s_at	0.0254998	1.35627
chr1	201946789	201947389	TMCC2	213096_at	0.0190622	1.36063
chr10	1045907	1046507	GTPBP4	218238_at	0.00884736	1.36165
chr21	33022048	33023035	SYNJ1	207594_s_at	0.0253655	1.37773
chr9	14858874	14859741	FREM1	228233_at	0.00623219	1.37941
chr12	119471197	119471797	RNF10	237062_at	0.0189225	1.381
chr8	30132855	30133541	DCTN6	203261_at	0.00059002	1.39256
chr5	6766598	6767837	POLS	202466_at	0.00059247	1.39842
chrX	102739353	102739953	MORF4L2	243857_at	0.0164831	1.40709
chr2	79450987	79451587	CTNNA2	205373_at	0.025292	1.43323
chr1	47403213	47404130	TAL1	206283_s_at	0.00506776	1.43986
chr1	75305637	75306240	LHX8	1569469_a_at	0.0187018	1.44224
chrX	103022850	103023452	MGC39900	1570039_at	0.104848	1.45199
chr1	238938699	238939437	PLD5	1563933_a_at	0.0684384	1.47065
chr1	119244120	119244721	TBX15	230438_at	0.0163359	1.47072
chr20	35043346	35043946	SAMHD1	1559883_s_at	0.030225	1.48121
chr12	101846990	101847590	PAH	205719_s_at	0.012325	1.49702
chr1	201008325	201008925	PLEKHA6	229245_at	0.0317765	1.50144
chr1	152103260	152103910	RUSC1	206949_s_at	6.94E-05	1.5359
chr3	112743047	112743887	CD96	1555120_at	0.068633	1.53839
chr1	150753069	150753771	SLC39A1	217778_at	0.0056635	1.55775
chr4	57388291	57388993	HOP	211597_s_at	0.0025591	1.55828
chr1	114408335	114409082	SYT6	240267_at	1.34E-05	1.61037
chr14	88144019	88144619	ZC3H14	213063_at	0.00021084	1.63237
chr21	40160614	40161331	PCP4	205549_at	0.0423096	1.64365
chr8	57521584	57522348	PENK	213791_at	0.0548262	1.67257
chr5	140509523	140510123	PCDHB6	221317_x_at	0.00130838	1.68852
chr12	27823213	27824647	KLHDC5	225963_at	0.00014326	1.68879
chr15	54543619	54544219	MNS1	219703_at	0.00118783	1.69351
chr11	400184	400784	SI-GIRR,TMEM16J	52940_at	5.89E-06	1.701
chr1	24488290	24488890	C1orf201	227694_at	0.00132508	1.72569

chrX	72003394	72003994	RP11-493K23.2	1557203_at	0.00162769	1.7336
chr1	158812452	158813052	NOS1AP	1563512_at	0.0186163	1.7396
chr7	121378088	121378794	AASS	214829_at	0.00299767	1.74312
chr12	6884560	6885160	LRRC23	217609_at	0.00198228	1.74508
chr1	209790376	209790976	RPS6KC1	218909_at	2.88E-05	1.76967
chr7	3221959	3222559	SDK1	229912_at	0.0110418	1.79224
chr12	6528203	6529100	HOM-TES-103	36030_at	0.00010309	1.808
chr22	28035688	28036939	RASL10A	206850_at	0.00237696	1.8206
chr21	41760785	41761385	TMPRSS2	211689_s_at	0.0387358	1.82328
chr15	75498185	75498785	HMG20A	218152_at	0.00025875	1.83528
chr1	143099571	143100783	POLR3C	210573_s_at	6.35E-05	1.90153
chr18	42780285	42780886	KATNAL2	233644_at	0.0147301	1.91923
chr3	2527773	2528373	CNTN4	229084_at	0.0154215	1.95947
chr8	37714100	37714716	ERLIN2	221542_s_at	2.20E-06	1.96522
chr1	206401702	206402302	C1orf107	220251_at	7.91E-05	1.97523
chr21	41387383	41387983	BACE2	222446_s_at	1.82E-06	1.98796
chr1	85437236	85437876	C1orf52	228135_at	2.45E-05	2.01527
chr1	46519028	46519628	NSUN4	230023_at	0.00013605	2.11681
chr12	8076126	8076726	FOXJ2	203734_at	2.52E-05	2.13285
chr11	1953181	1953781	MRPL23	213897_s_at	0.00045013	2.13541
chr1	181745708	181746415	RNF2	205215_at	0.00158488	2.1428
chr12	26096228	26096877	RASSF8	225946_at	4.39E-06	2.14295
chr21	40589872	40590472	DSCAM	237268_at	0.00289323	2.15044
chr1	150752586	150753891	CREB3L4	226455_at	0.00271977	2.18488
chr8	54303832	54304432	OPRK1	207553_at	0.00013005	2.20323
chr1	168185804	168186484	BAT2D1	211944_at	0.00011694	2.21104
chrX	57500790	57501390	ZXDB	228005_at	0.00033431	2.22067
chr8	69096326	69096926	DEPDC2	1569934_at	0.0196139	2.22233
chr2	88311619	88312219	FLJ10916	219044_at	0.00011748	2.26623
chr12	4788103	4788703	KCNA6	1553347_s_at	0.00430296	2.30744
chr6	341139	341739	IRF4	204562_at	0.00196537	2.3101
chr12	3517141	3517741	PRMT8	207772_s_at	1.22E-05	2.35432
chr12	32913157	32913757	PKP2	207717_s_at	0.0027062	2.36807
chr16	17472053	17472739	XYLT1	213725_x_at	0.00031043	2.40426
chr12	8269236	8269836	FAM90A1	220535_at	0.00144652	2.40674
chr12	4257687	4258287	CCND2	200953_s_at	4.54E-06	2.4361
chr1	57188335	57188935	DAB1	226020_s_at	0.00010492	2.44504
chr7	73613026	73614323	GTF2I	210891_s_at	1.98E-06	2.45087
chr6	10501595	10502195	TFAP2A	204653_at	0.00733902	2.46069
chr20	33357451	33358051	C20orf44	222470_s_at	7.38E-06	2.46622

chr12	3251895	3252495	TSPAN9	220968_s_at	0.00033876	2.48506
chr5	140459918	140460518	PCDHB3	221410_x_at	0.00083844	2.5333
chrX	85108354	85108954	CHM	1569183_a_at	0.0137303	2.61588
chrX	48508826	48509426	TIMM17B	203342_at	1.74E-05	2.6265
chr11	41437818	41438468	LRRC4C	232226_at	0.00115391	2.68331
chr7	26935501	26936244	HOXA3	235521_at	0.0342767	2.78603
chr1	176442787	176443387	C1orf76	222095_s_at	0.00071046	2.79637
chr1	223390824	223392072	CABC1	218168_s_at	1.73E-06	2.80221
chr12	6947347	6947947	PHB2	201600_at	2.00E-05	2.81442
chr12	27754474	27755145	MRPS35	217942_at	7.94E-05	2.82236
chr1	45625805	45626443	TESK2	205486_at	3.27E-05	2.86543
chr1	114843120	114843720	DENND2C	230769_at	6.33E-05	2.9361
chr3	14698726	14699326	C3orf20	232257_s_at	0.00016912	2.97394
chr9	1039854	1040456	DMRT2	223704_s_at	0.00109809	2.98249
chr21	39849740	39850340	B3GALT5	239994_at	0.00067462	3.01009
chr1	204039146	204039746	CR2	205544_s_at	0.00069682	3.13796
chr7	143498594	143499750	ARHGEF5	202715_at	3.32E-05	3.15464
chr7	70209879	70210479	WBSCR17	227434_at	0.00115437	3.16588
chr7	126485123	126485723	GRM8	216255_s_at	0.00027597	3.23161
chr12	6579995	6580595	CHD4	201182_s_at	1.18E-05	3.24499
chr1	54828431	54829031	TTC4,C1orf175	46167_at	2.66E-06	3.29226
chr1	164320394	164320994	RCSD1	225763_at	0.00038196	3.31491
chrX	99468197	99468869	PCDH19	227282_at	0.00025035	3.33151
chr12	12760462	12761062	CDKN1B	209112_at	1.88E-05	3.34642
chr15	67463631	67464231	PAQR5	242871_at	4.25E-05	3.34723
chr3	14442263	14442863	SLC6A6	228754_at	2.81E-05	3.39787
chr1	27951707	27952307	SMPDL3B	205309_at	0.00027754	3.40446
chr12	12093546	12094163	BCL2L14	221241_s_at	4.14E-05	3.43144
chr11	56859790	56860427	SSRP1	200957_s_at	3.19E-07	3.77766
chr12	15360453	15361053	PTPRO	1554199_at	0.00012723	3.84674
chr12	5614632	5615232	TMEM16B	220111_s_at	0.00047901	3.92117
chr1	116921016	116921616	IGSF3	202421_at	2.12E-05	3.99686
chr3	193608614	193609214	FGF12	207501_s_at	0.00017318	4.01025
chr1	35323677	35324277	SFPQ	201586_s_at	3.20E-06	4.11031
chr7	26969436	26970036	HOXA7	206848_at	1.64E-05	4.18729
chr9	68704394	68704994	PIP5K1B	205632_s_at	0.00104982	4.2115
chr1	203195437	203196037	DYRK3	238250_at	2.52E-05	4.50235
chr9	112906146	112906746	ZFP37	207068_at	2.59E-05	4.60709
chr12	5473027	5473627	NTF3	206706_at	0.00084964	4.66223
chr1	153334486	153335086	IQGAP3	229538_s_at	6.68E-05	5.01197
chr2	233665004	233665604	NGEF	227240_at	1.52E-06	5.34726

chr1	50283458	50284075	ELAVL4	206051_at	3.80E-05	5.53421
chr9	100270852	100271452	TMEFF1	205123_s_at	7.94E-05	5.59892
chr2	131072457	131073057	CFC1	223753_s_at	0.00365482	5.65054
chr21	41655320	41655927	MX2	204994_at	3.04E-05	5.71669
chr2	2305242	2305842	MYT1L	205888_s_at	0.00087086	5.74391
chr6	46567662	46568262	DSCR1L1	203498_at	1.23E-05	6.25566
chr4	83768472	83769072	FLJ12993	229623_at	0.00014904	6.27006
chr7	22169673	22170545	RAPGEF5	204681_s_at	1.51E-05	6.51325
chr1	40585228	40585910	ZNF643	207219_at	0.00028113	6.67017
chr12	31122095	31122695	DDX11	208159_x_at	4.16E-06	6.91972
chr1	156716697	156717297	IGSF9	229276_at	6.66E-05	6.93401
chr12	2973663	2974263	TEAD4	41037_at	2.38E-06	7.00861
chr12	34065983	34066764	ALG10	1552304_at	2.59E-05	7.22779
chr12	32259947	32260547	BICD1	1554020_at	3.37E-05	7.82314
chr7	95047010	95047610	DYNC1I1	205348_s_at	0.00017107	8.54967
chr1	24390619	24391321	GRHL3	232116_at	6.50E-05	8.59386
chr1	65486481	65487551	DNAJC6	204720_s_at	0.0001051	8.80073
chr6	122972323	122972923	PKIB	231120_x_at	1.73E-05	9.5959
chr14	22946561	22947161	MYH6	204737_s_at	1.29E-05	10.2781
chr21	40160621	40161221	LOC150084	243027_at	9.35E-06	10.9202
chr12	53268583	53269223	PPP1R1A	205478_at	3.82E-06	11.0893
chr12	16649123	16649847	LMO3	204424_s_at	0.00027128	11.8634
chr12	31371158	31371758	FAM60A	223038_s_at	1.47E-07	12.9728
chr7	38846676	38847276	POU6F2	233772_at	3.37E-05	13.0159
chr7	31504752	31505352	C7orf16	220231_at	0.00021283	13.047
chr12	21447757	21448363	SLCO1A2	207308_at	2.71E-05	14.002
chr1	235875359	235876006	CHRM3	1559633_a_at	1.95E-06	15.9288
chr12	32028759	32029359	C12orf35	218614_at	1.00E-07	17.2325
chr12	6334754	6335354	SCNN1A	203453_at	7.07E-06	22.0019
chr1	43494054	43494654	CDC20	202870_s_at	6.23E-08	22.6948
chr8	25392625	25393225	CDCA2	226661_at	2.98E-07	23.2232
chr3	27736715	27737315	EOMES	231776_at	0.00032009	33.2001
chr1	154962310	154962910	CD1D	205789_at	5.06E-06	34.6742
chr20	54636651	54637251	TFAP2C	205286_at	1.21E-06	47.4313
chrX	69931639	69932239	SLC7A3	230597_at	1.01E-06	56.5338
chr15	87199558	87200158	ACAN	n.a		
chr12	43730651	43731861	DBX2	n.a		
chr6	26332862	26333462	HIST1H3E	n.a		
chr1	146596884	146597484	HIST2H2BF	n.a		
chr1	149008701	149009302	HRNR	n.a		
chr11	126375601	126376771	KIRREL3	n.a		

chr21	30666302	30666902	KRTAP13-2	n.a
chrX	37186514	37187417	LANCL3	n.a
chr9	122471268	122471868	OR1B1	n.a
chr1	243941225	243941825	OR2B11	n.a
chr1	244549525	244550125	OR2L3	n.a
chr1	244668829	244669429	OR2M2	n.a
chr1	244691911	244692511	OR2M3	n.a
chr1	244941640	244942240	OR2T2	n.a
chr1	245128500	245129100	OR2T2,OR2T35	n.a
chr1	245048715	245049315	OR2T29	n.a
chr1	244977431	244978031	OR2T5	n.a
chr11	4893369	4893969	OR51G2	n.a
chr11	56137454	56138054	OR5M1	n.a
chr15	63128866	63129473	OSTbeta	n.a
chrX	119796446	119797047	RP6-166C19.11, RP6-166C19.10, RP6-166C19.9, CT47.8, RP6-166C19.5, RP6-166C19.4, RP6-166C19.3, RP6-166C19.2	n.a
chrX	102337521	102338121	TCEAL5	n.a
chr7	49589995	49590618	VWC2	n.a

Hypomethylated promoters

Chr.#	Promoter Start	Promoter End	Gene Symbol	Affy Probeset ID	P-value (Anno-va)	Fold Change
chr2	189698034	189698634	COL3A1	215076_s_at	3.17E-08	-1675.82
chr6	86251169	86251769	NT5E	203939_at	1.16E-08	-298.898
chr4	159405476	159406076	C4orf18	223204_at	1.30E-06	-180.628
chr12	52707916	52708561	HOXC6	206858_s_at	5.89E-08	-69.3609
chr10	94441605	94442205	HHEX	215933_s_at	2.01E-05	-25.9174
chr3	87072508	87073108	VGLL3	227399_at	2.05E-06	-17.8056
chr4	81357212	81357812	ANTXR2	225524_at	3.77E-07	-17.7698
chr3	147335463	147336063	PLOD2	202619_s_at	3.70E-08	-17.4504
chr17	41656827	41657453	KIAA1267	238774_at	1.40E-05	-16.609
chr9	5524486	5525086	PDCD1LG2	220049_s_at	0.000236	-16.4719
chr3	106745636	106746236	ALCAM	201952_at	5.34E-06	-12.7222

chr8	77779375	77779975	ZFHX4	241700_at	4.09E-07	-12.6772
chr5	64805155	64805755	ADAMTS6	1570351_at	5.04E-06	-10.2418
chr8	95004108	95004708	PPM2C	222572_at	1.02E-06	-9.13518
chr14	51590661	51591261	NID2	204114_at	3.13E-05	-8.55414
chr8	82067916	82068516	PAG1	225626_at	3.29E-05	-8.1713
chr6	11886780	11887765	C6orf105	229070_at	0.0010102	-7.89045
chr3	172570079	172570679	TNIK	240254_at	5.56E-05	-7.34269
chr16	73266734	73267334	MLKL	238025_at	9.36E-05	-6.74339
chr8	91088635	91089235	DECR1	202447_at	3.45E-07	-6.40996
chr8	104405981	104406581	FZD6	203987_at	5.66E-05	-5.83588
chr10	11098336	11098936	CUGBP2	202157_s_at	3.78E-08	-5.60164
chr6	56525392	56525992	DST	215016_x_at	2.18E-06	-5.3122
chr15	54173371	54173971	RFXDC2	1563364_at	0.0006167	-4.88561
chr11	9817607	9818207	SBF2	240326_at	0.000396	-4.12252
chr18	59593092	59593695	SERPINB7	206421_s_at	0.0006218	-4.0352
chr15	30716001	30716601	SCG5	203889_at	1.24E-05	-3.97063
chr10	61819394	61820386	ANK3	237839_at	0.0007007	-3.91859
chr8	116492823	116493423	TRPS1	222651_s_at	6.54E-05	-3.48174
chr3	123900360	123901220	PARP14	224701_at	0.0001855	-3.42235
chr4	184088663	184089263	ODZ3	233291_at	0.0002901	-3.258
chr3	157873227	157873827	TIPARP	212665_at	6.78E-05	-3.20152
chr8	82915132	82915733	SNX16	219793_at	0.0001564	-3.13071
chr3	71436494	71437101	FOXP1	224837_at	1.57E-06	-3.01297
chr18	37858793	37859393	PIK3C3	204297_at	3.04E-05	-2.93991
chr11	17147295	17148364	PIK3C2A	241905_at	0.0054888	-2.92735
chr5	130722587	130723187	CDC42SE2	229026_at	3.41E-05	-2.87585
chr1	93865709	93866309	BCAR3	239908_at	0.0006185	-2.85607
chr11	130281100	130281700	SNX19	202358_s_at	5.46E-06	-2.70345
chr12	131969328	131969930	GOLGA3	202106_at	8.77E-05	-2.70058
chr18	70473782	70474382	ZNF407	233228_at	0.0021123	-2.57881
chr6	13065048	13065648	PHACTR1	238297_at	9.42E-05	-2.48574
chr8	103313609	103314209	RRM2B	223342_at	0.0001103	-2.447
chr6	114289163	114289763	MARCKS	201668_x_at	4.77E-05	-2.43328
chr6	158279183	158279783	SNX9	223028_s_at	1.98E-05	-2.43066
chr3	112308285	112308885	PVRL3	227503_at	0.0004195	-2.40044
chr4	53302817	53303417	USP46	238726_at	0.0002875	-2.36675
chr6	36947124	36947724	C6orf89	224987_at	2.09E-06	-2.26821
chr5	88063376	88063976	MEF2C	209199_s_at	0.0009769	-2.26068
chr5	77973641	77974241	LHFPL2	212658_at	3.09E-05	-2.24945
chr10	3172875	3173475	PITRM1	205273_s_at	3.03E-05	-2.2452
chr2	61404827	61405427	USP34	233595_at	0.0002076	-2.21887

chr4	104356026	104356626	LOC133308	229491_at	5.88E-05	-2.1996
chr7	143011249	143011849	KIAA0738	212979_s_at	0.0015312	-2.18047
chr4	48524056	48524656	FRYL	212546_s_at	4.38E-07	-2.17365
chr11	12919908	12920652	TEAD1	224955_at	0.000198	-2.16535
chr11	95756878	95757478	CCDC82	223301_s_at	0.0003264	-2.16313
chr7	47394882	47395482	TNS3	217853_at	2.93E-05	-2.14554
chr7	130635851	130636451	MKLN1	233219_at	0.0004873	-2.12059
chr5	96145320	96145920	ARTS-1	210385_s_at	0.0014124	-2.08412
chr6	12232992	12233592	HIVEP1	204512_at	0.0002618	-2.07407
chr4	88169751	88170351	C4orf36	238767_at	0.0003844	-1.99552
chr12	83809500	83810100	SLC6A15	206376_at	0.0003495	-1.98785
chr11	124981019	124981619	STT3A	202223_at	0.0001089	-1.9607
chr16	79306030	79306630	CDYL2	1553647_at	0.00032	-1.89822
chr1	6191982	6192697	RPL22	214042_s_at	1.72E-06	-1.86823
chr2	161090783	161091383	RBMS1	225265_at	0.0001199	-1.85319
chr1	19308937	19309537	KIAA0090	212395_s_at	0.0004047	-1.85033
chr5	77752910	77753510	SCAMP1	212416_at	3.65E-05	-1.84099
chr5	156752295	156752895	CYFIP2	215785_s_at	0.0006223	-1.81638
chr6	111750491	111751091	REV3L	208070_s_at	0.0019614	-1.80456
chr6	135818508	135819108	AHI1	221569_at	0.0001658	-1.7533
chr9	6002871	6003471	RANBP6	213019_at	1.78E-05	-1.75174
chr8	126138120	126138720	KIAA0196	201985_at	0.0004142	-1.74055
chr11	85107510	85108543	SYTL2	225496_s_at	0.0063599	-1.73384
chr5	139922576	139923176	APBB3	204650_s_at	0.0007028	-1.70523
chr6	83903954	83904554	DOPEY1	213271_s_at	0.0002256	-1.70296
chr5	70301841	70302441	NAIP	204860_s_at	4.10E-05	-1.65911
chr1	223707548	223708148	CDC42BPA	203794_at	0.0002575	-1.64574
chr17	526297	526897	VPS53	221707_s_at	3.29E-05	-1.60144
chr2	190272058	190272752	SLC40A1	223044_at	0.0048415	-1.5951
chr1	21778245	21778845	USP48	220079_s_at	0.002179	-1.54907
chr17	77999118	77999718	C17orf62	218130_at	9.53E-05	-1.53372
chr1	3779337	3779937	KIAA0562	204074_s_at	0.0004533	-1.50859
chr19	48066537	48067137	PSG1	210196_s_at	0.0018969	-1.49825
chr5	942869	943661	BRD9	220155_s_at	0.0011228	-1.48517
chr12	77941380	77942008	SYT1	203998_s_at	0.0133901	-1.48316
chr5	82409510	82410110	TMEM167	226276_at	0.000275	-1.47974
chr4	184561664	184562264	WWC2	222738_at	0.0105947	-1.47666
chr8	114458458	114459058	CSMD3	240228_at	0.0313051	-1.46487
chr1	12271490	12272090	VPS13D	212326_at	0.0006916	-1.44059
chr8	101301233	101301833	SPAG1	210117_at	0.0257172	-1.42321
chr2	100180463	100181162	AFF3	1565034_s_at	0.0044166	-1.41065

chr6	52212062	52212662	IL17F	234408_at	0.007835	-1.38599
chr22	17796314	17796914	MRPL40	203152_at	0.0046355	-1.38181
chr18	12248281	12248881	CIDEA	221295_at	0.0919727	-1.36342
chr19	48458773	48459373	PSG9	210126_at	0.00199	-1.35906
chr22	31203885	31204538	FBXO7	1554423_a_at	0.0005049	-1.3577
chr18	18968011	18968626	CABLES1	225532_at	0.0008339	-1.33842
chr1	19199386	19199986	ZUBR1	211950_at	0.0043924	-1.33687
chr19	53601111	53601711	GRIN2D	229883_at	0.0028669	-1.33329
chr19	63741989	63742589	ZBTB45	229862_x_at	0.0048397	-1.32499
chr6	127809823	127810423	KIAA0408,C6orf174	205496_at	0.0544141	-1.31991
chr6	169880192	169880792	WDR27	228326_at	0.005025	-1.31746
chr19	47929001	47929601	PSG3	203399_x_at	0.0094809	-1.26313
chr10	50336330	50336930	ERCC6	230108_at	0.0036094	-1.2504
chr8	125567020	125567755	RNF139	209510_at	0.0002523	-1.24172
chr15	49578664	49579264	DMXL2	215761_at	0.0455168	-1.23463
chr8	16094407	16095208	MSR1	208422_at	0.0012102	-1.22189
chr5	60495366	60495966	DKFZP686E2158	224740_at	0.0082463	-1.2193
chr1	5862601	5863201	NPHP4	216344_at	0.0460262	-1.21314
chr5	140025844	140026444	WDR55	219809_at	0.0040867	-1.20983
chr6	43302047	43302647	C6orf108	39817_s_at	0.000904	-1.20578
chr9	130617833	130618922	ABL1	202123_s_at	0.0004275	-1.20571
chr6	158502846	158503446	SERAC1	1569864_at	0.282363	-1.20076
chr6	22405122	22406209	PRL	205445_at	0.397208	-1.19827
chr6	26216243	26216843	HIST1H1T	207982_at	0.028728	-1.18876
chr4	184980491	184981091	FLJ12716	218179_s_at	0.246195	-1.18398
chr5	180598803	180599403	GNB2L1	200651_at	0.0145909	-1.17475
chr7	142691303	142691903	TAS2R41	1553558_at	0.0242593	-1.17375
chr5	178940092	178940692	RUFY1	218243_at	0.003189	-1.17002
chr11	69776786	69777386	PPFIA1	202065_s_at	0.0104695	-1.16674
chr6	108330795	108331395	SEC63	201914_s_at	2.90E-05	-1.16186
chr6	2062690	2063290	GMDS	214106_s_at	0.0024021	-1.13694
chr17	60439673	60440273	GNA13	224761_at	0.0030706	-1.13585
chrX	11070690	11071290	AMELX	208410_x_at	0.0020067	-1.13011
chr1	211114117	211114717	PTPN14	205503_at	0.220298	-1.09613
chr11	102102890	102103490	MMP8	207329_at	0.232354	-1.08512
chr6	131939004	131939604	ARG1	231662_at	0.260112	-1.0812
chr4	151860179	151861008	MAB21L2	210303_at	0.132716	-1.06967
chr5	90702141	90703264	ARRDC3	224797_at	0.755816	-1.06694
chr8	16903811	16905019	FGF20	220394_at	0.459798	-1.05083
chr2	228069388	228070040	TM4SF20	220639_at	0.722399	-1.03587

chr22	22356474	22357702	Rgr	235816_s_at	0.875429	-1.01845
chr6	292603	293203	DUSP22	218845_at	0.865269	-1.01191
chr6	50039676	50040277	DEFB114	1567878_at	0.95416	1.00205
chr19	48661480	48662180	LYPD3	204952_at	0.782884	1.01381
chr9	111601267	111601868	C9orf84	233504_at	0.641805	1.02224
chrX	154007754	154008589	VBP1	201472_at	0.0040304	1.05942
chr11	13697974	13698574	MLSTD2	224865_at	0.284475	1.07221
chr22	36568875	36570088	EIF3S6IP	217719_at	0.0125926	1.08105
chr6	150161912	150163179	PCMT1	208857_s_at	0.191904	1.08157
chr6	47757102	47757702	GPR111	1553036_at	0.306729	1.0935
chr1	16387118	16387718	FBXO42	221812_at	0.0215619	1.09565
chr19	57750022	57750622	ZNF808	1554476_x_at	0.350974	1.10081
chr6	133179134	133179734	RPS12	213377_x_at	0.0001096	1.10831
chr7	129502030	129502630	CPA2	206212_at	0.173627	1.12546
chr7	17840626	17841226	PRPS1L1	215486_at	0.19292	1.13034
chr18	9547279	9547879	PPP4R1	201594_s_at	0.0054405	1.13212
chrX	37416236	37416836	CYBB	233538_s_at	0.252004	1.14535
chr1	11251189	11251789	FRAP1	202288_at	0.0012096	1.15311
chr10	98731342	98731942	C10orf12	219601_s_at	0.0543077	1.15639
chr5	68700380	68700980	RAD17	210826_x_at	0.0035992	1.16441
chr6	116396496	116397096	FRK	207178_s_at	0.142464	1.18424
chr6	168083287	168083887	MLLT4	238871_at	0.0040206	1.20607
chr19	45211792	45212392	ZNF546	240429_at	0.136951	1.21426
chr11	93104847	93106154	JOSD3	218750_at	0.001805	1.21902
chr17	74336583	74337183	USP36	224978_s_at	8.36E-05	1.22242
chr19	61597342	61597942	ZNF582	241602_at	0.0037252	1.2402
chr8	88954610	88955210	WDR21C	1553199_at	0.011549	1.24029
chr6	74245909	74246509	MTO1	218716_x_at	0.0007592	1.25057
chr6	150115275	150116017	LATS1	1570425_s_at	0.023289	1.28404
chr4	186673248	186673848	LRP2BP	207797_s_at	0.0066698	1.29018
chr8	141685068	141685668	EIF2C2	213310_at	0.0002577	1.29469
chr5	14747466	14748066	FAM105B	229268_at	0.0003866	1.29702
chr1	35689581	35690181	KIAA0319L	222468_at	0.008991	1.30522
chr19	55215617	55216217	VRK3	221998_s_at	0.001667	1.3146
chr2	95209311	95210151	MRPS5	224333_s_at	0.0012771	1.32027
chr3	126494226	126494826	ZNF148	203319_s_at	0.0033445	1.32634
chr5	142586513	142587113	ARHGAP26	205068_s_at	0.0001504	1.35089
chr3	138146622	138147767	NCK1	211063_s_at	0.0007847	1.35682
chr9	130483367	130484738	FUBP3	212824_at	0.01122	1.39981
chr10	35504030	35504630	CREM	207630_s_at	4.71E-05	1.40062
chr18	58392137	58392737	ZCCHC2	222816_s_at	0.0181652	1.40265

chr8	64285559	64286159	YTHDF3	1564053_a_at	0.0053051	1.40865
chr19	49207066	49207666	ZNF230	1557322_at	0.016799	1.42095
chr8	8925157	8925757	THEX1	226416_at	0.0001286	1.45581
chr11	77090343	77090943	RSF1	222541_at	0.0010482	1.46326
chr16	3433179	3433779	FLJ14154	45526_g_at	0.0003014	1.47048
chr14	44650899	44651499	PRPF39	220553_s_at	0.0688403	1.47335
chr13	43867766	43868366	C13orf21	228044_at	0.0020176	1.47978
chr3	184164333	184164933	DCUN1D1	218583_s_at	0.0001041	1.48515
chr18	20259771	20260780	IMPACT	222698_s_at	0.0024569	1.48842
chr11	107549198	107549798	NPAT	209798_at	0.0016663	1.50727
chr14	89825640	89826240	C14orf102	227575_s_at	0.0007178	1.5088
chr6	64452729	64453329	PHF3	217952_x_at	6.75E-05	1.52272
chr9	92163435	92164035	NOL8	218244_at	0.0003533	1.54786
chr19	57139288	57139888	ZNF613	219851_at	0.0231674	1.57354
chr15	39775763	39776363	MGA	212945_s_at	0.0002494	1.58766
chr6	43433400	43434000	ZNF318	203521_s_at	0.0175365	1.59677
chr6	97451415	97452015	C6orf66	219006_at	0.0002196	1.59908
chr5	6660367	6660967	NSUN2	223076_s_at	0.000159	1.60008
chrX	128772356	128772956	UTP14A	221514_at	0.0009089	1.63022
chr19	63749815	63750675	TRIM28	200990_at	4.85E-05	1.64802
chr6	121654938	121655538	C6orf170	232038_at	0.0322307	1.66563
chr2	71556507	71557107	ZNF638	211257_x_at	0.000347	1.67477
chr19	63332112	63332712	ZNF329	219765_at	0.001193	1.70271
chr6	4717705	4718305	CDYL	203099_s_at	8.40E-06	1.7031
chr5	150059302	150059902	RBM22	222527_s_at	0.0002369	1.72343
chr1	231729705	231730305	ARID4B	221230_s_at	0.0002048	1.72707
chr11	68077731	68078331	SAPS3	222467_s_at	3.79E-05	1.75264
chr7	102383863	102384463	NAPE-PLD	226041_at	0.0009867	1.75624
chr18	12978760	12979360	CEP192	218827_s_at	0.0005368	1.77455
chr10	120808702	120809332	EIF3S10	200597_at	7.04E-05	1.78736
chr7	115932572	115933899	MET	211599_x_at	0.0001047	1.80436
chr20	21262078	21262678	XRN2	233878_s_at	2.43E-06	1.83293
chr6	46765983	46766583	TDRD6	240137_at	0.0009271	1.83732
chr3	72972889	72973489	SHQ1	219083_at	3.90E-05	1.86269
chr22	23277680	23278280	SNRPD3	202567_at	7.66E-05	1.86345
chr2	127762560	127763160	ERCC3	202176_at	0.0001798	1.86796
chr2	207464027	207464627	FASTKD2	216996_s_at	6.07E-05	1.89461
chr4	84748133	84748733	CCDC98	226521_s_at	7.46E-05	1.90452
chr5	43330614	43331214	HMGCS1	205822_s_at	0.0003179	1.911
chr5	139888044	139888644	ANKHD1,MASK-BP3	208773_s_at	2.56E-05	1.9263

chr5	176570577	176571177	NSD1	219084_at	0.0002243	1.92929
chr18	54680297	54681208	ZNF532	225021_at	0.0001319	1.93269
chr6	84961367	84961967	KIAA1009	206006_s_at	0.0004486	1.93446
chr12	110602128	110602728	ACAD10	238655_at	0.0007095	1.96716
chr6	161623555	161624246	AGPAT4	228667_at	0.0005569	1.99293
chr20	13703803	13704403	ESF1	218859_s_at	0.0022079	1.99538
chr19	1308879	1309479	MUM1	223347_at	7.84E-05	2.00325
chr22	45604423	45605023	TBC1D22A	227044_at	0.0009597	2.05222
chr19	57188162	57188762	ZNF615	241827_at	0.0005957	2.08284
chr4	154535493	154536268	TRIM2	214248_s_at	0.0002036	2.1049
chr8	81573810	81575165	ZBTB10	233899_x_at	0.0018363	2.14749
chr16	56032240	56032878	CIAPIN1	208424_s_at	7.21E-06	2.16628
chr11	92916453	92917053	C11orf75	219806_s_at	1.39E-05	2.16698
chr1	10298061	10298661	KIF1B	209234_at	8.30E-05	2.17549
chr1	23385402	23386002	HNRPR	208766_s_at	3.63E-06	2.18055
chr6	79732453	79733053	PHIP	212542_s_at	2.39E-05	2.19809
chr11	108098626	108099226	DDX10	204977_at	4.52E-06	2.20203
chr19	45232891	45233491	ZNF780B	215570_s_at	0.0114908	2.20707
chr14	77244998	77245598	C14orf156	221434_s_at	0.0001302	2.22431
chr4	144795596	144796212	SMARCA5	213251_at	3.03E-05	2.31593
chr16	32594819	32595419	TP53TG3	220167_s_at	0.0003327	2.31612
chr6	71291711	71292311	KIAA1411	223497_at	0.0042843	2.37689
chr5	40868494	40869094	RPL37	224766_at	0.0008741	2.42583
chr2	172116697	172117297	METTL8	220007_at	0.0004455	2.43511
chr16	66906099	66907485	PRMT7	219408_at	0.0006949	2.45846
chr11	85665123	85665723	EED	209572_s_at	1.40E-05	2.50244
chr11	20073303	20073903	NAV2	218330_s_at	0.0007017	2.52454
chr4	10122799	10123895	KIAA1729	226909_at	0.0001107	2.59892
chr19	57484629	57485344	ZNF766	227284_at	5.10E-05	2.60629
chr5	145842611	145843374	TCERG1	202396_at	4.63E-05	2.69185
chr19	57211366	57211966	ZNF614	227045_at	0.0001083	2.69649
chr4	57211803	57212512	ARL9	229062_at	0.0007089	2.72526
chr3	114700417	114701017	CCDC52	234995_at	1.23E-06	2.73527
chr1	199629233	199629881	KLHL12	225068_at	1.91E-05	2.7708
chr7	93810841	93811441	CASD1	219342_at	8.83E-05	2.77516
chr4	22122922	22123522	GPR125	210473_s_at	4.13E-05	2.82958
chr19	63138452	63139054	ZNF418	243439_at	4.63E-06	2.85726
chr1	91116053	91117479	ZNF644	1553725_s_at	0.000394	2.86385
chr5	81318647	81319247	ATG10	1570523_s_at	4.95E-05	2.95055
chr5	118718995	118719715	TNFAIP8	210260_s_at	9.36E-07	3.1267
chr16	3433391	3434037	ZNF597	230542_at	0.0003399	3.1918

chr2	152124781	152125381	RIF1	236620_at	4.41E-05	3.25235
chr6	15361432	15362032	JARID2	203298_s_at	1.15E-05	3.34959
chr16	69126209	69126809	SF3B3	200687_s_at	1.44E-05	3.43828
chr8	124181874	124182474	WDR67	214061_at	9.14E-06	3.57095
chr19	55239634	55240234	ZNF473	213124_at	0.0004979	3.61807
chr19	57517711	57518311	ZNF480	222283_at	0.0007243	3.70783
chr4	426991	427591	ABCA11	220159_at	7.01E-05	4.38114
chr2	64591510	64592110	HSPC159	226188_at	5.32E-07	5.55806
chr3	161611749	161612515	SMC4	201663_s_at	6.95E-07	6.12805
chr2	203294496	203295096	ALS2CR13	1553220_at	5.20E-05	7.35694
chr5	128822994	128824103	ADAMTS19	1553180_at	1.87E-05	7.37068
chr11	28072871	28073471	KIF18A	221258_s_at	7.41E-07	8.84765
chr7	93930731	93931331	PEG10	212092_at	2.90E-06	9.36424
chr4	129164803	129165403	PLK4	204887_s_at	5.31E-08	9.54
chr19	57610926	57611526	ZNF528	232315_at	2.62E-06	9.55066
chr1	36004002	36004602	EIF2C1	228120_at	2.14E-07	11.3943
chr4	121340687	121341287	MAD2L1	1554768_a_at	8.18E-08	12.9464
chr5	137547422	137548022	KIF20A	218755_at	8.03E-07	20.6695
chr1	143509861	143510461	NBPF11,NBPF15,NBPF10	n.a		
chr10	94082751	94083351	39877	n.a		
chr19	53000328	53000928	TPRX1	n.a		
chr19	58650282	58650882	ZNF761	n.a		
chr5	140844425	140845025	PCDHGC3,PCDHGB4,PCDHGA8,PCDHGA12,PCDHGC5,PCDHGC4,PCDHGB7,PCDHGB6,PCDHGB5,PCDHGB3,PCDHGB2,PCDHGB1,PCDHGA11,PCDHGA10,PCDHGA9,PCDHGA7,PCDHGA6,PCDHGA5,PCDHGA4,PCDHGA3,PCDHGA2,PCDHGA1	n.a		
chr8	82536212	82536835	PERF15	n.a		

Supplementary Table 2.2 Differentially methylated CpG islands associated with genes and expression data of the corresponding genes.

Hypermethylation

Gene Symbol	Affy Probeset ID	P-value (Annova)	Fold Change
EID1	211698_at	0.000000676	-244.542
CXCL5	214974_x_at	0.0000105	-88.6218
PCDH10	228635_at	0.000000825	-53.0055
OSMR	205729_at	0.0000238	-46.8265
PTGER4	204897_at	0.000166471	-14.8193
TRHDE	219937_at	0.0000178	-12.6039
HTRA1	201185_at	0.000000737	-9.03052
TPM1	210987_x_at	0.000000505	-7.72639
JUN	201466_s_at	0.00000275	-6.48124
GRIK2	213845_at	0.000403575	-5.10917
NPAL3	225876_at	0.0000177	-4.09295
OSR1	228399_at	0.0010305	-3.68572
H2AFJ	224301_x_at	0.0000226	-2.99456
BOLA1	219345_at	0.0000334	-2.74018
SCRN1	201462_at	0.0000436	-2.23554
SPAG6	228207_at	0.00301116	-1.96703
C20ORF23	219570_at	0.000541584	-1.87178
DIRAS3	215506_s_at	0.00131237	-1.73675
DNALI1	205186_at	0.00717893	-1.63706
KTI12	225642_at	0.000240378	-1.62077
SLC6A5	210810_s_at	0.00145759	-1.48957
BIRC3	210538_s_at	0.0168651	-1.38544
EID3	231292_at	0.139438	-1.37579
HOXA6	208557_at	0.00556848	-1.30142
FOXD3	241609_at	0.0356657	-1.26392
SLC24A6	218749_s_at	0.0307705	-1.26077
CDX4	221340_at	0.0242089	-1.22667
FOXA2	210103_s_at	0.0910759	-1.21764
IL1RAPL2	221112_at	0.00128094	-1.21264
KCND3	211827_s_at	0.0208893	-1.19117
KLHL28	220374_at	0.0022046	-1.18506
LOC387856	229626_at	0.120937	-1.16633
DMRTB1	225495_x_at	0.0322984	-1.16118
CSTF2T	212905_at	0.0783426	-1.1354
FGF6	208417_at	0.1652	-1.10634

WNT2	205648_at	0.26502	-1.0955
SLC2A2	206535_at	0.734153	-1.03382
C20ORF85	229542_at	0.903475	-1.01025
C14ORF39	1561985_at	0.2643	1.05499
COL14A1	228750_at	0.0524737	1.13256
NPY	206001_at	0.28104	1.1368
CYP24A1	206504_at	0.0878528	1.15346
ONECUT1	1560952_at	0.117835	1.16619
HIST2H4B	207046_at	0.0100029	1.20035
OXCT2	235275_at	0.0152751	1.22975
POLS	202466_at	0.00059247	1.39842
TBX15	230438_at	0.0163359	1.47072
KIAA1024	215081_at	0.00534215	1.47076
SYT6	240267_at	0.0000134	1.61037
PENK	213791_at	0.0548262	1.67257
AASS	214829_at	0.00299767	1.74312
WT1	206067_s_at	0.0158927	1.85737
LANCL2	218219_s_at	0.00101825	1.87004
POLR3C	210573_s_at	0.0000635	1.90153
KATNAL2	233644_at	0.0147301	1.91923
NKX2-5	206578_at	0.000227729	2.1657
KCNA6	1553347_s_at	0.00430296	2.30744
IRX1	230472_at	0.00371003	3.13884
FGF12	207501_s_at	0.000173181	4.01025
IRX3	229638_at	0.0000101	4.14638
NTF3	206706_at	0.000849635	4.66223
FAM19A4	242348_at	0.0000486	7.78963
ADAMTS9	1562275_at	0.0000319	8.16595
SOX7	228698_at	0.00000387	8.5605
PKIB	231120_x_at	0.0000173	9.5959
PCDHAC1	223435_s_at	0.00000676	17.3996
SLC10A4	239913_at	0.00000102	22.1169
TFAP2C	205286_at	0.00000121	47.4313
HIST2H2BF	n.a		
LBXCOR1	n.a		

Remark: 79 DMRs are mapped to 70 CGIs that are gene-associated because some CGIs are very long and overlap with several DMRs

Hypermethylation

Gene Symbol	Affy Probeset ID	P-value (Annova)	Fold Change
MSX1	205932_s_at	8.70E-07	10.5518
SYDE2	242245_at	0.000434746	2.36734
TP53TG3	220167_s_at	0.000332719	2.31612
FOXC1	1553613_s_at	0.000238135	2.19014
ZNF793	244180_at	0.0358029	1.70432
WDR21C	1553199_at	0.011549	1.24029
NKX2-2	206915_at	0.315184	1.07122
REXO1L1	1553009_s_at	0.775598	1.01994
SGCE	204688_at	0.643748	-1.02459
CABLES1	225532_at	0.000833948	-1.33842
PCDHGC4	211066_x_at	1.19E-05	-2.50772

Remark: 13 DMRs are mapped to 11 CGIs that are gene-associated because some CGIs are very long and overlap with several DMRs

Supplementary Table 2.3 Primer sequences used in this study

Methylation control genes for MeDIP

RASSF1A-F	CAGGGGCTACACTCTCCCAGT
RASSF1A-R	CAGGACTGAGCCCAGACTTCA
NPY-F	CTCTCACCCCTCGGAGACG
NPY-R	CCCCTAGACAGACGGGTCGTA
ACTB-F	AACGCCCCAGGCCTTGTCTT
ACTB-R	CCCGTGATGAAGGCTACAAACCT

qPCR primers for Hyper-genes

APOLD1-F	TCAGACATACTGCCCCATCA
APOLD1-R	CATACATGCAAAAACGGTGC
ALG10-F	TCTCTTCAGTGTTGGCAACTTC
ALG10-R	AGACTCTCTGGATACTTGAGGCA
C20orf85-F	CTGGGTGGTCTGTGGGACT
C20orf85-R	CAAAATCGAGCTTCCTGAGC
CDX4-F	GGAATTCCTTTTCCAGCTCC
CDX4-R	CTATGCATGGATGCGCAAG
CXCL5-F	TTGTCTTGATCCAGAAGCCC
CXCL5-R	AAACTTTTCCATGCGTGCTC
DDR2-F	AGGATCCTGCTCCACAGAGA
DDR2-R	AGGAACAGCACCAAGAGCAT
EID1-F	GCCGGCTACAGAGTATCAGC
EID1-R	GATCAAACGGGGTCTTCTCA
EOMES-F	CACATTGTAGTGGGCAGTGG
EOMES-R	CGCCACCAAACTGAGATGAT
FAM60A-F	AGGGCTGGACCCAGTCTAAA
FAM60A-R	GAGGCACTTGAGGTGGTACTG
FGF12-F	GCCATGAATGGTGAAGGCTA
FGF12-R	ATTCTTGCTGGCGGTACAGT
GRHL3-F	TAGAGACAAGCGGTCAGCAG
GRHL3-R	CTGGGTCGTTCTTTAGCAGC
GRIK2-F	GCACCGTTAAACTCCTGCTC
GRIK2-R	ATTGGGCCAGATTCCACATA
H2AFJ-F	GACATGATCACCTCTCGCCT
H2AFJ-R	GCGGCCGTAAAGAGTTTGTA
MAN2B2-F	GTCAACGTGCCAGGAGAACT

MAN2B2-R	GCACCTTGATGACCAGATCC
MNS1-F	AGCAATCTGAGCTGCCCTT
MNS1-R	AGGACGAAAAGATGAGGCAA
NLRP3-F	TGCTGAGTACCGAGGACAAA
NLRP3-R	TCTGTGTGTGGGACTGAAGC
NPY-F	TCCAGCCCAGAGACACTGATT
NPY-R	AGGGTCTTCAAGCCGAGTTCT
NTF3-F	GAAACGCGATGTAAGGAAGC
NTF3-R	GGTTTGGGATGTTTTGCACT
OSR1-F	CCTTCAGCTAAAGCCCCAG
OSR1-R	CCCATTTCGGTAGTTGCAGT
PCDH10-F	GGAGTACACGACCTCACCGT
PCDH10-R	CCCGTCTACACTGTGTCCCT
PKIB-F	CCAGGGACAGGAAAGATAGGA
PKIB-R	CCACGTCAGTCATTTTTGATG
RBMS3-F	GGGAGCTGGTGCATAGGAC
RBMS3-R	AGATTCCAGCTACATGGGCA
RGAG1-F	TCTGACTCAGCCAGTGCTCTT
RGAG1-R	CAACATATGGAGCGCCTACC
RPS4Y1-F	TCCTGTCATCAAGGTGAACG
RPS4Y1-R	GGCTCCACCAATCACCATAC
TFAP2C-F	AAAGCCGCTCATGTGACTCT
TFAP2C-R	GGAAATTCGGCTTCACAGAC
TIAM1-F	AAAGGCTGTGCATTCAATCCTG
TIAM1-R	TCAGTGCACACAATCTTTTGCC
TMEM29-F	GAATCCAGCATCGCCTTCCAA
TMEM29-R	CCCATCATCGGCTGTTCTGAG
XAGE1-F	AGCTTGCGTTGTTTCAGCTT
XAGE1-R	AAATCTGGATTTGGGTTCCG
ZNF643-F	CCTGTGGGAGGATGTGACTAA
ZNF643-R	TCACCTCCCGGTACAGATTC
ZSWIM2-F	TTGGGGAGTTTGAACTCGAT
ZSWIM2-R	AAGCTTCCAAGGAACCATGA

qPCR primers for Hypo-genes

CCDC82-F	AGCGTTGATCAGAAGAGCCT
CCDC82-R	TGAGGAGGGTGATGAAGAGAA
EIF2C1-F	CACACACTGCGTAGCCATTC
EIF2C1-R	CAGGGCTGCAGCTCATTATT

MAB21L2-F	CTCGCAATACGTTGAGGGAT
MAB21L2-R	GAAAGTAGGGCTTAACGGGG
SLC40A1-F	ATGGGGGCTAAGATGTTGGT
SLC40A1-R	CCAAAGGGATTGGATTGTTG
VBP1-F	TCTTTTCCAACAATGCCTGA
VBP1-R	TGCAAAGCTTCAGTTCCTCC
ZFHX4-F	TAGCAGGATTCGGGATTCAC
ZFHX4-R	TGCATACCACCAATCACAGG
ZNF480-F	CCACTCCGCCTGAGAGAAT
ZNF480-R	ATGCTGTGATGAAAAAGCCC
ZNF615-F	TGCTGCAGAGGATCATCAAT
ZNF615-R	CCAAATTGGAACGAGGAGAA
ZNF780B-F	TCAGGTCCATATTTTGACTCCA
ZNF780B-R	TCCATTTCTAAGCCAGATGTGA

Bisulfite sequencing primers

BSS-PCDH10-B-F	ATTTGTTGATGTAAATAGGGGAA
BSS-PCDH10-B-R	ATCTTTCAAAATCTTAATTCCCTC
BSS-APOLD1-F	GGATTTAATTTTTATATGATGAAAGGG
BSS-APOLD1-R	TCCAACAACAATCCCTAAAAAC
BSS-RGAG1-F	ATTAAGGGTTTTGGTTAGGTTT
BSS-RGAG1-R	TACCCACAATACCTTACACAAA
BSS-HOXA7-F	TTTTTTTAAGTTGGGGGAAA
BSS-HOXA7-R	AAACTTAAAATCCTCCCCATC
BSS-HOXC10-2-F	TTTGTTTAAGGAAAAATGTGGG
BSS-HOXC10-2-R	CATCTCAAAAACCCACAAAACT
BSS-GAD1-F	GAATGATTTAGTATTGGTATTTGG
BSS-GAD1-R	TAAAAACACAAAAAACATCCTAA
BSS-ZSWIM2-F	GTTTTGGTGTTAGTTGAGTTTTT
BSS-ZSWIM2-R	ATCACAAAATAATTTCAACATCC
BSS-OSR1-F	TTTAGTTAGAAAGGATGAGTGAGTT
BSS-OSR1-R	CTACACACTTCATTAATACCCAAC
BSS-Chr1-Hyper-F	TAATAGTTTTTTGGTTTTTGTTT
BSS-Chr1-Hyper-R	ATAAATCCCTTATCACAAACA
BSS-Chr1-Hypo-F	GGGGTTGTAGAGTTAGTGAATA
BSS-Chr1-Hypo-R	ATACTTCCCAATCCTATTTCTT
BSS-Chr1-Iso-F	TGGGGATAGGTTTAGTTTTAT
BSS-Chr1-Iso-R	TAAAATTTACCACCTACCCC
BSS-mir-199a-2-F	GGGTTTTTTGGTTTTTAGTGTG

BSS-mir-199a-2-R	CTCACTTTCAATTAACCACAACC
BSS-mir-124a-2-F	GAGTAGAGATAGGAGTTGGGTTTATG
BSS-mir-124a-2-R	TATTTTCCCAACTTAACCCAAA
BSS-mir-184-F	AGAGTTGTATGTTTGAATTTTTGTGTG
BSS-mir-184-R	TCAAAAAAAAACCCTAAACCCA

MSP primers

MSP-APOLD1-U-F	GTAGTGGTTGTTTTGTTGAATTT
MSP-APOLD1-U-R	AAACATAAACACACAATCAACCT
MSP-APOLD1-M-F	GTGGTTGTTTCGTTGAATTC
MSP-APOLD1-M-R	AAACATAAACACGCGATCAA

snoRNA qPCR primers

ACA8-F	GCATGGTATCTGCACTCAGC
ACA8-R	AGCACAAAGCCAAGAAAACC
ACA33-F	GCCAATGAATCTGCTTACCTG
ACA33-R	GCCATTCTCAGGGACCTTAAC
HBII-240-F	AAAATAAATGTCTGAACATATGAATGC
HBII-240-R	CGCTTATCTCAGTTAAATGCTGAA

References

Ahuja N, Mohan AL, Li Q, Stolker JM, Herman JG, Hamilton SR, Baylin SB, Issa JP (1997) Association between CpG island methylation and microsatellite instability in colorectal cancer. *Cancer Res* **57:** 3370-4

Akama TO, Okazaki Y, Ito M, Okuizumi H, Konno H, Muramatsu M, Plass C, Held WA, Hayashizaki Y (1997) Restriction landmark genomic scanning (RLGS-M)-based genome-wide scanning of mouse liver tumors for alterations in DNA methylation status. *Cancer Res* **57:** 3294-9

Albers P, Albrecht W, Algaba F, Bokemeyer C, Cohn-Cedermark G, Horwich A, Klepp O, Laguna MP, Pizzocaro G (2005) Guidelines on testicular cancer. *Eur Urol* **48:** 885-94

Aleman A, Cebrian V, Alvarez M, Lopez V, Orenes E, Lopez-Serra L, Algaba F, Bellmunt J, Lopez-Beltran A, Gonzalez-Peramato P, Cordon-Cardo C, Garcia J, del Muro JG, Esteller M, Sanchez-Carbayo M (2008) Identification of PMF1 methylation in association with bladder cancer progression. *Clin Cancer Res* **14:** 8236-43

Alves G, Tatro A, Fanning T (1996) Differential methylation of human LINE-1 retrotransposons in malignant cells. *Gene* **176:** 39-44

Andrews PW (1998) Teratocarcinomas and human embryology: pluripotent human EC cell lines. Review article. *APMIS* **106:** 158-67; discussion 167-8

Arnold CN, Goel A, Niedzwiecki D, Dowell JM, Wasserman L, Compton C, Mayer RJ, Bertagnolli MM, Boland CR (2004) APC promoter hypermethylation contributes to the loss of APC expression in colorectal cancers with allelic loss on 5q. *Cancer Biol Ther* **3:** 960-4

Bachellerie JP, Cavaille J, Huttenhofer A (2002) The expanding snoRNA world. *Biochimie* **84:** 775-90

Bachman KE, Herman JG, Corn PG, Merlo A, Costello JF, Cavenee WK, Baylin SB, Graff JR (1999) Methylation-associated silencing of the tissue inhibitor of metalloproteinase-3 gene suggest a suppressor role in kidney, brain, and other human cancers. *Cancer Res* **59:** 798-802

Balch C, Yan P, Craft T, Young S, Skalnik DG, Huang TH, Nephew KP (2005) Antimitogenic and chemosensitizing effects of the methylation inhibitor zebularine in ovarian cancer. *Mol Cancer Ther* **4:** 1505-14

Ballestar E, Esteller M (2002) The impact of chromatin in human cancer: linking DNA methylation to gene silencing. *Carcinogenesis* **23:** 1103-9

Barski A, Zhao K (2009) Genomic location analysis by ChIP-Seq. *J Cell Biochem* **107:** 11-8

Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116:** 281-97

Bell AC, Felsenfeld G (2000) Methylation of a CTCF-dependent boundary controls imprinted expression of the Igf2 gene. *Nature* **405:** 482-5

Bibikova M, Lin Z, Zhou L, Chudin E, Garcia EW, Wu B, Doucet D, Thomas NJ, Wang Y, Vollmer E, Goldmann T, Seifart C, Jiang W, Barker DL, Chee MS, Floros J, Fan JB (2006) High-throughput DNA methylation profiling using universal bead arrays. *Genome Res* **16:** 383-93

Bird AP (1986) CpG-rich islands and the function of DNA methylation. *Nature* **321:** 209-13

Blanchard F, Tracy E, Smith J, Chattopadhyay S, Wang Y, Held WA, Baumann H (2003) DNA methylation controls the responsiveness of hepatoma cells to leukemia inhibitory factor. *Hepatology* **38:** 1516-28

Bosl GJ, Motzer RJ (1997) Testicular germ-cell cancer. *N Engl J Med* **337:** 242-53

Bourc'his D, Xu GL, Lin CS, Bollman B, Bestor TH (2001) Dnmt3L and the establishment of maternal genomic imprints. *Science* **294:** 2536-9

Boyes J, Bird A (1991) DNA methylation inhibits transcription indirectly via a methyl-CpG binding protein. *Cell* **64:** 1123-34

Brandt J, Veith AM, Volff JN (2005) A family of neofunctionalized Ty3/gypsy retrotransposon genes in mammalian genomes. *Cytogenet Genome Res* **110:** 307-17

Bruniquel D, Schwartz RH (2003) Selective, stable demethylation of the interleukin-2 gene enhances transcription by an active process. *Nat Immunol* **4:** 235-40

Burger H, Nooter K, Boersma AW, Kortland CJ, Stoter G (1998) Expression of p53, Bcl-2 and Bax in cisplatin-induced apoptosis in testicular germ cell tumour cell lines. *Br J Cancer* **77:** 1562-7

Cairns P (2007) Gene methylation and early detection of genitourinary cancer: the road ahead. *Nat Rev Cancer* **7:** 531-43

Carr IM, Valleley EM, Cordery SF, Markham AF, Bonthron DT (2007) Sequence analysis and editing for bisulphite genomic sequencing projects. *Nucleic Acids Res* **35:** e79

Casey G, Neville PJ, Liu X, Plummer SJ, Cicek MS, Krumroy LM, Curran AP, McGreevy MR, Catalona WJ, Klein EA, Witte JS (2006) Podocalyxin variants and risk of prostate cancer and tumor aggressiveness. *Hum Mol Genet* **15:** 735-41

Chakrabarty A, Tranguch S, Daikoku T, Jensen K, Furneaux H, Dey SK (2007) MicroRNA regulation of cyclooxygenase-2 during embryo implantation. *Proc Natl Acad Sci U S A* **104:** 15144-9

Chan AW, Chan MW, Lee TL, Ng EK, Leung WK, Lau JY, Tong JH, Chan FK, To KF (2005) Promoter hypermethylation of Death-associated protein-kinase gene associated with advance stage gastric cancer. *Oncol Rep* **13:** 937-41

Chan MW, Chan LW, Tang NL, Tong JH, Lo KW, Lee TL, Cheung HY, Wong WS, Chan PS, Lai FM, To KF (2002) Hypermethylation of multiple genes in tumor tissues and voided urine in urinary bladder cancer patients. *Clin Cancer Res* **8:** 464-70

Cheah MS, Wallace CD, Hoffman RM (1984) Hypomethylation of DNA in human cancer cells: a site-specific change in the c-myc oncogene. *J Natl Cancer Inst* **73:** 1057-65

Chedin F, Lieber MR, Hsieh CL (2002) The DNA methyltransferase-like protein DNMT3L stimulates de novo methylation by Dnmt3a. *Proc Natl Acad Sci U S A* **99:** 16916-21

Chen R, Alvero AB, Silasi DA, Kelly MG, Fest S, Visintin I, Leiser A, Schwartz PE, Rutherford T, Mor G (2008) Regulation of IKKbeta by miR-199a affects NF-kappaB activity in ovarian cancer cells. *Oncogene* **27:** 4712-23

Cheung HH, Lee TL, Davis AJ, Taft DH, Rennert OM, Chan WY Genome-wide DNA methylation profiling reveals novel epigenetically regulated genes and non-coding RNAs in human testicular cancer. *Br J Cancer*

Cheung HH, Lee TL, Rennert OM, Chan WY (2009) DNA methylation of cancer genome. *Birth Defects Res C Embryo Today* **87:** 335-50

Chim CS, Wong AS, Kwong YL (2003) Epigenetic inactivation of INK4/CDK/RB cell cycle pathway in acute leukemias. *Ann Hematol* **82:** 738-42

Cho NY, Kim BH, Choi M, Yoo EJ, Moon KC, Cho YM, Kim D, Kang GH (2007) Hypermethylation of CpG island loci and hypomethylation of LINE-1 and Alu repeats in prostate adenocarcinoma and their relationship to clinicopathological features. *J Pathol* **211:** 269-77

Choi IS, Estecio MR, Nagano Y, Kim do H, White JA, Yao JC, Issa JP, Rashid A (2007) Hypomethylation of LINE-1 and Alu in well-differentiated neuroendocrine tumors (pancreatic endocrine tumors and carcinoid tumors). *Mod Pathol* **20:** 802-10

Cokus SJ, Feng S, Zhang X, Chen Z, Merriman B, Haudenschild CD, Pradhan S, Nelson SF, Pellegrini M, Jacobsen SE (2008) Shotgun bisulphite sequencing of the Arabidopsis genome reveals DNA methylation patterning. *Nature* **452:** 215-9

Costello JF, Fruhwald MC, Smiraglia DJ, Rush LJ, Robertson GP, Gao X, Wright FA, Feramisco JD, Peltomaki P, Lang JC, Schuller DE, Yu L, Bloomfield CD, Caligiuri MA, Yates A, Nishikawa R, Su Huang H, Petrelli NJ, Zhang X, O'Dorisio MS, Held WA, Cavenee WK, Plass C (2000) Aberrant CpG-island methylation has non-random and tumour-type-specific patterns. *Nat Genet* **24:** 132-8

Crockford GP, Linger R, Hockley S, Dudakia D, Johnson L, Huddart R, Tucker K, Friedlander M, Phillips KA, Hogg D, Jewett MA, Lohynska R, Daugaard G, Richard S, Chompret A, Bonaiti-Pellie C, Heidenreich A, Albers P, Olah E, Geczi L, Bodrogi I, Ormiston WJ, Daly PA, Guilford P, Fossa SD, Heimdal K, Tjulandin SA, Liubchenko L, Stoll H, Weber W, Forman D, Oliver T, Einhorn L, McMaster M, Kramer J, Greene MH, Weber BL, Nathanson KL, Cortessis V, Easton DF, Bishop DT, Stratton MR, Rapley EA (2006) Genome-wide linkage screen for testicular germ cell tumour susceptibility loci. *Hum Mol Genet* **15:** 443-51

Dai Z, Zhu WG, Morrison CD, Brena RM, Smiraglia DJ, Raval A, Wu YZ, Rush LJ, Ross P, Molina JR, Otterson GA, Plass C (2003) A comprehensive search for DNA amplification in lung cancer identifies inhibitors of apoptosis cIAP1 and cIAP2 as candidate oncogenes. *Hum Mol Genet* **12:** 791-801

Daskalos A, Nikolaidis G, Xinarianos G, Savvari P, Cassidy A, Zakopoulou R, Kotsinas A, Gorgoulis V, Field JK, Liloglou T (2009) Hypomethylation of retrotransposable elements correlates with genomic instability in non-small cell lung cancer. *Int J Cancer* **124:** 81-7

Datta J, Kutay H, Nasser MW, Nuovo GJ, Wang B, Majumder S, Liu CG, Volinia S, Croce CM, Schmittgen TD, Ghoshal K, Jacob ST (2008) Methylation mediated silencing of MicroRNA-1 gene and its role in hepatocellular carcinogenesis. *Cancer Res* **68:** 5049-58

del Senno L, Barbieri R, Amelotti F, Bernardi F, Buzzoni D, Marchetti G, Patracchini P, Piva R, Rossi M, Conconi F, et al. (1986) Methylation and expression of c-myc and c-abl oncogenes in human leukemic K562 cells before and after treatment with 5-azacytidine. *Cancer Detect Prev* **9:** 9-15

Del Senno L, Maestri I, Piva R, Hanau S, Reggiani A, Romano A, Russo G (1989) Differential hypomethylation of the c-myc protooncogene in bladder cancers at different stages and grades. *J Urol* **142:** 146-9

Detich N, Theberge J, Szyf M (2002) Promoter-specific activation and demethylation by MBD2/demethylase. *J Biol Chem* **277:** 35791-4

Dulaimi E, Ibanez de Caceres I, Uzzo RG, Al-Saleem T, Greenberg RE, Polascik TJ, Babb JS, Grizzle WE, Cairns P (2004) Promoter hypermethylation profile of kidney cancer. *Clin Cancer Res* **10:** 3972-9

Eads CA, Danenberg KD, Kawakami K, Saltz LB, Blake C, Shibata D, Danenberg PV, Laird PW (2000) MethyLight: a high-throughput assay to measure DNA methylation. *Nucleic Acids Res* **28:** E32

Esquela-Kerscher A, Slack FJ (2006) Oncomirs - microRNAs with a role in cancer. *Nat Rev Cancer* **6:** 259-69

Estecio MR, Gharibyan V, Shen L, Ibrahim AE, Doshi K, He R, Jelinek J, Yang AS, Yan PS, Huang TH, Tajara EH, Issa JP (2007) LINE-1 hypomethylation in cancer is highly variable and inversely correlated with microsatellite instability. *PLoS One* **2:** e399

Esteller M (2007) Epigenetic gene silencing in cancer: the DNA hypermethylome. *Hum Mol Genet* **16 Spec No 1:** R50-9

Esteve PO, Chin HG, Pradhan S (2005) Human maintenance DNA (cytosine-5)-methyltransferase and p53 modulate expression of p53-repressed promoters. *Proc Natl Acad Sci U S A* **102:** 1000-5

Fabre C, Grosjean J, Tailler M, Boehrer S, Ades L, Perfettini JL, de Botton S, Fenaux P, Kroemer G (2008) A novel effect of DNA methyltransferase and histone deacetylase inhibitors: NFkappaB inhibition in malignant myeloblasts. *Cell Cycle* **7:** 2139-45

Fan M, Yan PS, Hartman-Frey C, Chen L, Paik H, Oyer SL, Salisbury JD, Cheng AS, Li L, Abbosh PH, Huang TH, Nephew KP (2006) Diverse gene expression and DNA methylation profiles correlate with differential adaptation of breast cancer cells to the antiestrogens tamoxifen and fulvestrant. *Cancer Res* **66:** 11954-66

Fandy TE (2009) Development of DNA methyltransferase inhibitors for the treatment of neoplastic diseases. *Curr Med Chem* **16:** 2075-85

Fang JY, Zhu SS, Xiao SD, Jiang SJ, Shi Y, Chen XY, Zhou XM, Qian LF (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

Feinberg AP, Ohlsson R, Henikoff S (2006) The epigenetic progenitor origin of human cancer. *Nat Rev Genet* **7:** 21-33

Feinberg AP, Vogelstein B (1983) Hypomethylation distinguishes genes of some human cancers from their normal counterparts. *Nature* **301:** 89-92

Felsberg J, Yan PS, Huang TH, Milde U, Schramm J, Wiestler OD, Reifenberger G, Pietsch T, Waha A (2006) DNA methylation and allelic losses on chromosome arm 14q in oligodendroglial tumours. *Neuropathol Appl Neurobiol* **32:** 517-24

Finocchiaro G, Mancuso FM, Cittaro D, Muller H (2007) Graph-based identification of cancer signaling pathways from published gene expression signatures using PubLiME. *Nucleic Acids Res* **35:** 2343-55

Florl AR, Steinhoff C, Muller M, Seifert HH, Hader C, Engers R, Ackermann R, Schulz WA (2004) Coordinate hypermethylation at specific genes in prostate carcinoma precedes LINE-1 hypomethylation. *Br J Cancer* **91:** 985-94

Foltz G, Ryu GY, Yoon JG, Nelson T, Fahey J, Frakes A, Lee H, Field L, Zander K, Sibenaller Z, Ryken TC, Vibhakar R, Hood L, Madan A (2006) Genome-wide analysis of epigenetic silencing identifies BEX1 and BEX2 as candidate tumor suppressor genes in malignant glioma. *Cancer Res* **66:** 6665-74

Friedman LM, Dror AA, Mor E, Tenne T, Toren G, Satoh T, Biesemeier DJ, Shomron N, Fekete DM, Hornstein E, Avraham KB (2009) MicroRNAs are essential for development and function of inner ear hair cells in vertebrates. *Proc Natl Acad Sci U S A* **106:** 7915-20

Frommer M, McDonald LE, Millar DS, Collis CM, Watt F, Grigg GW, Molloy PL, Paul CL (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

Fruhwald MC, O'Dorisio MS, Dai Z, Rush LJ, Krahe R, Smiraglia DJ, Pietsch T, Elsea SH, Plass C (2001) Aberrant hypermethylation of the major breakpoint cluster region in 17p11.2 in medulloblastomas but not supratentorial PNETs. *Genes Chromosomes Cancer* **30:** 38-47

Garcia-Manero G, Daniel J, Smith TL, Kornblau SM, Lee MS, Kantarjian HM, Issa JP (2002) DNA methylation of multiple promoter-associated CpG islands in adult acute lymphocytic leukemia. *Clin Cancer Res* **8:** 2217-24

Gaudet F, Hodgson JG, Eden A, Jackson-Grusby L, Dausman J, Gray JW, Leonhardt H, Jaenisch R (2003) Induction of tumors in mice by genomic hypomethylation. *Science* **300:** 489-92

Gonzalgo ML, Jones PA (2002) Quantitative methylation analysis using methylation-sensitive single-nucleotide primer extension (Ms-SNuPE). *Methods* **27:** 128-33

Griffiths EA, Gore SD (2008) DNA methyltransferase and histone deacetylase inhibitors in the treatment of myelodysplastic syndromes. *Semin Hematol* **45:** 23-30

Gupta A, Godwin AK, Vanderveer L, Lu A, Liu J (2003) Hypomethylation of the synuclein gamma gene CpG island promotes its aberrant expression in breast carcinoma and ovarian carcinoma. *Cancer Res* **63:** 664-73

Hajkova P, Erhardt S, Lane N, Haaf T, El-Maarri O, Reik W, Walter J, Surani MA (2002) Epigenetic reprogramming in mouse primordial germ cells. *Mech Dev* **117:** 15-23

Han L, Witmer PD, Casey E, Valle D, Sukumar S (2007) DNA methylation regulates MicroRNA expression. *Cancer Biol Ther* **6:** 1284-8

Hanabata T, Tsukuda K, Toyooka S, Yano M, Aoe M, Nagahiro I, Sano Y, Date H, Shimizu N (2004) DNA methylation of multiple genes and clinicopathological relationship of non-small cell lung cancers. *Oncol Rep* **12:** 177-80

Hark AT, Schoenherr CJ, Katz DJ, Ingram RS, Levorse JM, Tilghman SM (2000) CTCF mediates methylation-sensitive enhancer-blocking activity at the H19/Igf2 locus. *Nature* **405:** 486-9

Hatada I, Hayashizaki Y, Hirotsune S, Komatsubara H, Mukai T (1991) A genomic scanning method for higher organisms using restriction sites as landmarks. *Proc Natl Acad Sci U S A* **88:** 9523-7

Hattori N, Nishino K, Ko YG, Ohgane J, Tanaka S, Shiota K (2004) Epigenetic control of mouse Oct-4 gene expression in embryonic stem cells and trophoblast stem cells. *J Biol Chem* **279:** 17063-9

Hayatsu N, Kaneko MK, Mishima K, Nishikawa R, Matsutani M, Price JE, Kato Y (2008) Podocalyxin expression in malignant astrocytic tumors. *Biochem Biophys Res Commun* **374:** 394-8

He L, Hannon GJ (2004) MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet* **5:** 522-31

Hesson L, Bieche I, Krex D, Criniere E, Hoang-Xuan K, Maher ER, Latif F (2004) Frequent epigenetic inactivation of RASSF1A and BLU genes located within the critical 3p21.3 region in gliomas. *Oncogene* **23:** 2408-19

Hirohashi S, Kanai Y (2003) Cell adhesion system and human cancer morphogenesis. *Cancer Sci* **94:** 575-81

Hoffman BG, Jones SJ (2009) Genome-wide identification of DNA-protein interactions using chromatin immunoprecipitation coupled with flow cell sequencing. *J Endocrinol* **201:** 1-13

Honda T, Tamura G, Waki T, Kawata S, Terashima M, Nishizuka S, Motoyama T (2004) Demethylation of MAGE promoters during gastric cancer progression. *Br J Cancer* **90:** 838-43

Hong FZ, Wang B, Li HM, Liew CT (2005) [Hypermethylation of fragile histidine triad gene and 3p14 allelic deletion in ovarian carcinomas]. *Zhonghua Bing Li Xue Za Zhi* **34:** 257-61

Horwich A, Shipley J, Huddart R (2006) Testicular germ-cell cancer. *Lancet* **367:** 754-65

Houshdaran S, Cortessis VK, Siegmund K, Yang A, Laird PW, Sokol RZ (2007) Widespread epigenetic abnormalities suggest a broad DNA methylation erasure defect in abnormal human sperm. *PLoS One* **2:** e1289

Huang Q, Gumireddy K, Schrier M, le Sage C, Nagel R, Nair S, Egan DA, Li A, Huang G, Klein-Szanto AJ, Gimotty PA, Katsaros D, Coukos G, Zhang L, Pure E, Agami R (2008) The microRNAs miR-373 and miR-520c promote tumour invasion and metastasis. *Nat Cell Biol* **10:** 202-10

Huang TH, Perry MR, Laux DE (1999) Methylation profiling of CpG islands in human breast cancer cells. *Hum Mol Genet* **8:** 459-70

Ichimi T, Enokida H, Okuno Y, Kunimoto R, Chiyomaru T, Kawamoto K, Kawahara K, Toki K, Kawakami K, Nishiyama K, Tsujimoto G, Nakagawa M, Seki N (2009) Identification of novel microRNA targets based on microRNA signatures in bladder cancer. *Int J Cancer* **125:** 345-52

Illingworth R, Kerr A, Desousa D, Jorgensen H, Ellis P, Stalker J, Jackson D, Clee C, Plumb R, Rogers J, Humphray S, Cox T, Langford C, Bird A (2008) A novel CpG island set identifies tissue-specific methylation at developmental gene loci. *PLoS Biol* **6:** e22

Iorio MV, Visone R, Di Leva G, Donati V, Petrocca F, Casalini P, Taccioli C, Volinia S, Liu CG, Alder H, Calin GA, Menard S, Croce CM (2007) MicroRNA signatures in human ovarian cancer. *Cancer Res* **67:** 8699-707

Irizarry RA, Ladd-Acosta C, Carvalho B, Wu H, Brandenburg SA, Jeddeloh JA, Wen B, Feinberg AP (2008) Comprehensive high-throughput arrays for relative methylation (CHARM). *Genome Res* **18:** 780-90

Jabbour E, Issa JP, Garcia-Manero G, Kantarjian H (2008) Evolution of decitabine development: accomplishments, ongoing investigations, and future strategies. *Cancer* **112:** 2341-51

Jacinto FV, Ballestar E, Ropero S, Esteller M (2007) Discovery of epigenetically silenced genes by methylated DNA immunoprecipitation in colon cancer cells. *Cancer Res* **67:** 11481-6

Jeronimo C, Usadel H, Henrique R, Oliveira J, Lopes C, Nelson WG, Sidransky D (2001) Quantitation of GSTP1 methylation in non-neoplastic prostatic tissue and organ-confined prostate adenocarcinoma. *J Natl Cancer Inst* **93:** 1747-52

Jiang C, Pugh BF (2009) Nucleosome positioning and gene regulation: advances through genomics. *Nat Rev Genet* **10:** 161-72

Johnson JM, Edwards S, Shoemaker D, Schadt EE (2005) Dark matter in the genome: evidence of widespread transcription detected by microarray tiling experiments. *Trends Genet* **21:** 93-102

Jones PA, Takai D (2001) The role of DNA methylation in mammalian epigenetics. *Science* **293:** 1068-70

Jurgens B, Schmitz-Drager BJ, Schulz WA (1996) Hypomethylation of L1 LINE sequences prevailing in human urothelial carcinoma. *Cancer Res* **56:** 5698-703

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

Kaneko Y, Shibuya M, Nakayama T, Hayashida N, Toda G, Endo Y, Oka H, Oda T (1985) Hypomethylation of c-myc and epidermal growth factor receptor genes in human hepatocellular carcinoma and fetal liver. *Jpn J Cancer Res* **76:** 1136-40

Kang GH, Lee HJ, Hwang KS, Lee S, Kim JH, Kim JS (2003) Aberrant CpG island hypermethylation of chronic gastritis, in relation to aging, gender, intestinal metaplasia, and chronic inflammation. *Am J Pathol* **163:** 1551-6

Kato K, Maesawa C, Itabashi T, Fujisawa K, Otsuka K, Kanno S, Tada H, Tatemichi Y, Kotani K, Oikawa H, Sugai T, Wakabayashi G, Masuda T (2009) DNA hypomethylation at the CpG island is involved in aberrant expression of the L1 cell adhesion molecule gene in colorectal cancer. *Int J Oncol* **35:** 467-76

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

Kerjaschki D, Sharkey DJ, Farquhar MG (1984) Identification and characterization of podocalyxin--the major sialoprotein of the renal glomerular epithelial cell. *J Cell Biol* **98:** 1591-6

Keshet I, Schlesinger Y, Farkash S, Rand E, Hecht M, Segal E, Pikarski E, Young RA, Niveleau A, Cedar H, Simon I (2006) Evidence for an instructive mechanism of de novo methylation in cancer cells. *Nat Genet* **38:** 149-53

Khulan B, Thompson RF, Ye K, Fazzari MJ, Suzuki M, Stasiek E, Figueroa ME, Glass JL, Chen Q, Montagna C, Hatchwell E, Selzer RR, Richmond TA, Green RD, Melnick A, Greally JM (2006) Comparative isoschizomer profiling of cytosine methylation: the HELP assay. *Genome Res* **16:** 1046-55

Kim DH, Kim JS, Park JH, Lee SK, Ji YI, Kwon YM, Shim YM, Han J, Park J (2003) Relationship of Ras association domain family 1 methylation and K-ras mutation in primary non-small cell lung cancer. *Cancer Res* **63:** 6206-11

Kim DH, Nelson HH, Wiencke JK, Christiani DC, Wain JC, Mark EJ, Kelsey KT (2001) Promoter methylation of DAP-kinase: association with advanced stage in non-small cell lung cancer. *Oncogene* **20:** 1765-70

Kim KH, Choi JS, Kim IJ, Ku JL, Park JG (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

Kimura H, Shiota K (2003) Methyl-CpG-binding protein, MeCP2, is a target molecule for maintenance DNA methyltransferase, Dnmt1. *J Biol Chem* **278:** 4806-12

Koch LK, Zhou H, Ellinger J, Biermann K, Holler T, von Rucker A, Buttner R, Gutgemann I (2008) Stem cell marker expression in small cell lung carcinoma and developing lung tissue. *Hum Pathol* **39:** 1597-605

Koch S, Mayer F, Honecker F, Schittenhelm M, Bokemeyer C (2003) Efficacy of cytotoxic agents used in the treatment of testicular germ cell tumours under normoxic and hypoxic conditions in vitro. *Br J Cancer* **89:** 2133-9

Konishi N, Tao M, Nakamura M, Kitahaori Y, Hiasa Y, Nagai H (1996) Genomic alterations in human prostate carcinoma cell lines by two-dimensional gel analysis. *Cell Mol Biol (Noisy-le-grand)* **42:** 1129-35

Kouzarides T (2007) Chromatin modifications and their function. *Cell* **128:** 693-705

Krausz C, Looijenga LH (2008) Genetic aspects of testicular germ cell tumors. *Cell Cycle* **7:** 3519-24

Krege S, Beyer J, Souchon R, Albers P, Albrecht W, Algaba F, Bamberg M, Bodrogi I, Bokemeyer C, Cavallin-Stahl E, Classen J, Clemm C, Cohn-Cedermark G, Culine S, Daugaard G, De Mulder PH, De Santis M, de Wit M, de Wit R, Derigs HG, Dieckmann KP, Dieing A, Droz JP, Fenner M, Fizazi K, Flechon A, Fossa SD, del Muro XG, Gauler T, Geczi L, Gerl A, Germa-Lluch JR, Gillessen S, Hartmann JT, Hartmann M, Heidenreich A, Hoeltl W, Horwich A, Huddart R, Jewett M, Joffe J, Jones WG, Kisbenedek L, Klepp O, Kliesch S, Koehrmann KU, Kollmannsberger C, Kuczyk M, Laguna P, Galvis OL, Loy V, Mason MD, Mead GM, Mueller R, Nichols C, Nicolai N, Oliver T, Ondrus D, Oosterhof GO, Ares LP, Pizzocaro G, Pont J, Pottek T, Powles T, Rick O, Rosti G, Salvioni

R, Scheiderbauer J, Schmelz HU, Schmidberger H, Schmoll HJ, Schrader M, Sedlmayer F, Skakkebaek NE, Sohaib A, Tjulandin S, Warde P, Weinknecht S, Weissbach L, Wittekind C, Winter E, Wood L, von der Maase H (2008) European consensus conference on diagnosis and treatment of germ cell cancer: a report of the second meeting of the European Germ Cell Cancer Consensus group (EGCCCG): part I. *Eur Urol* **53:** 478-96

Kuromitsu J, Kataoka H, Yamashita H, Muramatsu M, Furuichi Y, Sekine T, Hayashizaki Y (1995) Reproducible alterations of DNA methylation at a specific population of CpG islands during blast formation of peripheral blood lymphocytes. *DNA Res* **2:** 263-7

Larsen F, Gundersen G, Lopez R, Prydz H (1992) CpG islands as gene markers in the human genome. *Genomics* **13:** 1095-107

Lee DY, Shatseva T, Jeyapalan Z, Du WW, Deng Z, Yang BB (2009) A 3'-untranslated region (3'UTR) induces organ adhesion by regulating miR-199a* functions. *PLoS One* **4:** e4527

Lee EJ, Lee BB, Kim JW, Shim YM, Hoseok I, Han J, Cho EY, Park J, Kim DH (2006a) Aberrant methylation of Fragile Histidine Triad gene is associated with poor prognosis in early stage esophageal squamous cell carcinoma. *Eur J Cancer* **42:** 972-80

Lee TL, Leung WK, Chan MW, Ng EK, Tong JH, Lo KW, Chung SC, Sung JJ, To KF (2002) Detection of gene promoter hypermethylation in the tumor and serum of patients with gastric carcinoma. *Clin Cancer Res* **8:** 1761-6

Lee TL, Yeh J, Van Waes C, Chen Z (2006b) Epigenetic modification of SOCS-1 differentially regulates STAT3 activation in response to interleukin-6 receptor and epidermal growth factor receptor signaling through JAK and/or MEK in head and neck squamous cell carcinomas. *Mol Cancer Ther* **5:** 8-19

Lei H, Oh SP, Okano M, Juttermann R, Goss KA, Jaenisch R, Li E (1996) De novo DNA cytosine methyltransferase activities in mouse embryonic stem cells. *Development* **122:** 3195-205

Leung WK, Yu J, Ng EK, To KF, Ma PK, Lee TL, Go MY, Chung SC, Sung JJ (2001) Concurrent hypermethylation of multiple tumor-related genes in gastric carcinoma and adjacent normal tissues. *Cancer* **91:** 2294-301

Li E, Bestor TH, Jaenisch R (1992) Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* **69:** 915-26

Li LC, Shiina H, Deguchi M, Zhao H, Okino ST, Kane CJ, Carroll PR, Igawa M, Dahiya R (2004) Age-dependent methylation of ESR1 gene in prostate cancer. *Biochem Biophys Res Commun* **321:** 455-61

Li LC, Zhao H, Nakajima K, Oh BR, Ribeiro Filho LA, Carroll P, Dahiya R (2001) Methylation of the E-cadherin gene promoter correlates with progression of prostate cancer. *J Urol* **166:** 705-9

Li Y, Tabatabai ZL, Lee TL, Hatakeyama S, Ohyama C, Chan WY, Looijenga LH, Lau YF (2007) The Y-encoded TSPY protein: a significant marker potentially plays a role in the pathogenesis of testicular germ cell tumors. *Hum Pathol* **38:** 1470-81

Licchesi JD, Herman JG (2009) Methylation-specific PCR. *Methods Mol Biol* **507:** 305-23

Lim JH, Kim SP, Gabrielson E, Park YB, Park JW, Kwon TK (2005) Activation of human cancer/testis antigen gene, XAGE-1, in tumor cells is correlated with CpG island hypomethylation. *Int J Cancer* **116:** 200-6

Lin EA, Kong L, Bai XH, Luan Y, Liu CJ (2009) miR-199a, a bone morphogenic protein 2-responsive MicroRNA, regulates chondrogenesis via direct targeting to Smad1. *J Biol Chem* **284:** 11326-35

Lind GE, Skotheim RI, Lothe RA (2007) The epigenome of testicular germ cell tumors. *APMIS* **115:** 1147-60

Lister R, Ecker JR (2009) Finding the fifth base: genome-wide sequencing of cytosine methylation. *Genome Res* **19:** 959-66

Lister R, O'Malley RC, Tonti-Filippini J, Gregory BD, Berry CC, Millar AH, Ecker JR (2008) Highly integrated single-base resolution maps of the epigenome in Arabidopsis. *Cell* **133:** 523-36

Liu L, van Groen T, Kadish I, Tollefsbol TO (2009) DNA methylation impacts on learning and memory in aging. *Neurobiol Aging* **30:** 549-60

Lujambio A, Calin GA, Villanueva A, Ropero S, Sanchez-Cespedes M, Blanco D, Montuenga LM, Rossi S, Nicoloso MS, Faller WJ, Gallagher WM, Eccles SA, Croce CM, Esteller M (2008) A microRNA DNA methylation signature for human cancer metastasis. *Proc Natl Acad Sci U S A* **105:** 13556-61

Lujambio A, Ropero S, Ballestar E, Fraga MF, Cerrato C, Setien F, Casado S, Suarez-Gauthier A, Sanchez-Cespedes M, Git A, Spiteri I, Das PP, Caldas C, Miska E, Esteller M (2007) Genetic unmasking of an epigenetically silenced microRNA in human cancer cells. *Cancer Res* **67:** 1424-9

Ma L, Teruya-Feldstein J, Weinberg RA (2007) Tumour invasion and metastasis initiated by microRNA-10b in breast cancer. *Nature* **449:** 682-8

Makrilia N, Kollias A, Manolopoulos L, Syrigos K (2009) Cell adhesion molecules: role and clinical significance in cancer. *Cancer Invest* **27:** 1023-37

Mares J, Kriz V, Weinhausel A, Vodickova S, Kodet R, Haas OA, Sedlacek Z, Goetz P (2001) Methylation changes in promoter and enhancer regions of the WT1 gene in Wilms' tumours. *Cancer Lett* **166:** 165-71

Maruyama R, Sugio K, Yoshino I, Maehara Y, Gazdar AF (2004) Hypermethylation of FHIT as a prognostic marker in nonsmall cell lung carcinoma. *Cancer* **100:** 1472-7

Mayer W, Niveleau A, Walter J, Fundele R, Haaf T (2000) Demethylation of the zygotic paternal genome. *Nature* **403:** 501-2

Meissner A, Mikkelsen TS, Gu H, Wernig M, Hanna J, Sivachenko A, Zhang X, Bernstein BE, Nusbaum C, Jaffe DB, Gnirke A, Jaenisch R, Lander ES (2008) Genome-scale DNA methylation maps of pluripotent and differentiated cells. *Nature* **454:** 766-70

Mendell JT (2005) MicroRNAs: critical regulators of development, cellular physiology and malignancy. *Cell Cycle* **4:** 1179-84

Migliore C, Petrelli A, Ghiso E, Corso S, Capparuccia L, Eramo A, Comoglio PM, Giordano S (2008) MicroRNAs impair MET-mediated invasive growth. *Cancer Res* **68:** 10128-36

Milicic A, Harrison LA, Goodlad RA, Hardy RG, Nicholson AM, Presz M, Sieber O, Santander S, Pringle JH, Mandir N, East P, Obszynska J, Sanders S, Piazuelo E, Shaw J, Harrison R, Tomlinson IP, McDonald SA, Wright NA, Jankowski JA (2008) Ectopic expression of P-cadherin correlates with promoter hypomethylation early in colorectal carcinogenesis and enhanced intestinal crypt fission in vivo. *Cancer Res* **68:** 7760-8

Miremadi A, Oestergaard MZ, Pharoah PD, Caldas C (2007) Cancer genetics of epigenetic genes. *Hum Mol Genet* **16 Spec No 1:** R28-49

Mohn F, Weber M, Schubeler D, Roloff TC (2009) Methylated DNA immunoprecipitation (Me-DIP). *Methods Mol Biol* **507:** 55-64

Morey SR, Smiraglia DJ, James SR, Yu J, Moser MT, Foster BA, Karpf AR (2006) DNA methylation pathway alterations in an autochthonous murine model of prostate cancer. *Cancer Res* **66:** 11659-67

Morozova O, Marra MA (2008) Applications of next-generation sequencing technologies in functional genomics. *Genomics* **92:** 255-64

Morrow EM, Yoo SY, Flavell SW, Kim TK, Lin Y, Hill RS, Mukaddes NM, Balkhy S, Gascon G, Hashmi A, Al-Saad S, Ware J, Joseph RM, Greenblatt R, Gleason D, Ertelt JA, Apse KA, Bodell A, Partlow JN, Barry B, Yao H, Markianos K, Ferland RJ, Greenberg ME, Walsh CA (2008) Identifying autism loci and genes by tracing recent shared ancestry. *Science* **321:** 218-23

Motiwala T, Ghoshal K, Das A, Majumder S, Weichenhan D, Wu YZ, Holman K, James SJ, Jacob ST, Plass C (2003) Suppression of the protein tyrosine phosphatase receptor type O gene (PTPRO) by methylation in hepatocellular carcinomas. *Oncogene* **22:** 6319-31

Muromoto R, Sugiyama K, Takachi A, Imoto S, Sato N, Yamamoto T, Oritani K, Shimoda K, Matsuda T (2004) Physical and functional interactions between Daxx and DNA methyltransferase 1-associated protein, DMAP1. *J Immunol* **172:** 2985-93

Nabeshima K, Inoue T, Shimao Y, Okada Y, Itoh Y, Seiki M, Koono M (2000) Front-cell-specific expression of membrane-type 1 matrix metalloproteinase and gelatinase A during cohort migration of colon carcinoma cells induced by hepatocyte growth factor/scatter factor. *Cancer Res* **60:** 3364-9

Nagai H, Kim YS, Yasuda T, Ohmachi Y, Yokouchi H, Monden M, Emi M, Konishi N, Nogami M, Okumura K, Matsubara K (1999) A novel sperm-specific hypomethylation sequence is a demethylation hotspot in human hepatocellular carcinomas. *Gene* **237:** 15-20

Nakata S, Sugio K, Uramoto H, Oyama T, Hanagiri T, Morita M, Yasumoto K (2006) The methylation status and protein expression of CDH1, p16(INK4A), and fragile histidine triad in nonsmall cell lung carcinoma: epigenetic silencing, clinical features, and prognostic significance. *Cancer* **106:** 2190-9

Nam EJ, Yoon H, Kim SW, Kim H, Kim YT, Kim JH, Kim JW, Kim S (2008) MicroRNA expression profiles in serous ovarian carcinoma. *Clin Cancer Res* **14:** 2690-5

Nambu S, Inoue K, Saski H (1987) Site-specific hypomethylation of the c-myc oncogene in human hepatocellular carcinoma. *Jpn J Cancer Res* **78:** 695-704

Nan HM, Song YJ, Yun HY, Park JS, Kim H (2005) Effects of dietary intake and genetic factors on hypermethylation of the hMLH1 gene promoter in gastric cancer. *World J Gastroenterol* **11:** 3834-41

Nan X, Meehan RR, Bird A (1993) Dissection of the methyl-CpG binding domain from the chromosomal protein MeCP2. *Nucleic Acids Res* **21:** 4886-92

Neff T, Armstrong SA (2009) Chromatin maps, histone modifications and leukemia. *Leukemia* **23:** 1243-51

Ng HH, Zhang Y, Hendrich B, Johnson CA, Turner BM, Erdjument-Bromage H, Tempst P, Reinberg D, Bird A (1999) MBD2 is a transcriptional repressor belonging to the MeCP1 histone deacetylase complex. *Nat Genet* **23:** 58-61

Nosho K, Yamamoto H, Takahashi T, Mikami M, Taniguchi H, Miyamoto N, Adachi Y, Arimura Y, Itoh F, Imai K, Shinomura Y (2007) Genetic and epigenetic profiling in early colorectal tumors and prediction of invasive potential in pT1 (early invasive) colorectal cancers. *Carcinogenesis* **28:** 1364-70

Oda M, Greally JM (2009) The HELP assay. *Methods Mol Biol* **507:** 77-87

Ogino S, Kawasaki T, Kirkner GJ, Suemoto Y, Meyerhardt JA, Fuchs CS (2007) Molecular correlates with MGMT promoter methylation and silencing support CpG island methylator phenotype-low (CIMP-low) in colorectal cancer. *Gut* **56:** 1564-71

Ogino S, Kawasaki T, Nosho K, Ohnishi M, Suemoto Y, Kirkner GJ, Fuchs CS (2008) LINE-1 hypomethylation is inversely associated with microsatellite instability and CpG island methylator phenotype in colorectal cancer. *Int J Cancer* **122:** 2767-73

Oka M, Rodic N, Graddy J, Chang LJ, Terada N (2006) CpG sites preferentially methylated by Dnmt3a in vivo. *J Biol Chem* **281:** 9901-8

Okano M, Bell DW, Haber DA, Li E (1999) DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* **99:** 247-57

Okazaki Y, Okuizumi H, Sasaki N, Ohsumi T, Kuromitsu J, Hirota N, Muramatsu M, Hayashizaki Y (1995) An expanded system of restriction landmark genomic scanning (RLGS Ver. 1.8). *Electrophoresis* **16:** 197-202

Ongenaert M, Van Neste L, De Meyer T, Menschaert G, Bekaert S, Van Criekinge W (2008) PubMeth: a cancer methylation database combining text-mining and expert annotation. *Nucleic Acids Res* **36:** D842-6

Ordway JM, Budiman MA, Korshunova Y, Maloney RK, Bedell JA, Citek RW, Bacher B, Peterson S, Rohlfing T, Hall J, Brown R, Lakey N, Doerge RW, Martienssen RA, Leon J, McPherson JD, Jeddeloh JA (2007) Identification of novel high-frequency DNA methylation changes in breast cancer. *PLoS ONE* **2:** e1314

Oswald J, Engemann S, Lane N, Mayer W, Olek A, Fundele R, Dean W, Reik W, Walter J (2000) Active demethylation of the paternal genome in the mouse zygote. *Curr Biol* **10:** 475-8

Oue N, Oshimo Y, Nakayama H, Ito R, Yoshida K, Matsusaki K, Yasui W (2003) DNA methylation of multiple genes in gastric carcinoma: association with histological type and CpG island methylator phenotype. *Cancer Sci* **94:** 901-5

Pang AL, Peacock S, Johnson W, Bear DH, Rennert OM, Chan WY (2009) Cloning, characterization, and expression analysis of the novel acetyltransferase retrogene Ard1b in the mouse. *Biol Reprod* **81:** 302-9

Paredes J, Albergaria A, Oliveira JT, Jeronimo C, Milanezi F, Schmitt FC (2005) P-cadherin overexpression is an indicator of clinical outcome in invasive breast carcinomas and is associated with CDH3 promoter hypomethylation. *Clin Cancer Res* **11:** 5869-77

Pattamadilok J, Huapai N, Rattanatanyong P, Vasurattana A, Triratanachat S, Tresukosol D, Mutirangura A (2008) LINE-1 hypomethylation level as a potential prognostic factor for epithelial ovarian cancer. *Int J Gynecol Cancer* **18:** 711-7

Paz MF, Wei S, Cigudosa JC, Rodriguez-Perales S, Peinado MA, Huang TH, Esteller M (2003) Genetic unmasking of epigenetically silenced tumor suppressor genes in colon cancer cells deficient in DNA methyltransferases. *Hum Mol Genet* **12:** 2209-19

Peltier HJ, Latham GJ (2008) Normalization of microRNA expression levels in quantitative RT-PCR assays: identification of suitable reference RNA targets in normal and cancerous human solid tissues. *RNA* **14:** 844-52

Pettersson E, Lundeberg J, Ahmadian A (2009) Generations of sequencing technologies. *Genomics* **93:** 105-11

Ramsahoye BH, Biniszkiewicz D, Lyko F, Clark V, Bird AP, 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 (2007) Susceptibility alleles for testicular germ cell tumour: a review. *Int J Androl* **30:** 242-50; discussion 250

Rapley EA, Crockford GP, Teare D, Biggs P, Seal S, Barfoot R, Edwards S, Hamoudi R, Heimdal K, Fossa SD, Tucker K, Donald J, Collins F, Friedlander M, Hogg D, Goss P, Heidenreich A, Ormiston W, Daly PA, Forman D, Oliver TD, Leahy M, Huddart R, Cooper CS, Bodmer JG, Easton DF, Stratton MR, Bishop DT (2000) Localization to Xq27 of a susceptibility gene for testicular germ-cell tumours. *Nat Genet* **24:** 197-200

Reilly JG, Thomas CA, Jr., Sen A (1982) DNA methylation in mouse cells in culture as measured by restriction enzymes. *Biochim Biophys Acta* **697:** 53-9

Ren B, Dynlacht BD (2004) Use of chromatin immunoprecipitation assays in genome-wide location analysis of mammalian transcription factors. *Methods Enzymol* **376:** 304-15

Robertson KD (2005) DNA methylation and human disease. *Nat Rev Genet* **6:** 597-610

Robertson KD, Ait-Si-Ali S, Yokochi T, Wade PA, Jones PL, Wolffe AP (2000) DNMT1 forms a complex with Rb, E2F1 and HDAC1 and represses transcription from E2F-responsive promoters. *Nat Genet* **25:** 338-42

Rodriguez BA, Huang TH (2005) Tilling the chromatin landscape: emerging methods for the discovery and profiling of protein-DNA interactions. *Biochem Cell Biol* **83:** 525-34

Roman-Gomez J, Jimenez-Velasco A, Agirre X, Cervantes F, Sanchez J, Garate L, Barrios M, Castillejo JA, Navarro G, Colomer D, Prosper F, Heiniger A, Torres A (2005a) Promoter hypomethylation of the LINE-1 retrotransposable elements activates sense/antisense transcription and marks the progression of chronic myeloid leukemia. *Oncogene* **24:** 7213-23

Roman-Gomez J, Jimenez-Velasco A, Agirre X, Prosper F, Heiniger A, Torres A (2005b) Lack of CpG island methylator phenotype defines a clinical subtype of T-cell acute lymphoblastic leukemia associated with good prognosis. *J Clin Oncol* **23:** 7043-9

Roman-Gomez J, Jimenez-Velasco A, Castillejo JA, Agirre X, Barrios M, Navarro G, Molina FJ, Calasanz MJ, Prosper F, Heiniger A, Torres A (2004) Promoter hypermethylation of cancer-related genes: a strong independent prognostic factor in acute lymphoblastic leukemia. *Blood* **104:** 2492-8

Rountree MR, Bachman KE, Baylin SB (2000) DNMT1 binds HDAC2 and a new co-repressor, DMAP1, to form a complex at replication foci. *Nat Genet* **25:** 269-77

Sahai E (2005) Mechanisms of cancer cell invasion. *Curr Opin Genet Dev* **15:** 87-96

Saito Y, Liang G, Egger G, Friedman JM, Chuang JC, Coetzee GA, Jones PA (2006) Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells. *Cancer Cell* **9:** 435-43

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A* **74:** 5463-7

Sanger F, Nicklen S, Coulson AR (1992) DNA sequencing with chain-terminating inhibitors. 1977. *Biotechnology* **24:** 104-8

Sarbia M, Geddert H, Klump B, Kiel S, Iskender E, Gabbert HE (2004) Hypermethylation of tumor suppressor genes (p16INK4A, p14ARF and APC) in adenocarcinomas of the upper gastrointestinal tract. *Int J Cancer* **111:** 224-8

Sato S, Yoshimizu T, Sato E, Matsui Y (2003) Erasure of methylation imprinting of Igf2r during mouse primordial germ-cell development. *Mol Reprod Dev* **65:** 41-50

Saxonov S, Berg P, Brutlag DL (2006) A genome-wide analysis of CpG dinucleotides in the human genome distinguishes two distinct classes of promoters. *Proc Natl Acad Sci U S A* **103:** 1412-7

Schneider-Stock R, Ocker M (2007) Epigenetic therapy in cancer: molecular background and clinical development of histone deacetylase and DNA methyltransferase inhibitors. *IDrugs* **10:** 557-61

Schopperle WM, Kershaw DB, DeWolf WC (2003) Human embryonal carcinoma tumor antigen, Gp200/GCTM-2, is podocalyxin. *Biochem Biophys Res Commun* **300:** 285-90

Schumacher A, Kapranov P, Kaminsky Z, Flanagan J, Assadzadeh A, Yau P, Virtanen C, Winegarden N, Cheng J, Gingeras T, Petronis A (2006) Microarray-based DNA methylation profiling: technology and applications. *Nucleic Acids Res* **34:** 528-42

Sharrard RM, Royds JA, Rogers S, Shorthouse AJ (1992) Patterns of methylation of the c-myc gene in human colorectal cancer progression. *Br J Cancer* **65:** 667-72

Shiozawa E, Takimoto M, Makino R, Adachi D, Saito B, Yamochi-Onizuka T, Yamochi T, Shimozuma J, Maeda T, Kohno Y, Kawakami K, Nakamaki T, Tomoyasu S, Shiokawa A, Ota H (2006) Hypermethylation of CpG islands in p16 as a prognostic factor for diffuse large B-cell lymphoma in a high-risk group. *Leuk Res* **30:** 859-67

Shivakumar L, Minna J, Sakamaki T, Pestell R, White MA (2002) The RASSF1A tumor suppressor blocks cell cycle progression and inhibits cyclin D1 accumulation. *Mol Cell Biol* **22:** 4309-18

Shuangshoti S, Hourpai N, Pumsuk U, Mutirangura A (2007) Line-1 hypomethylation in multistage carcinogenesis of the uterine cervix. *Asian Pac J Cancer Prev* **8:** 307-9

Siu LL, Chan JK, Wong KF, Kwong YL (2002) Specific patterns of gene methylation in natural killer cell lymphomas : p73 is consistently involved. *Am J Pathol* **160:** 59-66

Skotheim RI, Lind GE, Monni O, Nesland JM, Abeler VM, Fossa SD, Duale N, Brunborg G, Kallioniemi O, Andrews PW, Lothe RA (2005) Differentiation of human embryonal carcinomas in vitro and in vivo reveals expression profiles relevant to normal development. *Cancer Res* **65:** 5588-98

Smallwood A, Esteve PO, Pradhan S, Carey M (2007) Functional cooperation between HP1 and DNMT1 mediates gene silencing. *Genes Dev* **21:** 1169-78

Smiraglia DJ, Plass C (2002) The study of aberrant methylation in cancer via restriction landmark genomic scanning. *Oncogene* **21:** 5414-26

Smiraglia DJ, Smith LT, Lang JC, Rush LJ, Dai Z, Schuller DE, Plass C (2003) Differential targets of CpG island hypermethylation in primary and metastatic head and neck squamous cell carcinoma (HNSCC). *J Med Genet* **40:** 25-33

Smiraglia DJ, Szymanska J, Kraggerud SM, Lothe RA, Peltomaki P, Plass C (2002) Distinct epigenetic phenotypes in seminomatous and nonseminomatous testicular germ cell tumors. *Oncogene* **21:** 3909-16

Smith JF, Mahmood S, Song F, Morrow A, Smiraglia D, Zhang X, Rajput A, Higgins MJ, Krumm A, Petrelli NJ, Costello JF, Nagase H, Plass C, Held WA (2007) Identification of DNA methylation in 3' genomic regions that are associated with upregulation of gene expression in colorectal cancer. *Epigenetics* **2:** 161-72

Solomon MJ, Larsen PL, Varshavsky A (1988) Mapping protein-DNA interactions in vivo with formaldehyde: evidence that histone H4 is retained on a highly transcribed gene. *Cell* **53:** 937-47

Somasiri A, Nielsen JS, Makretsov N, McCoy ML, Prentice L, Gilks CB, Chia SK, Gelmon KA, Kershaw DB, Huntsman DG, McNagny KM, Roskelley CD (2004) Overexpression of the anti-adhesin podocalyxin is an independent predictor of breast cancer progression. *Cancer Res* **64:** 5068-73

Song F, Smith JF, Kimura MT, Morrow AD, Matsuyama T, Nagase H, Held WA (2005) Association of tissue-specific differentially methylated regions (TDMs) with differential gene expression. *Proc Natl Acad Sci U S A* **102:** 3336-41

Sorensen AL, Collas P (2009) Immunoprecipitation of methylated DNA. *Methods Mol Biol* **567:** 249-62

Suzuki M, Shigematsu H, Iizasa T, Hiroshima K, Nakatani Y, Minna JD, Gazdar AF, Fujisawa T (2006) Exclusive mutation in epidermal growth factor receptor gene, HER-2, and KRAS, and synchronous methylation of nonsmall cell lung cancer. *Cancer* **106:** 2200-7

Takeda T, Go WY, Orlando RA, Farquhar MG (2000) Expression of podocalyxin inhibits cell-cell adhesion and modifies junctional properties in Madin-Darby canine kidney cells. *Mol Biol Cell* **11:** 3219-32

Tate PH, Bird AP (1993) Effects of DNA methylation on DNA-binding proteins and gene expression. *Curr Opin Genet Dev* **3:** 226-31

Teodoridis JM, Hall J, Marsh S, Kannall HD, Smyth C, Curto J, Siddiqui N, Gabra H, McLeod HL, Strathdee G, Brown R (2005) CpG island methylation of DNA damage response genes in advanced ovarian cancer. *Cancer Res* **65:** 8961-7

Thu KL, Vucic EA, Kennett JY, Heryet C, Brown CJ, Lam WL, Wilson IM (2009) Methylated DNA immunoprecipitation. *J Vis Exp*

To KF, Leung WK, Lee TL, Yu J, Tong JH, Chan MW, Ng EK, Chung SC, Sung JJ (2002) Promoter hypermethylation of tumor-related genes in gastric intestinal metaplasia of patients with and without gastric cancer. *Int J Cancer* **102:** 623-8

Toyota M, Suzuki H, Sasaki Y, Maruyama R, Imai K, Shinomura Y, Tokino T (2008) Epigenetic silencing of microRNA-34b/c and B-cell translocation gene 4 is associated with CpG island methylation in colorectal cancer. *Cancer Res* **68:** 4123-32

Tsai WC, Hsu PW, Lai TC, Chau GY, Lin CW, Chen CM, Lin CD, Liao YL, Wang JL, Chau YP, Hsu MT, Hsiao M, Huang HD, Tsou AP (2009) MicroRNA-122, a tumor suppressor microRNA that regulates intrahepatic metastasis of hepatocellular carcinoma. *Hepatology* **49:** 1571-82

Tsang WP, Wong TW, Cheung AH, Co CN, Kwok TT (2007) Induction of drug resistance and transformation in human cancer cells by the noncoding RNA CUDR. *RNA* **13:** 890-8

Tsukamoto N, Morita K, Karasawa M, Omine M (1992) Methylation status of c-myc oncogene in leukemic cells: hypomethylation in acute leukemia derived from myelodysplastic syndromes. *Exp Hematol* **20:** 1061-4

Ueda T, Volinia S, Okumura H, Shimizu M, Taccioli C, Rossi S, Alder H, Liu CG, Oue N, Yasui W, Yoshida K, Sasaki H, Nomura S, Seto Y, Kaminishi M, Calin GA, Croce CM (2009) Relation between microRNA expression and progression and prognosis of gastric cancer: a microRNA expression analysis. *Lancet Oncol*

Varambally S, Cao Q, Mani RS, Shankar S, Wang X, Ateeq B, Laxman B, Cao X, Jing X, Ramnarayanan K, Brenner JC, Yu J, Kim JH, Han B, Tan P, Kumar-Sinha C, Lonigro RJ, Palanisamy N, Maher CA, Chinnaiyan AM (2008) Genomic loss of microRNA-101 leads to overexpression of histone methyltransferase EZH2 in cancer. *Science* **322:** 1695-9

Vire E, Brenner C, Deplus R, Blanchon L, Fraga M, Didelot C, Morey L, Van Eynde A, Bernard D, Vanderwinden JM, Bollen M, Esteller M, Di Croce L, de Launoit Y, Fuks F (2006) The Polycomb group protein EZH2 directly controls DNA methylation. *Nature* **439:** 871-4

Vladimirova V, Mikeska T, Waha A, Soerensen N, Xu J, Reynolds PC, Pietsch T (2009) Aberrant methylation and reduced expression of LHX9 in malignant gliomas of childhood. *Neoplasia* **11:** 700-11

Waha A, Koch A, Hartmann W, Milde U, Felsberg J, Hubner A, Mikeska T, Goodyer CG, Sorensen N, Lindberg I, Wiestler OD, Pietsch T (2007) SGNE1/7B2 is epigenetically altered and transcriptionally downregulated in human medulloblastomas. *Oncogene* **26:** 5662-8

Wang JC (2005) Finding primary targets of transcriptional regulators. *Cell Cycle* **4:** 356-8

Wang Q, Williamson M, Bott S, Brookman-Amissah N, Freeman A, Nariculam J, Hubank MJ, Ahmed A, Masters JR (2007) Hypomethylation of WNT5A, CRIP1 and S100P in prostate cancer. *Oncogene* **26:** 6560-5

Wang SS, Smiraglia DJ, Wu YZ, Ghosh S, Rader JS, Cho KR, Bonfiglio TA, Nayar R, Plass C, Sherman ME (2008) Identification of novel methylation markers in cervical cancer using restriction landmark genomic scanning. *Cancer Res* **68:** 2489-97

Watts GS, Futscher BW, Holtan N, Degeest K, Domann FE, Rose SL (2008) DNA methylation changes in ovarian cancer are cumulative with disease progression and identify tumor stage. *BMC Med Genomics* **1:** 47

Weber B, Stresemann C, Brueckner B, Lyko F (2007a) Methylation of human microRNA genes in normal and neoplastic cells. *Cell Cycle* **6:** 1001-5

Weber M, Davies JJ, Wittig D, Oakeley EJ, Haase M, Lam WL, Schubeler D (2005) Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. *Nat Genet* **37:** 853-62

Weber M, Hellmann I, Stadler MB, Ramos L, Paabo S, Rebhan M, Schubeler D (2007b) Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. *Nat Genet* **39:** 457-66

Widschwendter M, Jiang G, Woods C, Muller HM, Fiegl H, Goebel G, Marth C, Muller-Holzner E, Zeimet AG, Laird PW, Ehrlich M (2004a) DNA hypomethylation and ovarian cancer biology. *Cancer Res* **64:** 4472-80

Widschwendter M, Siegmund KD, Muller HM, Fiegl H, Marth C, Muller-Holzner E, Jones PA, Laird PW (2004b) Association of breast cancer DNA methylation profiles with hormone receptor status and response to tamoxifen. *Cancer Res* **64:** 3807-13

Willingham AT, Gingeras TR (2006) TUF love for "junk" DNA. *Cell* **125:** 1215-20

Woodson K, Hayes R, Wideroff L, Villaruz L, Tangrea J (2003) Hypermethylation of GSTP1, CD44, and E-cadherin genes in prostate cancer among US Blacks and Whites. *Prostate* **55:** 199-205

Worley LA, Long MD, Onken MD, Harbour JW (2008) Micro-RNAs associated with metastasis in uveal melanoma identified by multiplexed microarray profiling. *Melanoma Res* **18:** 184-90

Wu J, Smith LT, Plass C, Huang TH (2006) ChIP-chip comes of age for genome-wide functional analysis. *Cancer Res* **66:** 6899-902

Xiong Z, Laird PW (1997) COBRA: a sensitive and quantitative DNA methylation assay. *Nucleic Acids Res* **25:** 2532-4

Yamagata Y, Maekawa R, Asada H, Taketani T, Tamura I, Tamura H, Ogane J, Hattori N, Shiota K, Sugino N (2009) Aberrant DNA methylation status in human uterine leiomyoma. *Mol Hum Reprod* **15:** 259-67

Yan PS, Efferth T, Chen HL, Lin J, Rodel F, Fuzesi L, Huang TH (2002) Use of CpG island microarrays to identify colorectal tumors with a high degree of concurrent methylation. *Methods* **27:** 162-9

Yan PS, Perry MR, Laux DE, Asare AL, Caldwell CW, Huang TH (2000) CpG island arrays: an application toward deciphering epigenetic signatures of breast cancer. *Clin Cancer Res* **6:** 1432-8

Yan PS, Venkataramu C, Ibrahim A, Liu JC, Shen RZ, Diaz NM, Centeno B, Weber F, Leu YW, Shapiro CL, Eng C, Yeatman TJ, Huang TH (2006) Mapping geographic zones of cancer risk with epigenetic biomarkers in normal breast tissue. *Clin Cancer Res* **12:** 6626-36

Yanagawa N, Tamura G, Honda T, Endoh M, Nishizuka S, Motoyama T (2004) Demethylation of the synuclein gamma gene CpG island in primary gastric cancers and gastric cancer cell lines. *Clin Cancer Res* **10:** 2447-51

Ying J, Gao Z, Li H, Srivastava G, Murray PG, Goh HK, Lim CY, Wang Y, Marafioti T, Mason DY, Ambinder RF, Chan AT, Tao Q (2007) Frequent epigenetic silencing of protocadherin 10 by methylation in multiple haematologic malignancies. *Br J Haematol* **136:** 829-32

Ying J, Li H, Seng TJ, Langford C, Srivastava G, Tsao SW, Putti T, Murray P, Chan AT, Tao Q (2006) Functional epigenetics identifies a protocadherin PCDH10 as a candidate tumor suppressor for nasopharyngeal, esophageal and multiple other carcinomas with frequent methylation. *Oncogene* **25:** 1070-80

Yu J, Cheng YY, Tao Q, Cheung KF, Lam CN, Geng H, Tian LW, Wong YP, Tong JH, Ying JM, Jin H, To KF, Chan FK, Sung JJ (2009) Methylation of protocadherin 10, a novel tumor suppressor, is associated with poor prognosis in patients with gastric cancer. *Gastroenterology* **136:** 640-51 e1

Yu J, Zhang H, Gu J, Lin S, Li J, Lu W, Wang Y, Zhu J (2004) Methylation profiles of thirty four promoter-CpG islands and concordant methylation behaviours of sixteen genes that may contribute to carcinogenesis of astrocytoma. *BMC Cancer* **4:** 65

Yu MY, Tong JH, Chan PK, Lee TL, Chan MW, Chan AW, Lo KW, To KF (2003) Hypermethylation of the tumor suppressor gene RASSFIA and frequent concomitant loss of heterozygosity at 3p21 in cervical cancers. *Int J Cancer* **105:** 204-9

Zhang B, Pan X, Cobb GP, Anderson TA (2007) microRNAs as oncogenes and tumor suppressors. *Dev Biol* **302:** 1-12

Zhang Q, Wang HY, Marzec M, Raghunath PN, Nagasawa T, Wasik MA (2005a) STAT3- and DNA methyltransferase 1-mediated epigenetic silencing of SHP-1 tyrosine phosphatase tumor suppressor gene in malignant T lymphocytes. *Proc Natl Acad Sci U S A* **102:** 6948-53

Zhang X, Yazaki J, Sundaresan A, Cokus S, Chan SW, Chen H, Henderson IR, Shinn P, Pellegrini M, Jacobsen SE, Ecker JR (2006) Genome-wide high-resolution mapping and functional analysis of DNA methylation in arabidopsis. *Cell* **126:** 1189-201

Zhang YJ, Chen Y, Ahsan H, Lunn RM, Chen SY, Lee PH, Chen CJ, Santella RM (2005b) Silencing of glutathione S-transferase P1 by promoter hypermethylation and its relationship to environmental chemical carcinogens in hepatocellular carcinoma. *Cancer Lett* **221:** 135-43

Zhao W, Liu H, Liu W, Wu Y, Chen W, Jiang B, Zhou Y, Xue R, Luo C, Wang L, Jiang JD, Liu J (2006) Abnormal activation of the synuclein-gamma gene in hepatocellular carcinomas by epigenetic alteration. *Int J Oncol* **28:** 1081-8

Zhou D, Qiao W, Yang L, Lu Z (2006) Bisulfite-modified target DNA array for aberrant methylation analysis. *Anal Biochem* **351:** 26-35

Zhu S, Wu H, Wu F, Nie D, Sheng S, Mo YY (2008) MicroRNA-21 targets tumor suppressor genes in invasion and metastasis. *Cell Res* **18:** 350-9

Zochbauer-Muller S, Fong KM, Virmani AK, Geradts J, Gazdar AF, Minna JD (2001) Aberrant promoter methylation of multiple genes in non-small cell lung cancers. *Cancer Res* **61:** 249-55